I, Jared Klarquist, hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Immunology.

It is entitled:
Type I IFN control of sterile inflammation: Uncovering mechanisms behind autoimmunity and antitumor immunity

Student’s name: Jared Klarquist

This work and its defense approved by:

Committee chair: Edith Janssen, Ph.D.
Committee member: Divaker Choubey, Ph.D.
Committee member: Claire Chougnet, Ph.D.
Committee member: Michael Jordan, M.D.
Committee member: Jonathan Katz, Ph.D.
Type I IFN control of sterile inflammation: Uncovering mechanisms behind autoimmunity and antitumor immunity

A dissertation submitted to the Graduate School of the University of Cincinnati in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Immunology Graduate Program, College of Medicine

2016

by

Jared Klarquist

A.B., Dartmouth College, 2003

Committee Chair: Edith M. Janssen, Ph.D.
Abstract

Dying cells elicit immune responses toward self antigens which can be either harmful or beneficial. These responses can help control deadly cancers, or they can destroy healthy tissues and initiate debilitating autoimmune diseases. We have sought to define novel mechanisms that drive sterile inflammatory responses toward self antigens which may be exploited in new cancer immunotherapies or in treatments for autoimmune diseases. Type I interferon (IFN) has been described as a central mediator in bridging innate and adaptive immune responses toward cell-associated antigens, but the pathways both up- and downstream of IFN signaling have remained unclear. Our studies demonstrate that the stimulator of IFN genes (STING) induces type I IFN production by dendritic cells (DCs) in response to dying cell-derived cytosolic DNA. Further, we report that the IFN produced by DCs results in a positive feedback loop, enhancing their ability to prime effective and long-lasting CD8 T cell responses toward tumor cell-associated antigens. Moreover, we found that STING is also critical to driving autoimmune responses in the bm12 chronic graft-versus-host disease model of systemic lupus erythematosus (SLE). Within the context of bm12 autoimmunity, we determined that type I IFN sensing by CD4 helper T cells protected them from being killed by highly active natural killer (NK) cells, thereby augmenting autoimmune disease. We also showed that direct sensing of IFN enhanced the development of germinal center B cells and plasmablasts in an NK-independent manner. Lastly, we defined the metabolic programs of key T cell and B cell subsets, uncovering a fundamental role for glycolysis in these autoimmune responses. Together, our studies shine light on previously undescribed pathways controlling anticancer immune responses and autoimmunity, providing opportunities for the development of nuanced treatment strategies which may prove both more specific and more effective than existing therapies.
Acknowledgements

First, I would like to express my deepest gratitude to my graduate studies advisor and mentor, Dr. Edith Janssen. Your ambition, cheerfulness, patience, and passion have helped shape my professional abilities and my excitement for science. You have also been a model for how to balance a successful scientific career and a strong commitment to family. Without your continued support, this dissertation would not have been possible. Thank you.

I would also like to thank my dissertation committee members: Dr. Claire Chougnet, Dr. Divaker Choubey, Dr. Michael Jordan, and Dr. Jonathan Katz. I appreciate the time you devoted to helping mentor me and your expert advice. And to my prior mentors, Dr. Hugo Rosen and Dr. Caroline Le Poole, I am grateful for your guidance and your investment in my career.

I owe my thanks to all the current and former members of the Janssen and Hoebe laboratories, especially to Maria Lehn and Cassie Hennies—your tireless work contributed greatly to the projects reported in this dissertation. To Dr. Kasper Hoebe, your mentorship through the years is sincerely appreciated; it has been critical to my progress and my development as a scientist.

Finally, I am grateful for my family. To my parents-in-law, thank you for the immeasurable support you have given me and my wife over the past nine years we have been together. To my brothers, thank you for your steadfast friendships. And to my parents, who have encouraged me personally and professionally since I was a child, and have done everything in their power to help me succeed, thank you. To my wife, Lori, the passion, dedication, and tenacity with which you have approached your career in medicine has provided invaluable inspiration to me. But foremost, your love for me and for our children has sustained me in undertaking this endeavor.
Abbreviations used in this dissertation

actmOVA membrane-targeted OVA driven by chicken beta actin promoter
ANA anti-nuclear antibody
APC antigen presenting cell
B16-OVA OVA-transfected B16 melanoma cell line
B16/F10 C57BL/6-derived melanoma cell line
B6 C57BL/6 mice
BCL-6 B-cell lymphoma 6 protein
BM bone marrow
bm12 B6(C)-H2-Ab1bm12/KhEgJ mice
cDC conventional DC
CFSE carboxyfluorescein succinimidyl ester
cGAMP cyclic guanosine monophosphate-adenosine monophosphate (cyclic GMP-AMP)
cGAS cyclic GMP-AMP synthase
cGVHD chronic graft-versus-host disease
CLR C-type lectin receptor
CTL cytotoxic T lymphocyte
CTLA4 cytotoxic T-lymphocytes associated protein 4
CTV or VT CellTrace Violet proliferation/tracking dye
CXCR5 Chemokine (C-X-C motif) receptor 5
DAMP damage-associated molecular pattern
DC dendritic cell
dsDNA double-stranded deoxyribonucleic acid
DT diptheria toxin
DTR human diptheria toxin receptor
FACS fluorescence-activated cell sorting
FAO fatty acid beta oxidation
FASL Fas ligand (aka CD95L)
FOXP3 forkhead box P3
GC germinal center
ICOS inducible T-cell COStimulator
IFN interferon
IFNAR type I IFN receptor
IL interleukin
IRAK IL-1R-associated kinase
IRF3 interferon regulatory factor 3
ISRE interferon-sensitive response element
LPS lipopolysaccharide
MAVS mitochondrial antiviral signaling (aka Cardif, IPS-1, Visa)
mDC merocytic DC (aka CD8/CD11b double-negative DC)
MHC  major histocompatibility complex
mOVA  membrane-targeted OVA
MS  multiple sclerosis
MyD88  myeloid differentiation primary response gene 88
NFκB  nuclear factor kappa-light-chain-enhancer of activated B cells
NK  natural killer
OTI  TCR transgenic mice specific for the MHC class I-restricted epitope OVA$_{257-264}$
OTII  TCR transgenic mice specific for the MHC class II-restricted epitope OVA$_{323-339}$
OVA  chicken egg ovalbumin
OXPHOS  oxidative phosphorylation
PAMP  pathogen-associated molecular pattern
PD-1  programmed cell death 1 (aka PDCD1)
pDC  plasmacytoid DC
PRR  pattern recognition receptor
RAE1  retinoic acid early transcript 1
RIG-I  DEXD/H-box helicase 58 (aka DDX58, RLR-1)
SAVI  STING-associated vasculopathy with onset in infancy
SLE  systemic lupus erythematosus
SMARTA  TCR transgenic mice specific for the MHC class II-restricted LCMV GP$_{61-80}$
SNP  single-nucleotide polymorphism
STAT  signal transducer and activator of transcription
STING  stimulator of interferon genes (aka Tmem173, ERIS, MPYS, Mita)
T-bet  T-box transcription factor TBX21
T1D  type I diabetes
TCR  T cell receptor
Tfh  T follicular helper
TGF-β  transforming growth factor-β
Th  T helper
TNF  tumor necrosis factor
TRAF  TNF-receptor-associated factor
TRAIL  TNF-related apoptosis-inducing ligand
TRAM  TRIF-related adaptor molecule
Treg  regulatory T cell
TREX1  three prime repair exonuclease 1
TRIF  TIR domain-containing adaptor inducing IFN
WT  wild type
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CHAPTER 8: Summary and Discussion

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Chapter 1. A brief overview of the major immunological concepts central to this dissertation
1.1 A crossroads: innate and adaptive immunity

Innate and adaptive immunity are the two primary arms which comprise the immune system. Each arm plays its own role, but the two are intricately linked and tightly controlled. The innate immune system provides the first line of defense against invading viruses, bacteria, and fungi. Upon sensing conserved, pathogen-associated molecular patterns (PAMPs), innate immune cells can quickly consume and digest microorganisms or infected cells. This occurs rapidly and helps contain infectious agents very early after invasion. The expediency of innate immune responses comes at a cost—they have relatively low specificity—but provides a crucial level of protection during the 4-7 day time period needed to develop adaptive immune responses. As their name implies, adaptive immune cells provide a much higher level of specificity toward infectious particles, even as pathogens evolve and new pathogens are encountered. Specialized cells of the innate immune system, in addition to providing early protection, also play key roles in initiating and directing adaptive immune responses. Dendritic cell (DCs), serve a particularly central function at this interface of innate and adaptive immunity and will be a primary subject in several chapters of this dissertation.

Immune responses can be very powerful and potentially harmful. Some activated immune cells are given the license and several tools by which to kill any cell which appears to be harboring an infection. Other immune cells can produce robust, damaging inflammation. Hence, to avoid unintentional destruction of healthy tissue, multiple systems work in concert to limit immune responses. First, adaptive immune cells undergo a process of selection, which leads to the demise of any overly self-reactive cell. The entire purpose of some specialized lymphocytes is to regulate the responses of others. And finally complex networks within each cell integrate numerous often opposing signals, such that the balance of these signals ultimately determines cellular activity. Dysregulation of signals that promote or dampen immunity can therefore lead to profound tissue
destruction that may result in autoimmunity. Nuances in these signals may also allow the
detection and immune targeting of cancer, the uncontrolled growth of otherwise healthy
cells. Subsequent chapters in this dissertation will explore novel mechanisms that regu-
late immunity to self, with the goal of uncovering new targets for the treatment of cancer
and autoimmune disease.

1.1.1 Brief introduction to cells of the immune system

The innate immune system is composed of many cell types. Essentially every cell, even
highly specialized cells of non-hematopoietic origin, can operate as part of the innate
immune system, sensing microbes and alerting other cells of danger. In fact, primary pro-
tection from infection is provided by the non-hematopoietic cells that make up epithelial
surfaces. Epithelia serve as physical barriers, but also secrete antimicrobial compounds,
and can physically expel microorganisms through the action of cilia. Notwithstanding,
several unique cells of hematopoietic origin serve specialized functions within the innate
immune system and are of particular relevance to the work discussed in this dissertation.
These include macrophages, DCs, and natural killer (NK) cells.

DCs were initially described by Ralph Steinman and Zanvil Cohn in 1973, nearly 100
years after Élie Metchnikoff first described macrophages\textsuperscript{1,2}. DCs were recognized as
morphologically distinct from macrophages, forming elaborate tree-like “dendritic” struc-
tures, yet the two cell types performed similar functions. Macrophages and DCs share
the common ability to quickly consume relatively large particles including microorganisms
and dying cells or large portions thereof in a process called phagocytosis. Subsequently,
these cells have varying abilities to initiate adaptive immune responses through a pro-
cess called antigen presentation, the subject of the following section. The clearance and
recycling of dying cell material via phagocytosis is also critically important for maintaining
immunological tolerance\textsuperscript{3,4}. Macrophages are thought to play particularly important roles
in promoting tolerance, whereas DCs are considered the antigen presenting cell most capable of initiating adaptive immune responses to foreign and self-antigens\textsuperscript{5–7}. Many different subtypes of DCs exist, performing distinct but overlapping roles in immunity. As previously mentioned, subsequent Chapters 2 and 3 will cover DCs and their functions in cancer and autoimmune disease in much further detail.

NK cells are chiefly known for their function as killers of infected cells. Cells respond to intracellular infections by downregulating inhibitors and upregulating activators of NK function, and the balance these signals determines whether or not the cell becomes an NK target\textsuperscript{8}. The prototypical molecular interaction between NK cells and their targets is the engagement of the NK cell's killer immunoglobulin receptor (KIR) family proteins by the target cells' major histocompatibility complex (MHC) class I molecules. Engagement of KIRs by MHC class I produces a signal within the NK cell necessary to inhibit its killer functions—when MHC class I molecules are absent or significantly reduced, NK cells will kill\textsuperscript{9}. Additional signals from target cells can activate NK cell killer functions, including RAE1, MICA, MICB, CD48, and cytokines such as IL-2, IL-12, IL-18, and type I IFN\textsuperscript{10} (Figure 1).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Signals passed from target cells to NK cells.}
\end{figure}

Once activated, NK cells can kill target cells directly or indirectly. Indirect NK killing can include signaling target cells to commit suicide by release of the death-inducing cytokine TNFα or by surface expression of TRAIL or FASL, whereas more direct means of killing can be accomplished by delivering cytolytic granules containing perforin and granzymes\textsuperscript{11,12}. It is becoming increasingly clear that infection is not the only cellular stressor that can cause cells to become targets of NK cell killing. Malignant transformation, nutritional deficiencies, and DNA damage are a few examples of additional stressors which alter the balance of NK ligand expression such that they will activate NK cell killer function upon encountering NK cells\textsuperscript{13}. Recently, an immunoregulatory role for NK cells has also been described, whereby NK cells can limit vigorous immune responses to viruses by killing proliferating T cells\textsuperscript{14}, although the precise mechanisms promoting NK cell activation in this context have not yet been elucidated.

T cells comprise the major arm of adaptive immunity that develops in the thymus. The term describes a number of different cell types which perform distinct functions, yet these cells all share a highly variable receptor which distinguishes them from other cells, the T cell receptor (TCR). The majority of T cells express a heterodimeric TCR composed of an alpha and beta subunit. Within alpha/beta T cells, two primary divisions exist: CD4\textsuperscript{+} T cells and CD8\textsuperscript{+} T cells (hereafter referred to as CD4 T cells and CD8 T cells), which are derived from the same precursor cells\textsuperscript{15}. CD4 and CD8 T cells are positively and negatively selected on MHC class I and II proteins such that T cells which leave the thymus alive react weakly with self antigens, thus preventing autoimmunity, but also ensuring their potential reactivity toward foreign antigens\textsuperscript{16}. CD4 T cells, commonly referred to as “helper” T cells, make critical contributions toward the activation and differentiation of both CD8 T cells and B cells. They also act as effector cells producing a variety of cytokines depending upon their specific differentiation program. Expression of certain master regulator transcription factors determines the differentiation fate of CD4 T cells with some
permanence, though some of these cells do exhibit a degree of plasticity (Figure 2)\textsuperscript{17}.

The primary function of CD8 T cells is to kill infected cells, cancer cells, or otherwise damaged cells; thus, they are also called cytotoxic T cells (CTLs). The effector functions of CTLs are similar to those of NK cells, though an important difference between the two cell types is the relatively high degree of specificity of CTL clones toward a limited number of foreign peptide-MHC combinations. CD8 T cells primarily respond to endogenous antigens presented by essentially any cell; typically they recognize non-self, infectious material presented by MHC class I. However, some specialized DCs are capable of presenting exogenous antigens to CD8 T cells, as will be discussed later in this section. In the absence of appropriate regulatory controls, CD4 and CD8 T cells can both react strongly with self antigens and mediate autoimmune disease.
B cells are hematopoietic cells that mature in the bone marrow. Their primary functions are mediated by their antigen receptor, the B cell receptor (BCR). The BCR consists of one of several different immunoglobulin proteins complexed with a signaling adapter heterodimer Ig-α/Ig-β (CD79)\(^{18}\). The BCR can be activated successfully with or without T cell help, in so-called T-dependent or T-independent responses; here, we will focus on T-dependent responses. Although many functional subtypes of B cell exist, the most relevant subtypes to this dissertation include plasmablasts and germinal center (GC) B cells. Upon recognition of their cognate antigen, naïve B cells either differentiate into short-lived plasmablasts or GC B cells (Figure 3)\(^{19}\). Plasmablasts differentiate in extra-follicular regions of secondary lymphoid organs in what represents the first step in the development of a B cell response. They undergo class switch recombination (CSR) and are the primary source of early antibody secretion, but because they arise so quickly, plasmablasts

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**Figure 3: T cell-dependent B cell differentiation.**
Adapted from Moens L and Tangye SG: *Frontiers in Immunology*\(^{20}\), copyright 2014. Open access article used here under the terms of the Creative Commons Attribution License.
have limited time for somatic hypermutation (SHM) and therefore usually have relatively low antibody affinities\textsuperscript{20}. The rapid proliferation of B cells interacting with antigen-specific T follicular helper CD4 T cells (Tfh) results in the formation of GCs\textsuperscript{19}. GC B cells are the early stage B cells found within these GCs, which undergo CSR and significant SHM\textsuperscript{19}. Eventually GC B cells differentiate into memory B cells or long-lived antibody secreting plasma cells, which mediate highly effective long-term protection\textsuperscript{19,20}. Since the later chapters in this dissertation examine B cell responses within the first two weeks of disease onset, these studies are primarily focused on plasmablasts and GC B cells, the cell types most active in this period.

1.1.2 Antigen presentation and T cell priming
Adaptive immune responses are initiated by antigen presentation, a process that includes both phagocytosis-dependent and -independent processes. Professional antigen presenting cells (APCs) express MHC class I and II and are responsible for most productive priming of CD4 and CD8 T cells, although essentially any nucleated cell can present antigen to CD8 T cells via MHC class I.

TCRs are highly specific to unique combinations of MHC protein structures and peptide sequences\textsuperscript{21}, and T cells can only become activated when their TCRs bind MHC-peptide combination with relatively high affinity\textsuperscript{22}. The priming of CD4 T cells generally requires phagocytosis of exogenous material by APCs and presentation of exogenous protein antigens. Professional APCs include DCs as well as B cells, which, upon antigen capture, can present exogenous antigens to CD4 T cells in a similar manner as DCs. After engulfment, phagocytosed material remains compartmentalized in the phagosome. Fusion of the phagosome with highly acidic lysosomes leads to the digestion of phagocytosed material by a cocktail of toxic agents including lysozymes, free radicals, and acid hydro-lases\textsuperscript{23}. Small fragments of digested protein between 14 and 20 amino acids in length
are loaded on class II MHC proteins to be presented to CD4 T cells via their TCRs\textsuperscript{24–27}. Presentation of peptides 8-10 amino acids in length from endogenous proteins are loaded on to MHC class I and presented to CD8 T cells\textsuperscript{27}. This process is dependent upon the proteasomal degradation of endogenous protein\textsuperscript{28} and subsequent trafficking of these peptides to the endoplasmic reticulum via TAP proteins\textsuperscript{29}.

DCs can also take up dying cell material and present exogenous cell-associated antigens on MHC class I to CD8 T cells through a process called cross-presentation\textsuperscript{30–32}. Cross-presentation is especially important to this dissertation, because it is by cross-presentation that DCs most effectively initiate immune responses toward self-antigens including antitumor immunity\textsuperscript{33} and autoimmunity\textsuperscript{34}. The exact mechanism by which exogenous peptide is shuttled onto MHC I is not known, though its dependency on TAP proteins has been established\textsuperscript{35}. Several cell types are known to cross-present antigen, including dendritic cells, macrophages, and even neutrophils\textsuperscript{36}, but they are not equal in their abilities. So-called “lymphoid,” or CD8α\textsuperscript{+} murine DCs have been described as the most efficient cross-priming DCs\textsuperscript{35}. While “myeloid,” or CD11b\textsuperscript{+} DCs phagocytose bacteria and proteins well, they only weakly phagocytose dying cells and cross-prime poorly\textsuperscript{32,37}. In mice, “plasmacytoid” DCs (pDCs) are extremely weak at taking up apoptotic cells and are not thought to cross-present cell-associated antigen, though human pDCs are capable\textsuperscript{32}. Although exactly what determines the cross-priming abilities of various DC subsets remains unclear, rapid phagolysosomal acidification was associated with the minimal and transient ability of CD11b\textsuperscript{+} DCs to cross-present antigen\textsuperscript{37}. Recently, our lab has documented the superior cross-priming ability of the CD8α\textsuperscript{+}CD11b\textsuperscript{−} “merocytic” DCs (mcDC)\textsuperscript{32,37–40}. mcDC acquire dead cell material through the process of merocytosis, wherein they take up small particles into non-acidic compartments followed by prolonged antigen storage and sustained antigen presentation. Upon uptake of dying cell material, mcDCs, but not CD8α\textsuperscript{+} or CD11b\textsuperscript{+} DCs, produce the type I IFN required for protective
immunity secondary to ablative tumor therapy\textsuperscript{37}, a process that was further dissected in Chapter 5 of this dissertation.

Antigen presentation and TCR engagement is only one of three signals provided by professional APCs that are required to stimulate productive T cell responses. In addition to TCR stimulation, T cells receive activating signals by the engagement of costimulatory molecules and inflammatory cytokines. Costimulation is accomplished by the engagement of several additional cell surface receptors on the T cell\textsuperscript{41}. The ligands CD80 and CD86, ICOS-L, and OX40-L expressed on APCs bind to CD28\textsuperscript{42,43}, ICOS\textsuperscript{44}, and OX40\textsuperscript{45} on T cells, respectively. Inhibition can also be achieved by engagement of related receptors on the T cells; CTLA4\textsuperscript{46} and PD-1\textsuperscript{47,48} both inhibit T cell responses when ligated by CD80/CD86 and PD-L1, respectively. Cytokines produced by APCs and other nearby cells provide a third signal which can positively or negatively affect T cell priming. IL-12 and type I IFN are the prototypical signal 3 cytokines that promote T cell activation, differentiation, and proliferation\textsuperscript{49,50}. Inhibition of T cell proliferation and effector functions by IL-10 has been well established\textsuperscript{51,52}, and is one mechanism thought to promote peripheral tolerance\textsuperscript{53}. Depending on multiple factors, including the balance of pro- and anti-inflammatory cues, antigen presentation by APCs can either prime naïve T cells toward mature cells with effector functions, or it can cause T cell anergy or deletion, and result in tolerance.

1.1.3 PAMPs, DAMPs, and PRRs

An important component to the innate immune system is the ability of almost any cell to sense danger. A large number of cytosolic and membrane-bound proteins serve as pattern recognition receptors (PRRs) to an even larger number of danger signals—both foreign and native in origin. Foreign signals derived from invading microbes are known as pathogen-associated molecular patterns (PAMPs). PAMPs comprise conserved protein, sugar, and lipid moieties which are distinct from host molecules and are essential for
pathogen survival. Damage-associated molecular patterns (DAMPs), or “alarmins,” are endogenous molecules that alert the immune system of damage, even in the absence of infection. Alarmins are released by cells that undergo an uncontrolled cell death and can be secreted by immune cells. Some hypothesize they exist because most trauma is accompanied by exposure to pathogens, and alarmins can therefore prime the immune system even earlier than it could otherwise recognize the invasion. However, alarmins also aid in tissue repair, and can be beneficial even in sterile settings. Different PAMPs and alarmins are detected by families of receptors which localize to the cell surface, the internal space of endosomes, and the cytosol.

A large family of innate sensors, the toll-like receptors (TLRs), play a critical role in early immune responses to pathogens. The TLRs include at least 11 proteins with differential specificities toward both PAMPs and alarmins (Table 1). Most TLRs are situated in the plasma membrane and recognize extracellular PAMPs and alarmins. TLR3, TLR7, TLR8, and TLR9 are found within endosomal membranes and respond to intracellular pathogens. TLR signaling is accomplished largely through the adapter molecule MyD88, but also TRIF and TRAM. Only TLR3 and TLR4 can induce MyD88-independent signaling. Downstream TLR signaling is mediated by IL-1R-associated kinases (IRAKs), TNF-receptor-associated factor (TRAF) proteins, and several other downstream mediators which ultimately induce the translocation of NFkB or IFN-regulatory factors (IRFs), including IRF3, IRF5, and IRF7. Depending on the signal, these receptors can induce the rapid secretion of IL-6, TNF, and IL-12, among other inflammatory cytokines, or type I IFN.

The C-type lectin receptors (CLRs) provide another layer of protection against most invading pathogens through the recognition of carbohydrates. Most classes of pathogens express carbohydrates that activate CLRs. High levels of mannoses on viruses, myco-
bacteria, and fungi are sensed by DC-SIGN (CD209) or Langerin (CD207/CLEC4K). Certain strains of bacteria and some helminths express fucose, which is recognized by the same receptors. Dectin1 (CLEC7A) recognizes glucans, including β-1,3-glucan, expressed by several mycobacteria species and fungi. CLEC9A binds the filamentous actin of dying cells, and thereby aids in the antigen presentation of cell-associated material. Recently, attention has been given to targeting CLEC9A in DC-based vaccination strategies, since growing evidence suggest that CLEC9A-expressing DCs are essential for inducing CTL responses. And, given its role in presenting cell-associated antigens, activating CLEC9A has special promise in eliciting anti-tumor immunity.

Another major family of innate sensors is the AIM2-like receptor (ALR) or p200 family, which share at least one partially conserved region of 200 amino acids. This family contains several sensors of cytoplasmic DNA, including IFI16 and AIM2. A p200 family
is present in mice and humans, encompassing several proteins with similar structure and function; however, the genes exhibit considerable genetic and functional diversity. Among them, AIM2 is the only evolutionarily conserved gene. AIM2 functions in complex with ASC and caspase1 in a so-called “inflammasome.” Upon activation, the AIM2 inflammasome induces the cleavage of pro-IL-1β into active IL-1β and initiates a highly inflammatory form of programmed cell death called pyroptosis. Several other innate dsDNA-sensors including p200 family members signal through the adaptor protein commonly referred to as the stimulator of interferon genes, or “STING” (TMEM173, also known as MPYS, MITA, and ERIS). STING is also a cytosolic DNA sensor in its own right, directly binding to cyclic guanosine monophosphate–adenosinemonophosphate (cGAMP). The cyclic dinucleotide cGAMP is generated by cGAMP synthase (cGAS) from bacterial or endogenous DNA which has entered the cytosol. Once activated, STING activates and serves as a scaffold for TBK1, which phosphorylates IRF3. Phosphorylated IRF3 then translocates to the nucleus, where it induces production of type I IFN. STING will be discussed further in Chapter 5, where we will examine its role in promoting immune responses to dying tumor cells.

An additional family of proteins involved in cytosolic nucleotide sensing is the RIG-I-like family of receptors, including most notably RIG-I and MDA5. These are dsRNA helicases that mediate their effects through the common signaling intermediate mitochondrial antiviral signaling protein (MAVS; also known as IPS-1, VISA or CARDIF). MAVS directs further signaling that ultimately results in the activation and nuclear translocation of IRF3 and NFκB and subsequent production of type I IFN and other inflammatory cytokines. A depiction of the major cytosolic nucleotide sensors and their signaling pathways is shown in Figure 4.
1.1.4 Type I IFN

Type I IFN is a dominant effector molecule downstream of myriad danger signals discussed in the previous section. Type I IFN comprises 13 IFNα proteins, IFNβ, IFNκ, and IFNω, all of which signal through a common receptor known as the interferon (alpha and beta) receptor, or IFNAR. Essentially all nucleated cells express IFNAR, a heterodimer including the two distinct subunits IFNAR1 and IFNAR2. A soluble form of IFNAR2 has also been reported in mouse and human fluids including urine and serum. Soluble mouse Ifnar2 was shown to competitively inhibit Ifnar1/2 signaling in the L929 type I IFN reporter cell line, but it was also shown to complex with type I IFN and Ifnar1 in Ifnar2−/− thymocytes, indicating that soluble Ifnar2 may act as both an agonist and an antagonist to IFNAR signaling.
Upon binding their ligand at the cell surface, the conformation of IFNAR1 and IFNAR2 change and the two proteins dimerize, leading to the autophosphorylation of tyrosine kinase 2 (TYK2) and Janus activated kinase (JAK) 2, signaling molecules associated with the intracellular domains of IFNAR1 and IFNAR2, respectively. Phosphorylated TYK2 and JAK2, now active, phosphorylate the signal transducer and activator of transcription proteins STAT1 and STAT2 which subsequently dimerize. The activated STAT proteins rapidly translocate to the nucleus and bind IFN-stimulated response elements (ISREs), initiating the transcription of hundreds of so-called IFN-sensitive genes (ISGs). Activation of alternative STAT pairs, such as STAT4/STAT4, can lead to dimerization and initiation of transcription at so-called gamma-interferon activated sites (GAS) (Figure 5). A number of JAK/STAT-independent mechanisms also exist which are important for IFNAR signal transduction, including roles for CRKL, MAP kinases, PI3K, and mTOR in mediating RNA translation, RNA stability, and pro- and anti-apoptotic signals. Type I IFN is known to initiate a positive feedback loop. In fact, signaling through the IFNAR is required for the expression of some type I IFNs. Two of the IFNs induced early by PRR stimulation, IFNα4 and IFNβ, induce IRF9 activation, which, in concert with STAT1/STAT2 heterodimer, is required for the transcription of other type I IFN genes, including those encoding for IFNα2, 5, 6, and 8.

Type I IFN plays well described, key roles in antiviral defenses by conferring direct antiviral effects, inhibiting cellular growth, controlling apoptosis, and by promoting anti-viral immune responses. Studies have shown both autocrine and paracrine functions of type I IFN in the priming of T cells; for example, IFN produced by DCs can augment signals 1 and 2 within DCs, increasing the antigen presenting capacity of DCs and their expression of cell surface costimulatory molecules, while it can concurrently serve as a signal 3 cytokine, enhancing CD8 T cell priming through its signaling within T cells. Furthermore, type I IFN can promote the activation of B cells, CSR, and is vital to the generation of an-
tibody secreting plasma cells\textsuperscript{87,88}. Moreover, type I IFN is a potent stimulator of NK cells, which can efficiently kill infected cells\textsuperscript{89}. Importantly, while damage or pathogen sensing by PRRs can induce inflammation by the release vast quantities type I IFN, constitutive or “tonic” IFN signaling is thought to prime cells to respond more strongly to diverse inflammatory signals and is also thought to contribute to immune homeostasis\textsuperscript{90,91}. Despite the vast literature on the roles of type I IFN in anti-viral immunity, surprisingly little is understood about its precise functions in sterile inflammatory processes, including cancer immunology, cancer immunotherapies, and systemic autoimmune diseases. The current

Figure 5: Canonical and alternative JAK/STAT signaling pathways induced by type I IFN. Adapted from Tomasello et al.: Frontiers in Immunology\textsuperscript{80}, copyright 2014. Open access article used here under the terms of the Creative Commons Attribution License.
knowledge on the role of type I IFN in these processes will be discussed later in this chapter, and later chapters will seek to uncover previously unknown mechanisms by which IFN functions in disease.

1.2 Cancer
The term “cancer” encompasses many diseases which vary widely in pathogenesis, pathology, and prognoses. The primary defining feature of cancer is the dedifferentiation and unrestricted growth of previously healthy cells. These transformed cells have the potential to invade tissues or organs at remote sites from the initial neoplasm, thereby disrupting critical, life-sustaining biological processes. Cancer is among the most common causes of death worldwide, accounting for over 8 million deaths annually\(^92\). Cancer treatment can be physically taxing, as well as financially burdensome. The most recent estimates for the national cost of cancer care in the US totaled $125 billion\(^93\). And, as treatments for other diseases succeed in extending our average lifespan, cancer incidence is expected to rise rapidly. Research into novel ways to prevent, halt, and eliminate cancer is desperately needed.

1.2.1 The (re-)emerging field of cancer immunotherapy
Cancer immunotherapy is any process which augments the body’s immune reactivity toward neoplasms. The ultimate goal of immunotherapy is to eliminate tumors either alone or in conjunction with surgery, ablative therapies such as radiation, or chemotherapies. Some evidence indicates that our cancer burden is reduced by a natural ability of the immune system to recognize and destroy tumors as they arise, through what has been termed “cancer immunosurveillance”\(^94\). Moreover, the success of radiotherapy, at least in some cases, is dependent upon a functional immune system\(^95,96\). Unfortunately, the natural ability of the immune system to protect against tumors is limited, and may even lead to the outgrowth of transformed cells with reduced immunogenicity, making the de-
development of effective immunotherapies more challenging\textsuperscript{97,98}. Hence, determining what naturally drives immune protection against neoplasms and might therefore be manipulated to enhance immunotherapies has been an area of intense investigation.

One of the earliest examples of immunotherapy occurred in 1891, at which time the function of the immune system was scarcely understood, when William Coley gave patients intratumoral injections of \textit{Streptococcus ‘erysipelatis’ (pyogenes)}, and was met with some success in treating several solid tumors\textsuperscript{99}. The bacterial infection causes an acute inflammation of the skin and, when in the vicinity of certain carcinomas and sarcomas, led to their destruction. In the many decades since these initial findings, immunotherapies remained largely a niche treatment, especially given the relatively high success rates of radiation therapies and chemotherapy, and the insufficient evidence on the utility of historical immunotherapies. However, recent advances in our understanding of immunology together with a profoundly enhanced ability to manipulate or even manufacture immune responses has reinvigorated the field, leading to several FDA approved immunotherapies and more on the horizon\textsuperscript{100,101}.

Direct stimulation of the immune system has been an approach with some efficacy, but also significant potential side effects. In 1992 the administration of high dose IL-2 was approved to treat patients with metastatic renal cancers, and has a reported response rate of between 15 and 25\%, with some cases of nearly complete tumor regression\textsuperscript{102–104}. Unfortunately, this treatment has also been responsible for a number of deaths, with significant toxicities including capillary leak syndrome, myocardial infarctions, and gastrointestinal toxicities\textsuperscript{102}. The mechanism by which IL-2 is thought to halt tumor progression or even eliminate tumors is by activating T cells and NK cells, among other lymphocytes with the IL-2 receptor. This approach has also been used for patients with aggressive melanomas, and has been especially effective when it was given in conjunction with a
melanocyte-specific gp100 peptide, though response rates are still low, at only 16%, and toxicity is also seen in these patients\textsuperscript{105}. In another attempt to broadly stimulate immunity towards tumors, a novel anti-CD28 agonist was used in patients with renal cancer. Rather than any protective effect, however, this treatment resulted in the rapid release of inflammatory cytokines, a “cytokine storm,” which nearly killed the study volunteers\textsuperscript{106}. This type of damaging acute inflammatory response has been noted in several other immunomodulatory treatments\textsuperscript{107–109}, underscoring the challenge that immunotherapy poses, but also its potential strength.

A logical alternative to directly stimulating the immune system is to remove inhibitors of its function by “immune checkpoint” blockade. This approach was considered particularly promising given the increasing evidence that the tumor microenvironment exerted strong inhibitory pressure on infiltrating lymphocytes\textsuperscript{110,111}. Indeed, the promise of immune checkpoint blockade was realized upon the success of clinical trials using ipilimumab, tremelimumab, and nivolumab, monoclonal antibodies targeting cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death 1 (PD-1), respectively\textsuperscript{112–114}. Adverse events also occur with these treatments, though their toxicity appears to be relatively low, with few study drug-related deaths reported. These treatments halted tumor progression, but also importantly led to tumor regression in a large number of cases\textsuperscript{115–117}. These targets were chosen specifically because of their increasingly well-established immunosuppressive roles in cancer. CTLA-4 is highly expressed on regulatory T cells (Tregs), and suppresses the otherwise co-stimulatory effect of CD80 and CD86 on APCs, possibly by capturing and removing these molecules from the surface of APCs\textsuperscript{118}. Tregs are found at high frequencies in a number of tumors, where they are thought to play an important role in promoting tumor outgrowth through immune suppression\textsuperscript{119}. PD-1 and its ligand PD-L1 also play critical roles in suppressing antitumor immune responses, through the induction of T cell anergy or exhaustion; importantly, many tumors express PD-L1, though
anti-PD-1 therapy appears to be nearly as successful in patients bearing PD-L1-negative tumors\textsuperscript{117}. The successes of these and similar immune checkpoint blockades have been instrumental in reviving interest and hope in immunotherapy, though there is still much room for improvement in terms of efficacy and safety.

1.2.2 Type I IFN in immunotherapy and cancer immunology

The immunomodulatory cytokine central to much of the work within this dissertation, type I IFN, has itself been the focus of immunotherapies, and has been shown to play key roles in antineoplastic immunity more broadly. Imiquimod, a potent TLR7 agonist, is used as a highly effective treatment for basal cell carcinoma\textsuperscript{120} and vulvar intraepithelial neoplasia\textsuperscript{121}. The use of CpG oligodeoxynucleotides, which potently stimulate IFN production via TLR9, has also been shown to prolong the survival of tumor-bearing mice, although its efficacy is most dramatic when combined with tumor ablative therapies\textsuperscript{122,123}. Treatment of chronic myeloid leukemia (CML) with recombinant IFNα was shown to be more effective than conventional chemotherapeutics\textsuperscript{124,125}, with upwards of 70% achieving complete hematological remission. Moreover, the 10 year survival in responders was greater than 70\%\textsuperscript{126}. While the therapeutic use of type I IFN has shown great efficacy, like many broadly immunomodulatory agents, its use is also associated with significant toxicities, especially flu-like symptoms, which many patients cannot tolerate; however, lower doses may be just as effective as higher doses and result in fewer toxicities\textsuperscript{127}.

Beyond its use as an immunotherapeutic agent, type I IFN also plays key roles in cancer immunology. Several key studies have underscored this point, and have elucidated some mechanisms by which IFN functions. Dunn et al. demonstrated that mice were more susceptible to carcinogen-induced tumors if they lacked IFNAR, and that mice treated with anti-IFNAR antibodies were much more susceptible to transplanted tumor outgrowth than mice given isotype controls\textsuperscript{128}. Fuertes et al. found an IFN signature in human metastatic
melanomas and infiltration of those tumors by T cells. In mouse modeling, the group went on to demonstrate that spontaneous antitumor CD8 T cell responses were defective in mice lacking either IFNAR or signal transducer and activator of transcription 1 (STAT1), a transcription factor immediately downstream of IFN signaling. Burnette et al. showed that radiation therapy of B16F10 tumors induced type I IFN, and that the sustained progression-free survival following radiotherapy was highly dependent on the ability of the animals ability to sense type I IFN. In work from our lab that is presented later in this dissertation, we show that the type I IFN released following tumor ablative therapies is produced by DCs, and that DCs themselves are the critical cell type which needs to sense IFN in order to prime effective antitumor CD8 T cell responses. Additionally, we showed that STING within DCs was required for IFN production, and that the ligand responsible for its induction was nuclear DNA from dying tumor cells. Studies like these, which provide further insight into the mechanisms which boost antitumor immunity, are needed to allow the rational design of novel, targeted immunotherapies with increased efficacy and reduced toxicity.

1.3 Autoimmunity
Autoimmunity describes the aberrant activity of an organism’s immune system to become highly activated by its own tissues. This can lead to destructive inflammation resulting in the loss of tissues and organs. But like cancer, autoimmunity describes myriad diseases with diverse outcomes, some more severe than others. Autoimmune diseases can be life-threatening, including responses toward major organs like type I diabetes or multiple sclerosis (MS), or in the case of systemic diseases such as systemic lupus erythematosus (SLE). Whether or not they are responsible for early mortality, many autoimmune diseases can be painful or otherwise debilitating.

Our lab recently began studying autoimmunity, because understanding what drives im-
mune responses to self-antigens in the context of cancer immunotherapies may provide insight into the mechanisms of autoimmunity. And, reciprocally, insights into autoimmune disease may provide avenues to exploit for novel anticancer immunotherapies. The study of SLE in particular was a natural choice for our lab, because, like antitumor immunity, SLE is a sterile inflammatory process involving atypical cell death and dying cell clearance, which also requires type I IFN.

1.3.1 Systemic lupus erythematosus
SLE is a systemic autoimmune disease affecting multiple systems defined most prototypically by the development of anti-nuclear antibodies (ANA), antibody complex formation, and subsequent nephritis. However, SLE can affect patients in different ways and likely encompasses many different conditions with a core set of shared clinical signs. Reported symptoms can also resemble those of distinct diseases. Therefore, a diagnosis of SLE relies upon a patient meeting several well-defined immunological and clinical classification criteria\textsuperscript{131}. Although SLE is usually not fatal, many patients live with significantly burdensome ailments\textsuperscript{132}. In addition to nephritis, clinical signs include cutaneous, oral, neurological, hematological, pulmonary, and cardiac disorders. Estimates of incidence vary—between 10 to 150 cases per 100,000 individuals in the US are affected, with the highest incidence among women and people with African or Hispanic ancestry\textsuperscript{133–135}.

1.3.2 SLE pathogenesis
The cause of SLE is not known. Given the heterogeneity of clinical symptoms patients develop, the pathogenesis of SLE almost certainly varies. The current understanding is that disease arises due to the unfortunate conspiroation of genetic risk factors and environmental triggers\textsuperscript{136,137}.

Most hypotheses as to what biological process initiates disease involve either dysregulat-
ed apoptosis or insufficient clearance of dying cells\textsuperscript{138–140}. Indeed, high levels of apoptotic cells are found in SLE patient serum\textsuperscript{141}, germinal centers\textsuperscript{142}, and in inflamed tissues, such as in the skin of patients with cutaneous lupus erythematosus\textsuperscript{142} and in the glomeruli of mice with an SLE-like disease\textsuperscript{143}. However, an abundance of apoptotic material alone cannot account for the development of systemic autoimmunity. Under physiological conditions, the clearance of dying cells is a tightly regulated process employing redundant mechanisms to prevent autoimmunity. Although it is not yet clear how immunological tolerance is broken in SLE, DCs likely play key roles. Perhaps the most prominent model proposes that initial injury is due to the unremitting output of type I IFN by pDCs which leads to persistent activation of cDCs and the subsequent expansion of autoreactive T cells\textsuperscript{144}. The known roles for DCs in SLE will be discussed in Chapter 3 of this dissertation.

Genome-wide association studies have linked a number of specific genes to increased risk for developing lupus\textsuperscript{145}. Alterations in these genes generally impart odds ratios of less than 1.5, and a combination of multiple polymorphisms is thought to be necessary to put an individual at significant risk for SLE. A few rare genetic variants may function on their own to greatly increase this risk, however. For example, rare mutations in the cytosolic exonuclease TREX1\textsuperscript{146–149}, and certain members of the complement system\textsuperscript{150} are believed to dramatically increase the risk for SLE. These and other associations have been supported by work in mice. Mice lacking C1q develop ANA with multiple specificities and glomerulonephritis\textsuperscript{143}. \textit{Trex1}-deficient mice develop a severe form of autoimmunity and ultimately succumb to inflammatory myocarditis\textsuperscript{151,152}.

Some likely environmental triggers of SLE have been identified, though this remains an area in need of investigation. Infection with Epstein-Barr virus was suggested by Harley and others as a possible environmental trigger for SLE, after recognized a striking sim-
ilarity between a short peptide within the splicesome, a common target of lupus ANAs, and an EBV peptide\textsuperscript{153}. A compelling association study supported this hypothesis—in a large study of adolescents, 99% of SLE patients had developed antibodies toward EBV, indicating prior infection, whereas anti-EBV antibodies were detected in only 70% of controls\textsuperscript{154}. SLE has also been associated with an increased sensitivity to sunlight; in controlled studies, patients developed erythema upon exposure to significantly lower levels of ultraviolet light and the erythema lasted longer compared with control subjects\textsuperscript{155}. A role for hormones in lupus etiology has long been suspected, especially since women are at a disproportionately higher risk for developing SLE. The current literature lacks the strength needed to confirm this hypothesis; however, hormones may indeed play a role in existing patients. Pregnancy and the postpartum period, for example, have been associated with increases in disease flares\textsuperscript{156,157}.

1.3.3 Type I IFN in SLE

Aberrant overproduction of type I IFN is a prominent feature associated with the development of SLE\textsuperscript{158–161}. Gene expression profiling of SLE patient peripheral blood mononuclear cells (PBMCs) identified the existence of a type I IFN gene signature which was found to correlate with disease severity\textsuperscript{162,163}. Furthermore, the use of IFN to treat hepatitis C virus and cancer has been associated with the induction of SLE\textsuperscript{164–166}, and the administration of IFN induced earlier onset, lethal disease in (NZB x NZW) F1 lupus-prone mice\textsuperscript{167}. Moreover, genetic deletion or antibody blockade of IFNAR in several mouse strains prone to spontaneous development of lupus-like diseases including (B6.Nba2 x NZW) F1, NZB, and BXSB.Yaa, or in pristane-induced lupus resulted in nearly complete abrogation of disease, demonstrating a vital role for type I IFN in murine models of SLE\textsuperscript{168–171}. Together, these findings strongly suggest type I IFN functions to promote human SLE pathogenesis and/or pathology.
Elevated levels of free nucleic acids and their uptake by APCs is one mechanism thought to induce the production of type I IFN. APCs, including DCs, may be exposed to higher levels of nucleic acid, due to reductions in serum DNAse I activity\textsuperscript{172}, which normally protect against sterile inflammation by degrading nuclear DNA released from apoptotic and necrotic cells. In fact, some SLE patients have been found to have mutations in DNAse I\textsuperscript{173}, and mice deficient in DNAse I develop an autoimmune disease resembling SLE\textsuperscript{174}. Some reports indicate that immune complexes (ICs), which are abundant in SLE patients, contain self DNAs and RNAs, and their uptake by DCs (particularly pDCs) may induce type I IFN via the engagement of TLR9 and TLR7, respectively\textsuperscript{175–177}. Toll-like receptors have been implicated in many studies, whether or not ICs are critical\textsuperscript{178–183}, and notably autoimmunity in the BXSB/Yaa lupus prone mouse has been causally linked to the translocation and duplication of TLR7\textsuperscript{184–186}.

Cytosolic nucleotide sensing has often been hypothesized to contribute to IFN production in SLE, although relatively little has been done to test these hypotheses. Certainly, the mouse studies on Trex1-deficiency show that cytosolic nucleotide sensing can mediate autoimmunity, and are highly suggestive that nucleotide sensing could be involved in SLE pathogenesis. The Ifi200 family of IFN-inducible genes, which includes the cytosolic DNA sensors Aim2 and Ifi204, has been linked to disease in the (NZB x NZW) F1 mice, is notably contained within the NZB-derived Nba2 locus, and is thought to play roles in those and additional mouse models of SLE\textsuperscript{187}. Indeed, we found that STING promoted autoimmunity in the bm12 inducible model of SLE (Chapter 5 of this dissertation), further implicating a possible role for cytosolic nucleotide sensing in SLE\textsuperscript{130}. Additionally, recent clinical studies revealed that activating STING mutations can induce inflammatory diseases with lupus-like features in patients, including high serum levels of type I IFN, autoantibodies, and a classic malar rash\textsuperscript{188,189}. Although these patients were diagnosed with a novel disease, STING-associated vasculopathy with onset in infancy (SAVI), and
not SLE, these case reports support a possible role for cytosolic nucleotide sensing and indeed STING in SLE pathogenesis.

Although many studies have assessed the necessity for IFN in SLE, what signals induce IFN, and what cell types produce IFN, relatively few have sought to characterize its mechanism of action in SLE. In 2001, Blanco et al. found that monocytes derived from patient peripheral blood differentiated into DCs, and efficiently presented antigen from dying cells to CD4 T cells. Furthermore, the serum of patients with SLE, but not control serum, could induce healthy donor monocytes to differentiate into DCs, and this process was dependent upon type I IFN. This led the authors to conclude that one role of IFN in lupus may be the constant induction of DCs\textsuperscript{190}. The role for type I IFN sensing by B cells may also be important in SLE, especially since type I IFN is known to promote B cell activation, class switching, and support the generation of antibody secreting plasma cells\textsuperscript{87,88,191}. However, no published reports have tested the role for IFN sensing specifically by B cells, an area of interest our lab is currently pursuing. In Chapter 5 of this dissertation, we provide the first study into the possible roles of IFN sensing by CD4 T cells in SLE. CD4 T cells that differentiate into T follicular helper cells (Tfh) deliver indispensable help to activated B cells in germinal center reactions. In the bm12 cGVHD model of SLE, transferred bm12 CD4 T cells all differentiate into Tfh. We found the ability of bm12 Tfh to sense type I IFN was critical to disease progression, and that the high levels of type I IFN induced in these animals provided Tfh protection from NK cell attack.

1.4 Cellular metabolism

The concept of cellular metabolism has been explored by researchers across multiple disciplines, revealing a complex network of molecular interactions which provide energy in the form of ATP, but also the basic components of cellular structure including nucleotides, amino acids, and lipids (Figure 6, left panel). Advances in the technology used
Figure 6: Cellular metabolism. A network depicting the integrated metabolic pathways immune cells use at various points during development and upon activation (left panel). A diagram highlighting the distinct relative contributions of the different pathways to different naïve and mature T cell subsets (right panel). Reprinted under the terms of the Creative Commons Attribution License from Buck M, O’Sullivan D, and Pearce E: Journal of Experimental Medicine 194, copyright 2015.

to interrogate these metabolic pathways, as well as an advanced understanding of various immune cells and the ability to purify them, has led to a recent flood of studies on the cellular metabolism of various immune cell subtypes. T cells have been particularly well studied. Naïve T cells rely predominantly on oxidative phosphorylation (OXPHOS) or fatty acid beta oxidation (FAO) with minimal utilization of glycolysis. Upon activation, T cells—in large part—undergo metabolic reprogramming to become much more dependent on aerobic glycolysis, FAO (Figure 6, right panel), and glutaminolysis. Glycolysis is not strictly required for the proliferation and differentiation of activated T cells, but it has been recognized as critical to their effector function. The switch to aerobic glycolysis, which was first described 60 years ago by Otto Warburg, who was studying cancer cells at the time, is not thought to better meet the energetic needs of...
the cells—especially considering the much higher efficiency of ATP production via the TCA cycle—but rather it is thought to meet these needs adequately while also providing the molecular substrates required in vast quantities during rapid proliferation and production of effector molecules, especially by shuttling glucose derivatives through the pentose phosphate pathway\textsuperscript{198}. Glutaminolysis is another major metabolic pathway upregulated in activated T cells which serves multiple purposes\textsuperscript{194}. It can contribute to energy production by converting glutamine into α-ketoglutarate, a TCA cycle intermediate. Alternatively, glutaminolysis can also contribute to the production of nucleic acids or amino acids. One notable exception to the paradigm of a metabolic switch toward aerobic glycolysis and glutaminolysis upon activation is regulatory T cells, which maintain their reliance on OXPHOS and FAO and do not switch to aerobic glycolysis\textsuperscript{199}. The metabolic program of T follicular helper cells has remained elusive until now. In Chapter 7, we thoroughly characterized the metabolic program of Tfh using cells isolated from the bm12 cGVHD model of SLE, and determined that these cells utilize both aerobic glycolysis and FAO, but not glutaminolysis. The metabolic program of B cell subsets \textit{in vivo} has not been well defined. However, \textit{in vitro} studies have shown increased glycolytic rates within B cells activated by LPS\textsuperscript{200} or anti-CD40 + anti-IgM\textsuperscript{201} stimulation. In Chapter 7 we also characterize the metabolic programs of plasmablasts and germinal center B cells directly \textit{ex vivo}, and demonstrate that these cell types are completely dependent upon glycolysis, with very little utilization of FAO or glutaminolysis.

1.5 Dissertation Aims

The immune system is a complex and powerful tool an organism wields to defend itself from invaders. Given its strength, the immune system is subject to tight control. Minor perturbations in signals that promote inflammation can lead to profound tissue destruction. This can be deleterious or helpful, depending on the context—immune responses to tumor antigens can help eliminate deadly tumors, whereas aberrant responses to healthy
tissue can cause debilitating autoimmune diseases. The subsequent chapters in this dissertation will explore novel mechanisms that regulate immunity toward self, with the goal of uncovering new targets for the treatment of certain cancers and autoimmune diseases. Briefly, in **Chapter 5**, we examine how STING-mediated DNA sensing promotes antitumor and autoimmune responses to dying cells via the induction of type I IFN. In **Chapter 6**, we further dissect the role of type I IFN autoimmune disease, and show that IFN protects Tfh from NK cell attack in the bm12 model of SLE. Finally, in **Chapter 7**, we originally hypothesized that type I IFN sensing may control CD4 T cell metabolism, but ultimately we examined cellular metabolism more globally in bm12 autoimmunity and found that Tfh, GC B cell, and plasmablasts all relied heavily upon glycolysis, such that inhibiting glycolysis completely abrogated disease.
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Chapter 2: Melanoma-infiltrating Dendritic Cells: limitations and opportunities of mouse models*

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Melanoma-infiltrating dendritic cells: Limitations and opportunities of mouse models.

Abstract
The infiltration of dendritic cells (DC) into melanomas has been suggested to play a tum-origenic role due to their capacity to induce tumor tolerance and promote angiogenesis and metastasis. However, studies have also shown that tumor-infiltrating DC (TIDC) induce anti-tumor responses and could therefore be targeted in a cost-effective therapeutic approach to obtain patient-specific DC that presenting relevant tumor-antigens, without the need for \textit{ex vivo} DC generation or tumor antigen identification. Unfortunately, little is known about the composition, nature and function of TIDC in human melanoma. The development of mouse melanoma models has contributed greatly to the molecular understanding of melanoma immunology in mice, but many questions on TIDC remain to be answered. Here we discuss the current knowledge of melanoma TIDC in various mouse models with regard to its translational potential and clinical relevance.

Introduction
The incidence of cutaneous malignant melanoma has been steadily increasing over the last decades. While complete surgical excision yields high 5 year survival for patients with localized tumors with less than 0.75 mm depth, the outcome is poor for patients with a greater depth of tumor invasion or metastases. Consequently, the development of novel therapeutic approaches is of great importance. Interestingly, melanomas are relatively immunogenic tumors and sensitive to CTL mediated lysis. As DC are the main antigen-presenting cell (APC) population capable of inducing CTLs, DC transfer, DC targeting and in situ DC induction, recruitment, and/or activation have become promising immunotherapeutic strategies. Topical or intratumoral administration of DC-activating agents—including IFNa, bacillus Calmette-Guerin (BCG), or purified toll-like receptor (TLR) ligands such as Imiquimod— are recommended as treatment options for patients with in-transit melanoma metastasis\textsuperscript{1-5}. While this approach is relatively successful in cutaneous metastases, efficacy is still poor in subcutaneous metastases. Improved understanding of the type, nature
and functionality of TIDC could lead to novel and more effective therapeutic approaches. To circumvent the ethical and TIDC availability constraints associated with human research, various animal models for melanoma have been established including models in *Xiphophorus, Danio*, guinea pigs, opossum, and small rodents, all of which have unique advantages and disadvantages. The relevance of the selected model depends on the questions to be answered and how closely the model resembles the histological, immunological and metastatic pattern observed in humans. To date, most work is performed in mice due to the availability of genetically modified mice, insights into mouse immunology, pathology and physiology, and the plethora of mouse-specific research tools. Here we will briefly review the current knowledge of TIDC obtained in the most common mouse melanoma models and the insight it has provided into the human disease.

**Selection of mouse model for melanoma tumors**

Melanoma models are generally divided into 3 different groups based on research focus; xenograft models, which allow for the study of tumor cell behavior; transplantation models to study melanoma immunology, and genetically modified animal models that focus on melanomagenesis. Strictly chemical carcinogen-induced melanoma models have decreased in popularity as they have relatively low relevance to human disease and therefore will not be discussed further.

*Xenograft models*: Orthotopic or ectopic transplantation of human cancer cells or solid tumors into immune compromised mice. The primary advantage of these models is the preservation of human cancer cell behavior including metastatic potential and preference; however, the absence of a complete immune system does not allow for full replication of interactions between tumors and immune cell subsets. While DC function is relatively normal in some immune compromised strains, various strains—including those on the NOD.Cg-Prkdc<sup>scid</sup>il2rg background—have defective DC development and function. In
addition, human tumor-derived mediators might affect mouse DC recruitment, retention, development and function different than the mouse homologues. The more recent development of human melanoma models in humanized mice\textsuperscript{10-12} circumvents these issues and provides an intriguing platform for clinically relevant TIDC studies.

\textit{Syngeneic transplantation models} have been around since the identification of the CloudmanS91 melanoma in BDA/2 mice, Harding-Passey melanoma in Balb/c x DBA/2F1 mice and B16 melanoma in C57BL/6 mice\textsuperscript{13-16}. The B16 melanoma is currently the most widely used model and has the advantage that it expresses at least 5 homologues of the best characterized human melanoma antigens (gp100/pmel17, MART-1/MelanA, tyrosinase, TRP-1/gp75 and TRP-2/DCT)\textsuperscript{17}, it is immunogenic and it has metastatic potential. The main drawback of this model is the rapid growth of the primary tumor that leads to problems with vascularization, necrosis, and swift mortality that precludes the assessment of prolonged tumor burden on TIDC behavior. Nevertheless, most TIDC studies have been performed in B16 melanoma models.

\textit{Genetically engineered models (GEMM):} The identification of genetic and epigenetic abnormalities in human melanomas has led to the development of genetically engineered mice with heritable predispositions to the development of melanoma. The use of (tissue-specific) expression of oncogenes including Ret, mutant forms of (N/K/H)Ras and BRaf, and HGF/SF with or without back-crossing to susceptible backgrounds (Ink4a/Arf\textsuperscript{f/f}, P53\textsuperscript{\textminus/\textminus}, p19\textsuperscript{\textminus/\textminus}, p16\textsuperscript{\textminus/\textminus}, Cdk4\textsuperscript{R24C/R24C}, etc.) has yielded models with different latency, penetrance of melanoma, and metastatic potential (reviewed in\textsuperscript{18,19}). Although melanocyte distribution differs between mouse and human, these models have great clinical relevance as they are based on genes known to be involved in the genesis and progression of human melanoma and can be easily combined with relevant environmental triggers such as UV irradiation to accelerate melanoma incidence. Only recently the field has begun to
use these models for TIDC studies.

**Dendritic cells**

DC are a heterogeneous population in terms of origin, morphology, phenotype and function. DC are derived from common myeloid and lymphoid precursors and rely heavily on Flt3L and/or GM-CSF for their development\textsuperscript{20-22}. DC express MHC class I and class II molecules together with a wide variety of membrane associated positive (CD40, CD80, CD86, CD137L, CD70) and negative (PDL1, PDL2) costimulatory molecules. In addition DC can produce a broad range of soluble pro-and anti-inflammatory mediators, including cytokines and chemokines. T cells interacting with DC via cognate TCR-peptide-MHC complexes will undergo apoptosis, anergy, or develop a regulatory phenotype if the degree of negative stimulation outweighs that of positive costimulation\textsuperscript{23}. If the positive sum of the signals surpasses an intrinsic threshold T cells will undergo proliferation, differentiate and acquire effector functions. Immature DC display great phagocytic capacity, relatively poor antigen presenting capacity and low levels of positive costimulatory molecules. Upon activation via innate receptors (TLRs, NLRs), proinflammatory cytokines or cross-linking of CD40, DC mature, reduce their phagocytic capacity, increase antigen presenting capacity, upregulate costimulatory molecules and cytokine production, and migrate to draining (lymphoid)areas where they interact with T cells\textsuperscript{24-26}.

Cells with DC characteristics have been repeatedly described in human melanoma samples. Depending on the study, the markers used, the localization and maturation status, DC infiltration is linked with a positive\textsuperscript{27-30} or negative\textsuperscript{31} prognostic outcome. The discrepancy in outcomes can be contributed to differences in clinical stages, the use of primary versus metastatic lesions, as well as use of markers that are non-specific or restrictive to a subpopulation of DC\textsuperscript{32}. 
Studies in several other tumor systems indicate that tumors inhibit dendropoiesis, decelerate DC differentiation and maturation, induce functional deficiencies, and accelerate cell death in DC or precursors\textsuperscript{33, 34}. Maintenance of an immature phenotype or promotion of tolerogenic phenotype could lead to anergy/deletion of tumor-specific T cells and the induction of cells with immunosuppressive functions such as Tregs. Slowed differentiation could contribute to the accumulation of myeloid-derived suppressor cells that are derived from precursors that under steady state conditions would differentiate into DC, macrophages and neutrophils\textsuperscript{35}. In addition, immature and pre-DC have been suggested to promote angiogenesis through the secretion of growth factors (i.e. Vegf) that directly act on the endothelium, secretion of mediators that enhance sensitivity of endothelial cells to growth factors\textsuperscript{36, 37}. Other experimental studies even suggest that DC precursors may undergo endothelial transdifferentiation or provide a scaffold for subsequent lining by endothelial cells\textsuperscript{38}.

**DC populations in mouse and man**

Recent genomic and proteomic approaches have discovered significant similarities between human and mouse DC populations\textsuperscript{39-43} thereby strengthening the relevance of TIDC research in mouse melanoma models. While several aspects of localization, surface marker expression, TLR expression, phagocytic potential and antigen presenting capacity are relatively comparable between some mouse and human DC subsets, they are not perfect matches and in some cases the equivalent populations are absent. We will briefly describe the mouse and human DC populations in the following sections.

**Mouse DC populations**

Under steady state conditions mouse DC express CD11c and MHC class II and are divided into plasmacytoid DC (pDC) and conventional DC (cDC)\textsuperscript{21}. pDC express intermediate levels of CD11c, and high levels of CD45RA (B220), PDCA1 (CD317) and TLR1,2,4,
7, and 9, and play an important role in infection due to their capacity to produce large amounts of type I IFNs\textsuperscript{44}. Antigen presentation by pDC is thought to be relatively poor\textsuperscript{45}. Conventional DC are further divided into blood-derived resident DC and migratory DC. Blood-derived resident cDC are present in lymphoid tissues and encompass: (\textit{i}) CD-11c\textsuperscript{high}MHCII\textsuperscript{+}CD8a+CD205+SIRPa-CD11b- (CD8aDC) that express XCL1, Clec9A and often CD103. CD8aDC express mRNA for most TLRs except 7 and 5 and are characterized by high TLR3 expression\textsuperscript{44}. These DC have the greatest potential to prime CTLs to cell-associated antigens via cross-presentation but have relatively low CD4 T cell activation potential\textsuperscript{46}; (\textit{ii}) CD11c\textsuperscript{high}MHCII\textsuperscript{+}CD8a-33D1+SIRPa+CD11b+ (CD11bDC), that predominantly activate CD4 T cells, have poor cross-presentation capacity and express most TLRs except TLR3; (\textit{iii}) CD11c\textsuperscript{high}MHCII\textsuperscript{-} cells that lack CD8a, CD4 and CD11b (generally termed “double” or “triple” negative DC) that may or may not express XCL1, Clec9A, and TLR3 and CD103. DC subsets in this population have been shown to potently prime both CD4 and CD8 T cells to cell-associated antigens\textsuperscript{47-49}.

Migratory DC can be found in many organs and migrate upon activation into draining lymphoid areas\textsuperscript{22, 50, 51}. As this review focuses on melanoma we will limit our description to skin-resident DC. Various populations of DC have been described in non-inflamed mouse skin. Langerhans cells (LC), CD11b+CD207+CD103- DC reside in the epidermis and express TLR2,4, and 9 but not 7\textsuperscript{52}. Cross-presentation of cell-associated antigens in LC has not been demonstrated but LC have the capacity to cross-present antigen associated with TLR ligands\textsuperscript{53, 54}. In the dermis CD11b+CD207- dermal DC (dDC) represent the major DC subset whereas, CD207+CD103+ dDC and CD207-CD11b- dDC represent ~20% of the dDC. dDC express most TLRs and especially the CD103+ dDC population has been associated with the cross-presentation of cell-associated antigens\textsuperscript{52, 55, 56}.

The sharing of multiple markers between DC subsets and their changes in expression
levels upon activation complicate the identification of DC subsets. Environmental cues associated with inflammation or tumors can change their surface characteristics, behavior or capacities adding another layer of complexity to identification of DC.

**Human DC populations**

Like mouse DC, human DC are generally divided into pDC and cDC. pDC are lineage negative (lin-) CD11c-HLA-DR+CD123+ BDCA2/4+ and express high levels of TLR 7 and 9. In contrast to mouse pDC human pDC have been shown to cross-present cell-associated antigens. cDC are further divided based on their expression of BDCA-1 (CD1c) and BDCA-3 (CD141). The BDCA-1+ DC has similarities with the mouse CD11b DC as it expresses SIRPa and CD11b, strongly responds to TLR1 and 6 stimuli and promotes induction of CD4 T cell responses. The BDCA-3+ DC has strong similarities with mouse CD8aDC and expresses Clec9A, XCR1, and high levels of TLR3. Recently it was shown that the BDCA-3 DCs have the greatest capacity for cross-presentation within the human DC repertoire.

LC are the only DC in healthy epidermis and comprise 2-8% of all epidermal cells. LC express high levels of CD1a, MHC class II, CD207, EpCAM, low levels of CD205, and DC immunoreceptor (DCIR). Both DCIR and CD205 are associated with antigen uptake and induction of antigen-specific T cell responses. LC express mRNA for TLR 1,2,5,6 and 9 (not 4 or 7/8). The number of dDC populations described in humans has recently been expanded. The major dDC population is BDCA-1/CD1c+, with most of these cells expressing CD11c and about 50% of the CD1c+ population expressing CD1a. CD1c dDC represent about 10% of all the CD11c+ dDC and demonstrate superior cross-presentation of soluble antigen compared to other DC populations. Most dDC express mRNA for TLR1,2,4,8,10 but the exact distribution among subsets needs further delineation.
Human melanoma TIDCs

Melanoma-infiltrating DC have been found in primary and metastatic lesions and encompass a broad spectrum of DC-like cells, including CD207+ LC, pDC, and CD1a+ DC (Table 1)\(^{27, 28, 31, 67-69}\). Due to differences in patient material, the low frequency of TIDC, the use of ambiguous analytical markers, and especially approaches that limits the number of analytical markers there is little consensus on the exact composition of the TIDC population\(^{32}\). However, there is a general agreement that the frequency of TIDC is higher in the peritumoral area than the intratumoral area and that the TIDC with the most mature phenotype (DC-LAMP+/CD83+/fascin+) tend to reside in the peritumoral area\(^{27, 31, 67, 68}\). It is thought that immature DC enter the tumors via the vasculature and—following further differentiation and activation—migrate towards the tumor edge. From there, the DC either locally form T cells clusters or continue migrating towards the draining LN where they interact with T cells. The relationship between the presence and location of different TIDC subsets and clinical outcome remains a puzzle as it not only depends on the type of the TIDC, but also their functionality and interactions with other cells, all aspects that are currently poorly understood.
Mouse melanoma in TIDCs
While mouse models have the advantage of ample tumor material that allows for easy selection of primary from metastatic lesions and selection of different tumor development stages, there is surprisingly little consensus in the field on TIDC frequency, composition, and function. Some of these discrepancies result from the use of different model systems or strain backgrounds. When we compared two xenograft models, 3 syngeneic transplantation models and 2 GEMMs we observed significant differences in TIDC frequency (data not shown) and composition between models (fig 1a). The highest frequency of TIDC was seen in syngeneic transplant models while GEMMs had significantly less TIDC. However, GEMMs showed greater diversity among the TIDC, with marked infiltration by pDC, LC and dDC. Xenografts showed the least variety, completely lacking LC and dDC while in syngeneic transplant models an occasional dDC (CD207+ EpCAM-) cell was found. Although a full comparison between studies is hard to make as not all studies used the same set of markers, a review of mouse melanoma literature showed similar findings in the different model systems (table 2).

The differences in TIDC composition between models and species highlight the importance of melanoma model validation for each type of study. While all models have significantly contributed to melanoma immunology knowledge, pre-clinical DC targeting studies would benefit from models that more accurately resemble TIDC composition seen in patients.

Mouse TIDC activation status
As in human melanoma, mature mouse TIDCs tend to reside in the peritumoral areas and total TIDCs seem to increase upon disease progression (fig.1b,c). Most studies assessing mouse TIDC activation and maturation status use flowcytometric analysis of CD11c+ cells from the entire tumor. Consequently, reports show a biphasic distribution
of the maturation markers CD40/CD80 and CD86\textsuperscript{70, 71, 73, 74}. Analysis after separation of the peritumoral and intratumoral areas of B16/F10 melanomas replicate the histological observations, showing significantly more mature TIDC in the peritumoral area compared to the intratumoral area (fig. 1d).

It is thought that the tumor environment promotes the recruitment of DC precursors and immature DC, but little is known on the ability of melanomas to support in situ differentiation\textsuperscript{76}. Diao et al showed that adoptively transferred immediate cDC precursors (Lin-CD-11c+MHCII-) were recruited to B16/F10 tumors where they proliferated and differentiated into cells with T cell priming capacity \textit{in vitro}, suggesting at least partial acquisition of DC-like functions\textsuperscript{77}. On the other hand, \textit{in vivo} data from Fainaru \textit{et al.} demonstrated that recruitment of immature DC promoted angiogenesis and tumor growth by enhancing

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<th>Table 2. Mouse melanoma TIDC characteristics</th>
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<td><strong>Model</strong></td>
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<td>Scl MV3 xenograft\textsuperscript{19}</td>
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<td>B16/F10, s.c.\textsuperscript{23}</td>
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<td>Tyr-N-Rasp\textsuperscript{36} DMBA/CH\textsubscript{12}O\textsuperscript{24}</td>
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Fig. 1. TIDC composition, location and maturation. (A) Composition of CD45+ Lin- CD11c+MHCI+ TIDC in different melanoma models. Tumors (400-600mm2) were harvested from Nu/J nude mice (MV3, A375; n=6/group), BDA/2 mice (CloudmanS91; n=5/group) and C57BL/6 mice (B16F1 and B16/F10 n=9/group), digested according to standard protocols106, 107, and analyzed by multicolor flowcytometry. β-actin::LSL-KRAS mice crossed onto a Tyr::CreERT2 background108 were repeatedly treated with Tamoxifen between 1 and 2 months of age. Tumors were harvested 4-6 months later (1-2 melanomas/mouse, 3 mice). MT::Ret transgenic mice109 were aged and spontaneous melanomas were harvested when they reached 200-300mm² (1-3 melanomas/mouse, 4 mice). (B) Representative confocal image of TIDC localization in a snap-frozen B16/F10 tumor seven days after s.c. injection of 2x10⁶ tumor cells in C57Bl/6 mice. CD11c, Red; CD11b, Green, nuclei, DAPI. (C). Relation between total TIDC frequency within the TIL and B16/F10 melanoma size in C57Bl/6 mice as determined by flowcytometry. (D) Differential expression of maturation markers on peritumoral and intratumoral TIDC. B16/F10 tumors (≈ 600mg, n=4-5/group) were harvested and the peritumoral area was collected using ophthalmic blades and peritumoral and intratumoral tissues were processed according to standard protocols106, 107. CD40, CD80 and CD86 expression were determined in the live CD45+ Lin- CD11c+ MHCI+ populations by multicolor flowcytometry.
endothelial cell migration and subsequent formation of vascular networks\textsuperscript{78}. Moreover, depletion of CD11c\textsuperscript{+} cells in CD11c-diphtheria toxin receptor (DTR) transgenic mice significantly reduced the tumor mass of intraperitoneally injected B16/F10 melanoma cells\textsuperscript{78}. While other tumor models suggest roles for endothelial like differentiation of DC-precur-
sors, Vegf-A, b-defensin, bFGF, and TGF\textsubscript{b1} in this type of processes, the mechanistic underpinning of DC-supported vasculogenesis in melanoma has not been clearly estab-
ished\textsuperscript{79, 80}.

**Mouse TIDC functionality**

In order to be a *bona fide* APC, DC will need to acquire antigens through one of the phagocytic pathways, process and present antigen, and communicate with T cells locally or upon migration to draining areas. Studies injecting beads into tumors revealed that a sizable fraction of the TIDC acquired one or more beads, indicating that that particulate uptake mechanisms were relatively intact\textsuperscript{71, 73}. However, Gerner *et al.* showed decreased uptake of intratumoral injected proteins compared to dDC from healthy tissue\textsuperscript{73}. Separating peritumoral and intratumoral TIDC, we found that *in vitro* uptake of protein and apoptotic cell material was higher in the peritumoral TIDC than the intratumoral TIDC (fig. 2a). Similar observations were made when peritumoral and intratumoral TIDC were analyzed 4 hours after *in vivo* intratumoral injection. Interestingly, co-administration of LPS decreased phagocytic uptake in the peritumoral TIDC but not the intratumoral TIDC (fig. 2b).

Most studies show decreased CD4 and CD8 T cell activating capacity of TIDC isolated from antigen expressing tumors or upon pulsing with antigen *in vivo* \textsuperscript{70, 73, 74}. However, sev-
eral studies indicate potent T cell priming capacity *in vitro* or *in vivo* \textsuperscript{71, 77, 81}. This discrepan-
cy can be partly explained by divergent subset composition, DC location, DC maturation state, isolation methods, and *in vitro* functional assessment protocols. When separating
TIDC based on GR1 expression, Diao et al. showed that GR1+ expressing TIDC had increased IL-10 production and reduced CD8 and CD4 T cell priming capacity compared to GR1- TIDC when loaded in vitro with antigen. In addition, CD8 T cells primed by GR1+ TIDC demonstrated significantly reduced cytokine production. Gerner et al. suggested that the TIDC’s decreased capacity for CD4 T cell activation resulted predominantly from reduced antigen uptake as antigen processing and presentation was unaltered. To
further dissect the antigen presenting and T cell priming/activating potential we isolated peritumoral and intratumoral TIDC from B16-OVA bearing mice and cultured them with an OVA\textsubscript{257-264}-specific reporter cell line (B3Z) and CFSE labeled OVA\textsubscript{257-264}-specific OT-1 T cells. We included brefeldinA in the isolation procedure to prevent turnover of MHC-I-peptide complexes while preserving the TIDC’s maturation state\textsuperscript{82}. Importantly, significant antigen presentation was observed only when brefeldinA was present during the isolation period, illustrating the importance of optimizing and standardizing TIDC isolation protocols. The total TIDC fraction poorly activated the B3Z cells (fig.2.c), suggesting a low frequency of OVA\textsubscript{257-264}-MHC complexes. Consequently, total TIDC-mediated OT-1 T cell activation and proliferation was poor as determined by CD69 upregulation and CFSE dilution (fig. 2.d,e). However, peritumoral TIDC displayed higher frequencies of OVA\textsubscript{257-264}-MHC complexes, and activated and induced proliferation in a sizable frequency of OT-1 T cells (fig. 2.c-e). Intratumoral TIDC had less OVA\textsubscript{257-264}-MHC complexes and activated OT-1 T cells without inducing proliferation. This lack of proliferation could be restored by addition of IL-2 but not blockade of IL-10 or TGFβ, suggesting the induction of T cell non-responsiveness. Importantly, treatment of peritumoral TIDC with TLR4 or 9 ligands significantly increased their potential to induce T cell proliferation while the same treatment did not improve that of intratumoral TIDC (data not shown). Together these observations show that differences in isolation protocols, TIDC subset criteria, and functional assays significantly complicate the comparison between studies and the extrapolative value of the findings.

While many studies indicate a decrease in maturation and functionality of melanoma TIDC, the mechanisms that underpin the changes in APC functions are still unclear. Increased expression of immunosuppressive cytokines and membrane-associated molecules by TIDC has been implicated in TIDC dysfunction\textsuperscript{72, 83}. Other tumor models suggest that tumor-derived cytokines or reduction in TIDC sensitivity to innate signals prevents
maturation, migration and thereby TIDC function. However, prolonged TIDC retention and maintenance of an immature phenotype was recently linked to lipid accumulation associated with increased scavenger receptor A expression and LXR-a mediated CCR7 down regulation. Norian et al. linked TIDC dysfunction to increased L-arginine metabolism in a spontaneous model of mammary carcinoma. More importantly, Zhang et al. linked the reduced B16 melanoma TIDC functionality to decreased metabolism resulting from increased SOCS3-pyruvate kinase M2 interactions. This observation and those in other tumor models clearly exemplify that the focus on basic immunological assays and parameters has become too restricted to determine the mechanisms of TIDC dysfunction. For a full appreciation of the developmental and functional defects in TIDC research approaches will have to incorporate disciplines beyond classical immunology.

**Scientific and therapeutic considerations**

Mouse models have been extensively used to test topical therapeutic therapies. Comparable to human melanoma, injections with GM-CSF, IFNa, Imiquimod, or BCG have shown various degree of clinical success in mice. In many of these approaches either increased numbers of DC or enhanced DCs maturation was observed in the tumor or tumor draining LN. In addition, other purified TLR ligands including poly(I:C), CpG, LPS, with or without additional immune-interfering therapies have been used successfully. Intratumoral administration of crude bacterial products, cytokines and stimulatory molecules expressed by viral vectors, microspheres or nanoparticles are well established in mouse models but have not been translated into the human system. While all these therapies were suggested to target TIDC or support TIDC functions, it is likely these approaches only partly activated TIDC as some targeted specific DC populations that are absent or poorly represented in the tumor or receptors that are poorly expressed in TIDC or rendered non-functional by the tumor. In those cases, it is more likely that other cells in the tumor environment are stimulated in a manner that promotes a DC activating/
restoring microenvironment.

In order to improve the clinical relevance and translational potential of mouse melanoma models for the design, optimization, and identification of novel therapeutic interventions that target TIDC either directly or indirectly we will have to overcome several hurdles. The further identification and characterization of human TIDC will be critical to identify and validate the best mouse models for each type of study. Eventually, the panel of specific markers used in both human and mouse DC studies must be standardized, even as investigators continue to discover new markers and DC populations\textsuperscript{32}. Furthermore, optimization and standardization of protocols for TIDC isolation and functional assessments will be essential for study-to-study comparisons and extrapolation of data between species and laboratories.

However, the greatest gain may be made by increased collaboration between different research disciplines that can provide better mouse models such as humanized mice for xenograft transplantation studies, GEMMs with TIDC patterns that resemble human TIDC profiles at different stages of disease, and new analytical platforms for extended TIDC analyses.

Although it is unlikely that mouse melanoma models will ever completely recapture the complexity of human melanoma in clinical situations, we have currently only scratched the surface of the mouse model potential in the analysis of TIDC dysfunction and therapy. Combining and integrating current models and standardizing analysis methods and expanding the disciplines of research will be instrumental to significantly improving the clinical relevance of mouse models and the identification of novel therapeutic targets.
Acknowledgments

This work is supported by NIH grant CA138617 (NCI) and AI079545 (NIAID) to EMJ.

Conflict of Interest

The authors have no conflict of interest.

Ethical Statement

All animal experiments were performed in strict accordance with animal protocols approved by the Institutional IACUC at CCHMC and LIAI that operate according the guidelines by the Association for Assessment and Accreditation of Laboratory Animal Care International and the recommendations in the Care and Use of Laboratory Animals of the National Institute of Health.
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Chapter 3: Dendritic cells in systemic lupus erythematosus: from pathogenic players to therapeutic tools*

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Abstract

System lupus erythematosus (SLE) is a multifactorial systemic autoimmune disease with a wide variety of presenting features. SLE is believed to result from dysregulated immune responses, loss of tolerance of CD4 T cells and B cells to ubiquitous self antigens, the subsequent production anti-nuclear and other auto-reactive antibodies. Recent research has associated lupus development with changes in the dendritic cell (DC) compartment, including altered DC subset frequency and localization, over-activation of mDCs and pDCs, as well as functional defects in DCs. Here we discuss the current knowledge on the role of DC dysfunction in SLE pathogenesis, with the focus on DCs as targets for interventional therapies.

Introduction

Systemic Lupus Erythematosus is a chronic autoimmune inflammatory disease that affects multiple organ systems, prototypically characterized by high levels of circulating autoantibodies and glomerulonephritis. Clinical symptoms also encompass musculoskeletal, dermatological, neuropsychiatric, pulmonary, gastrointestinal, cardiac, vascular, endocrine and hematologic manifestations. The reported incidence of SLE nearly tripled over the last 40 years due to improved detection of mild disease¹, but SLE prevalence estimates still vary considerably, ranging from 10 to 150 cases per 100,000, depending on geography, race and gender²-⁵. In the United States, the prevalence of SLE is higher among Asians, African Americans, African Caribbeans, and Hispanic Americans compared with Caucasians ⁶-⁹. Similarly, in European countries SLE prevalence is higher among people of Asian and African descent⁶-⁹. Interestingly, SLE is reported infrequently in Africa¹⁰. Mortality rates are relatively low, at 10-50 per 10,000,000 of the general population and show correlation with renal and cardiovascular manifestations as well as infection¹¹. Importantly, patients commonly experience profound fatigue and joint pain, and a decreased quality of life¹²-¹⁵.
The precise etiology of SLE remains unclear, and likely varies, considering its diverse clinical manifestations. Nevertheless, SLE is believed to result from dysregulated immune responses, loss of tolerance of CD4 T cells and B cells to ubiquitous self antigens, and the subsequent production anti-nuclear and other auto-reactive antibodies. This dysregulation is associated with high serum levels of type I IFN, observed in greater than 70% of patients\textsuperscript{16,17}. Current “standard of care” treatments encompass high-dose corticosteroids, anti-malarials, and immunosuppressive drugs that are associated with significant adverse side effects. As these treatments suppress symptoms and do not cure the disease, new therapies are needed.

Contemporary treatment strategies have been shifting emphasis toward the identification of immunological processes, both soluble and cellular, in order to redirect aberrant immune responses. Dendritic cells have recently been recognized as important players in the induction and progression of autoimmune diseases, including SLE\textsuperscript{18}. Human and mouse studies have associated lupus development with altered DC subset frequency and localization, over-activation of mDCs or pDCs, and functional defects in DCs\textsuperscript{19,20}.

However, full dissection of the relative contribution of the causes and the consequences of the dysfunctionality in the different DC subpopulations is needed to understand the processes that govern SLE development, progression, remission and relapses, in order to design interventional treatments that have the potential to redirect the immune system and eventually lead to a cure for this disease.

**DC populations in humans**

DCs are a heterogenous population of professional antigen presenting cells, which bridge innate and adaptive immunity. In the absence of exogenous triggers, DCs contribute to
the clearance of dying cells and the maintenance of tolerance. During infection, or in the context of autoimmunity, however, DCs play a pivotal role in the activation of CD4 and CD8 T cells. DCs were initially identified by Ralph Steinman and lack typical lineage markers for T cells (CD3) B cells (CD20) and NK cells (CD56) while expressing high levels of MHC class II\textsuperscript{21,22}. Within this population comparative studies have identified a small number of subsets that have homologues in several mammalian species\textsuperscript{23,24}.

**Myeloid DCs: BDCA1+ DCs and BDCA3+ DCs**

Myeloid DCs are considered “conventional” or “classical” DCs and are characterized by expression of CD11c, CD11b and lack of CD14 and CD16. Within this population we currently distinguish two populations based on the expression of the marker CD1c/BDCA1 and BDCA3/CD141\textsuperscript{25}.

The BDCA1+ DCs are the major myeloid DC population and are found in blood, lymphoid organs, and most tissues. BDCA1+ DCs express a wide variety of pattern recognition receptors including TRL1-8, lectins, and cytokines, allowing them responsiveness to a diverse array of environmental cues. BDCA1+ DCs are strong stimulators of naïve CD4 T cell responses, which can be shaped differently depending on which innate stimuli are present\textsuperscript{23}.

The BDCA3+ DCs make up >10% of the mDCs and have been found in lymphoid and non-lymphoid tissues as well as blood and bone marrow. BDCA3+DCs express high levels of TLR3, XCR1, CLEC9 and have been shown to display an increased capacity to phagocytose dying cells and cross-present cell-associated antigens to CD8 T cells compared to other DCs subsets\textsuperscript{26-28}.

**Plasmacytoid DCs**
pDCs lack the classic mDC markers CD11b and CD11c, and express high levels of CD123, CD303 (BDCA2) and CD304 (BDCA4). pDCs are known for their capacity to produce vast amounts of type I IFNs in response to viruses and/or virus-derived nucleic acids predominantly via engagement of TLR7 and TLR9. pDCs have been shown to prime CD4 T cells and cross-prime CD8 T cells, especially in the context of infection. Several studies implicate pDCs in the induction and maintenance of tolerance through the induction of regulatory T cells (Tregs).

**Monocyte-associated DCs**

There are currently several populations of DCs that are thought to develop from monocytes rather than common DC precursors. These cells display a variety of phenotypes and functions, but there is no consensus on their exact classification or their role in vivo.

CD14+ DCs are observed in several non-lymphoid tissues, including the skin. These cells express CD11c, but lack BDCA1 or BDCA3. The CD14+ DCs express low levels of costimulatory molecules or chemokine receptors that promote migration. While these cells have been suggested to be poor at stimulating naïve T cells, they have been found to support the formation of T follicular helper cells and to provide direct help to B cells.

Inflammatory DCs (iDCs) have been suggested to originate from classic CD14+ blood monocytes under inflammatory conditions. These cells may express some of the myeloid DC markers and seem prone to produce pro-inflammatory cytokines. In vitro studies suggest that different types of inflammatory stimuli give rise to populations with distinct pro-inflammatory phenotypes. TNFa/iNOS expressing inflammatory DCs have been found in skin lesions of patients with psoriasis and atopic dermatitis.

SlanDCs encompass a subset of monocytes with high expression of MHC class II, CD16,
and 6-sulpho LacNAc (slan). SlanDCs were shown to express TRL7 and TLR8 and to produce IL-12, IL-23 and TNF, preferentially promoting Th1 and Th17 cell differentiation. This population has been isolated from the inflamed skin of psoriatic patients and SLE patients with cutaneous lupus, the colon and draining lymph nodes of patients with inflammatory bowel diseases, as well as CSF samples and inflammatory brain lesions of patients with MS. Interestingly, SlanDC infiltration in tumors is associated with tolerance and poor prognosis, indicating either diversity within the slanDC population or heterogeneity in its function.

**Tissue DCs**

Non-lymphoid tissue resident DCs are present in most tissues in steady state and have been associated initially with induction of tolerance to self antigens. These cells migrate at a very low rate to the draining LN under steady state conditions, but show significant increased migration under inflammatory conditions. Several studies have identified networks of tissue resident DCs in the skin, lung, gut, and liver. Each of these networks consists of several subpopulations with different capacities for phagocytosis, antigen processing and presentation, migration, and the type of immune response they promote. Due to accessibility, skin DCs, especially Langerhans cells (LC), have been the most studied tissue-DC in the context of SLE.

**DC activation of T cells**

One of the defining features of DCs is the expression of class I and class II major histocompatibility proteins and the processing and presentation of peptide antigens to T cells. DCs predominantly present self-antigens in low quantities resulting in immunologic tolerance. Once activated, however, DCs mature in a process that usually involves migration to a draining lymph node and the priming of T cells. The factors governing the functional result of T cell priming are multifactorial, including the relative concentration of sur-
face peptide/MHC, costimulatory molecule expression, and cytokine release. Ultimately, the combination of these signals will result in either T cell anergy, deletion, or activation, proliferation and differentiation.

A wide variety of cell surface costimulatory proteins expressed by DCs can signal both activation (41-BB, CD40, CD70, CD80, CD83, CD86, GITRL, ICOSL, LTBR, OX40L) and inhibition (PDL1, PDL2) of an engaged T cell (reviewed in 54,55). In addition, secretion of pro- and anti-inflammatory cytokines by DCs contributes to the outcome of T cell priming. DCs can produce a wide variety of cytokines; which cytokines are produced depends upon environmental signals as well as upon the DC subtype. Cytokine production is driven by input from paracrine and autocrine cytokine signaling, as well as input from innate pattern recognition receptors (PRRs) including toll-like receptors (TLRs). The combination of these signals not only influences whether a T cell becomes activated, but also plays a key role in directing T cell differentiation toward various effector fates.

3. Role of DCs in SLE development and progression

Although it is not certain how immunological tolerance is broken in SLE, DCs are thought to play key roles56. Perhaps the most prominent model proposes that the initial injury is due to a build up of dying cells, a result of either dysregulated apoptosis or insufficient clearance of dying cells by DCs and other phagocytes57-59. Indeed, high levels of apoptotic cells are found in SLE patient serum, germinal centers, and in inflamed tissues, such as the skin and kidney60,61. Mounting evidence indicates that self RNA and DNA from these dying cells induces the unremitting output of type I IFN by pDCs62 via engagement of TLR9 or TLR763,64, and potentially via other cytosolic nucleotide sensing pathways such as RIG-I/IPS1 and STING (TMEM173)65-67. Type I IFNs produced by DCs promote their own activation and maturation in an autocrine manner, including increased IFN output and increased surface expression of CD80, CD86 and MHC class II, making them better
at activating T cells\textsuperscript{62,68-70}. Furthermore, type I IFNs directly promote B cell activation, antibody production, and T cell survival and expansion\textsuperscript{71-73}. Altogether, these data suggest that DCs are key players in SLE pathogenesis, and point to DCs as promising therapeutic targets.

4. DC abnormalities in SLE patients.

Several reports indicate that the frequency, composition, and phenotype of DCs in SLE patients differ from that of healthy individuals (see Table 1 and 2). However, it is difficult to compare results between laboratories, given differences in disease activity and manifestations, the effect of various drug treatments on DC development and phenotype, and the variations in analytical parameters.

Studies have shown reduced\textsuperscript{74-81}, normal\textsuperscript{80,82} and increased\textsuperscript{83} levels of CD11c\textsuperscript{+} mDC frequencies in PBMC from lupus patients compared to healthy controls. Similarly, pDC levels were found to be unaffected, reduced\textsuperscript{74-78,84,85}, or increased\textsuperscript{79,86}. Decreased frequencies of pDCs or mDCs were most often associated with active disease and to a lesser degree with non-active disease\textsuperscript{75}. Interestingly, studies showing peripheral pDCs decreases observed
a concomitant infiltration of pDCs in nephritic kidneys, suggesting that active pDCs may have migrated to the sites of inflammation\textsuperscript{78,82}. Similarly, Fiore et al. showed that besides pDCs, BDCA1+ DCs and BDCA3+ DCs were increased in the renal tubulointerstitium of patients with lupus nephritis\textsuperscript{78}. Increased numbers of pDCs and inflammatory/slanDCs are also found in cutaneous lesions of lupus patients, further suggesting migration of DCs to target organs\textsuperscript{87,88}. It is likely that DCs that reside in or have been recruited into the affected tissues will display different characteristics than those circulating in the periphery. Consequently, these populations should be included in further assessments in order to understand their contribution to disease pathogenesis and allow for a rational design of DC-targeting therapeutics.

### SLE-associated dysfunction in primary DCs

The few published maturation and functionality studies with primary human DCs have given conflicting results. Earlier reports indicated that DC from SLE patients have normal or even reduced levels of costimulatory molecules and are poor stimulators of allogeneic T cells in mixed lymphocyte reactions. Scheinecker et al. reported that in SLE patients B7+...
and CD40+ DCs were reduced and that DC-enriched APC from SLE patients displayed a diminished T cell-stimulatory capacity in both the allogeneic and the antigen-specific MLR, as compared with healthy individuals\textsuperscript{76}. On the other hand, Mozaffarian et al. showed increased CD80/CD86 and reduced PDL-1 expression on mDC during disease flares and an upregulation of PDL-1 during remission\textsuperscript{89}. Similarly, Gerl et al.\textsuperscript{81} published that monocytes and mDCs from SLE patients expressed higher levels of CD86 and BAFF, but not CD83 and CD40. Upon further assessment of their migratory capacity, they found that pDCs and mDCs from SLE patients had normal expression of CCR1, CCR5 and CCR7 but reduced expression of the chemokine receptor ChemR23 (CMKLR1). However, pDCs from the SLE patients showed an increased basal and CCL19-specific migration in vitro.

Assessment of peripheral monocytes, total DCs, BDCA1+ DCs and CD14\textsuperscript{-}lowCD16+ DCs by Henriques et al. showed that a higher percentage of SLE monocytes and CD14\textsuperscript{-}lowCD16+ DCs produced pro-inflammatory cytokines as well a higher amount of cytokines produced per cell, particularly in active disease. Data from Kwok et al.\textsuperscript{90} seemed to indicate that type I IFN production by pDC upon TLR9 engagement was diminished in SLE patients, leading them to hypothesize that the persistent presence of endogenous IFNa–inducing factors induces TLR tolerance in pDCs of SLE patients, resulting in impaired production of IFNa. Studies by Jin et al.\textsuperscript{79,91} also suggested deficiencies in TLR9 recruitment/signaling and production of pro-inflammatory cytokines in pDCs from SLE patients, however, they also showed that SLE pDC had an increased ability to stimulate T cells. Importantly, while pDCs from healthy donors induced suppressive T regulatory cell features (Foxp3 expression) in T cell cultures upon addition of apoptotic PMNs, SLE pDCs failed to do so.

These studies indicate that SLE is associated with phenotypic and functional changes in DCs and that these changes can affect different aspects of the DCs’ functional program.
in distinct and divergent ways.

**SLE-associated dysfunction in *in vitro* generated DCs**

Due to the paucity of DCs in leukopenic SLE patients, many studies have used in vitro generated monocyte-derived DCs (moDCs) to gain insight in DC generation, phenotype and function in the context of SLE.

Initial studies suggest that monocyte-derived DCs had a reduced pro-inflammatory and T cell stimulatory activity\(^9^2\) while later studies suggested accelerated differentiation and maturation concomitant with increased activity to maturation stimuli\(^9^3\). MoDCs from SLE patients expressed higher levels of HLA-DR and activating FcγRs, but decreased expression of inhibitory FcγR and expression levels correlated with disease severity\(^9^2,9^4\). In addition, moDCs spontaneously overexpressed activating costimulatory molecules including CD40, CD80, CD86, and showed increased production of stimulatory cytokines (IL-6, IL-8, BAFF/BlyS), eventually resulting in an increased capacity to activate T cells in an MLR\(^9^3,9^5\). Similarly, Nie et al.\(^9^6\) demonstrated substantial phenotypic and functional aberrations in DCs generated from (Flt3)-ligand and GM-CSF/IL-4 stimulated bone marrow aspirates. Both immature and mature DCs from SLE donors expressed higher levels of CCR7, CD40 and CD86 and induced stronger T-cell proliferation.

**Nature versus nurture**

Drawing causative relationships between DCs frequencies, maturation status, functionality, and disease is complex as it is not clear whether aberrations in DC frequency and functionality are the driver or a result of the disease. It is likely that genetic alterations in DCs predispose for the development of accelerated maturation and abnormal behavior. Evidence for this intrinsic defect is supported by the observations that moDCs from SLE patients, either generated from PBMC or bone marrow, display accelerated maturation
and increased pro-inflammatory status compared to moDC from healthy donors. On the other hand, serum of SLE patients has been shown to contain pro- and anti-inflammatory stimuli like type I IFN, type I IFN-inducing factors, and IL-10 that alter DC differentiation, maturation, and functionality, even in DCs from healthy donors. This raises the question whether the aberrant behavior of DCs in SLE patients is a result from an intrinsic defect, a result of their development in an inflammatory environment, or a combination of these two. To further confound the interpretation of human clinical data, various classic SLE treatments, including anti-malarials, corticosteroids, and immunosuppressive drugs significantly affect DC number, maturity and functionality.

**Mouse models to dissect the role of DCs in SLE pathogenesis**

The availability of mouse models provides an exciting opportunity to gain cellular and molecular insight in the role of different DC populations in the development and progression of SLE. There are a variety of spontaneous models, including the F1 hybrid between the New Zealand Black (NZB) and New Zealand White (NZW) strains (NZB/W F1) and its derivatives, the MRL/lpr, and BXSB/Yaa strains, as well as inducible models such as the pristane-induced model and chronic graft-versus-host-disease models (cGVHD). In recent years the number of models has been expanded with genetically modified mice, targeted in genes that can promote, resist, and modify lupus susceptibility. All of these models display their own variation of lupus-like disease reminiscent of symptoms observed in patients, including, autoantibody production, lymphoid activation and hyperplasia, lupus nephritis, and skin manifestations. Although all of these models have been instrumental in the identification of several main concepts in this disease, none of the models can completely recapitulate the complexity and variety of human disease. However, careful pairing of models with patient groups with the similar clinical manifestations can ensure the translational relevance of these preclinical models.
Mouse models have several advantages, (i) the relative homology between human and mouse DCs, (ii) the opportunity to genetically or pharmacologically eliminate specific DC populations during specific stages of disease, (iii) access to all target tissues for the assessment of tissue associated or infiltrating DCs, (iv) the opportunity to assess the effects of common treatments on these parameters, as well as (v) a plethora of biological and pharmacological tools to dissect the relative contribution of specific molecules and mediators to the development and progression of disease.

**Similarities between mouse and human DCs**

Recent genomic, proteomic and functional analyses of mouse and human DCs have identified high homology between the most present highest frequent DC populations. Like in human DCs, mouse DCs lineages encompass conventional DCs, pDCs, CD14+ DCs, tissue DCs and monocyte derived/inflammatory DCs.

Conventional mouse DCs encompass three main subpopulations which are found in circulation as well as in secondary lymphoid organs: (1) Cd11c$^{\text{high}}$MHCIICd8α-33D1+Sirpα+Cd11b+ (Cd11b DCs), which express most TLRs except Tlr3, display a preference for activation of CD4 T cells, and have high homology with the human BDCA1+ DCs; (2) Cd11chighMHCIICd8α+CD205+Sirpα-Cd11b- (Cd8α DCs), which express Xcl1, CD141, Clec9A and express mRNAs coding for most TLRs except Tlr5 and Tlr7, and are characterized by high Tlr3 expression and (3) Cd11chighMHCI+ cells that lack Cd8α, Cd4 and Cd11b (generally termed “double” or “triple” negative) DCs that, like CD8a DC, express Xcl1, CD141, Clec9A and Tlr3. These latter two populations have a high capacity to phagocytose dying cells and cross-present cell-associated or particulate antigens to CD8 T cells. Based on their genomic and functional analysis these two populations are considered to be homologues to the human BDCA3+ DCs.

Like human pDCs, mouse pDCs produce vast amounts of type I IFN in response to virus-
es via TRL7/9 mediated pathways. Compared to their human counterparts, mouse pDCs show relatively poor capacity for phagocytosis and antigen presentation. However, both populations have been implied in the maintenance of peripheral tolerance.

Various types of inflammatory and monocyte-derived DCs have been identified in mice as well. Tissue infiltrating CD14+ DC-like cells have been found under inflammatory conditions. Inflammatory DCs have been shown to arise after a wide variety of immunological insults, including pathogenic infection, experimental sterile inflammation, and models of inflammatory diseases such as RA, colitis experimental autoimmune encephalomyelitis and allergic asthma (reviewed in).

**The role of DCs in mouse SLE models**

Recent studies indicate an important role for DCs in the development and progression of SLE-like disease in mouse models. Similar to human disease, DCs from lupus-prone mice display a range of alterations in their numbers and their functionality. Splenic DCs from NZB/W F1 showed enhanced maturation and a stronger ability to attract B cells and present antigens to T cells than DCs from control mice. pDCs from SLE-prone mice showed increased type I IFN producing capacity upon TLR9 stimulation and increased cell survival compared to pDCs from C57BL/6 mice. Enhanced mDC and pDC activity has also been reported in male BXSB/Mp mice that express an extra copy of Tlr7 on the Y chromosome.

Importantly, depletion studies have now shown causal relationships between DC subsets and disease manifestations. Constitutive depletion of pDCs in lupus prone mice either through genetic ablation of IRF8, a transcription factor required for pDC and Cd8aDC development, or by diphtheria toxin treatment of mice expressing the diphtheria toxin receptor on pDCs resulted in markedly reduced type I IFN production, a reduced IFN signa-
ture, reduced auto-antibody production and reduction in the severity of kidney pathology glomerulonephritis. Importantly, transient pDC depletion during the early stages of disease was sufficient to significantly alter the course of the disease, suggesting a more prominent role for pDCs in the induction of the disease than in disease pathogenesis at later stages of disease. Diphtheria toxin treatment of Cd11c-DTA MLR. Fas<sup>lpr</sup> mice resulted in reduced T cell differentiation, plasma blast numbers and autoantibody levels. Interestingly, these mice developed interstitial kidney infiltrates but failed to progress to glomerular or interstitial nephritis, suggesting that DCs play a role in the development of tissue damage. In line with this observation, this group also showed that Cd11c depletion, but not LC depletion, resulted in significantly reduced dermatitis, demonstrating that DCs other than LCs control dermatitis in this model.

Besides the opportunity to assess the relative and temporal contribution of different DC populations to the development of specific disease manifestations, mouse models also allow for the identification of specific processes in DCs which affect disease development. Targeted deletion of regulatory molecules associated with SLE susceptibility in humans, including Shp1, A20, Blimp-1, Lyn or Eat-2, specifically in Cd11c+ cells resulted in increased DC activity and development of inflammatory and autoimmune phenotypes characterized by the production of auto-reactive antibodies, and several manifestations of SLE, including severe glomerulonephritis.

Together these observations indicate that mouse models provide a useful platform for the identification, dissection and targeting of DC intrinsic and extrinsic processes that facilitate the development, progression, and possibly a cure for SLE.

**DC targeted therapies for SLE**

Based on the general role of DC in the regulation of peripheral tolerance to self antigens,
the dysregulation of DCs observed in SLE, and the emerging evidence of the contribution of DCs in the initiation and perpetuation of SLE pathogenesis, is it not surprising that DC-targeting therapeutic strategies have become a topic of interest. Especially strategies that would promote self-antigen presentation in a tolerogenic context could be promising for the generation of an abortive or suppressive environment for the auto-reactive T and B cells and restoration of peripheral tolerance\textsuperscript{133,134}. In recent years several ex vivo models have been established for the generation of human DCs with stable tolerogenic functions (reviewed in\textsuperscript{135}). Generally, these resulting tolerogenic monocyte-derived DCs express low levels of positive costimulatory molecules and high levels of immune suppressive mediators (PDL-1, IL-10, etc.). Upon pulsing with specific antigens these DCs are anticipated to promote antigen-specific tolerance via the induction of T cell anergy, T cell apoptosis, skewing of T cell phenotypes to more Th2 or regulatory phenotypes, as well as the expansion of regulatory T cells.

Tolerogenic DC therapy is still in its infancy and little data is available on its in vivo potential. The first studies showed that transfer of antigen-loaded tolerogenic DCs could induce antigen-specific regulatory CD8 T cells and inhibit effector functions in antigen-specific CD8 T cells\textsuperscript{136,137}. A clinical trial in patients with type I diabetes using DCs treated with anti-sense oligonucleotides to silence costimulatory molecules was less successful, and although the treatment was well tolerated, only very limited tolerance outcomes were reported\textsuperscript{138}. A subsequent trial in T1D patients indicated that transfer of IL-10 and TGFb\textsubscript{1} generated tolerogenic DCs pulsed with pancreatic islet cells induced antigen-specific T cell hyporesponsiveness and was associated with better glycemic control\textsuperscript{139}. Similarly, transfer of a single dose of tolerogenic DCs –derived by ex vivo treatment with NF-kB inhibitors– into patients with active RA resulted in a modest improvement in disease activity 3 and 6 months after injection\textsuperscript{140}. Currently there are several trials addressing the therapeutic potential of tolerogenic DCs in Multiple Sclerosis, Rheumatoid Arthritis, Type
I Diabetes, and allergic asthma\textsuperscript{141}.

To date no tolerogenic DC transfer studies have been published in preclinical models or SLE patients. However, \textit{in vitro} data indicate that tolerogenic DCs can be generated from SLE patients\textsuperscript{83,142,143}, and that apoptotic cells can be used as source to load the DCs with auto-antigens\textsuperscript{143}. The insight obtained from currently ongoing tolerogenic DC treatment strategies in other chronic inflammatory diseases will help to identify critical parameters such as dose, route, and duration of treatment leading to the most efficacious outcome\textsuperscript{144,145}. However, a better understanding of the role of DCs in disease pathogenesis is critically needed in order to select the type of tolerogenic DC that can successfully counteract the dysfunctional adaptive immune responses that maintain the disease.

\textbf{Acknowledgements}

This work was supported in part by the Lupus Research Institute (to E.M.J), NCI grant CA138617 (to E.M.J), Charlotte Schmidlapp Award (to E.M.J.), the Albert J. Ryan Fellowship (to J.K.), the National Basic Research Program of China (973 program; 2014CB541902, to N.S.), the National Natural Science Foundation of China (no.81230072; no.81025016; no.81401331, to N.S.), the Program of the Shanghai Commission of Science and Technology (# 12JC1406000 to N.S.), and the Special Fund for Public Benefit Research from the Ministry of Health (no. 201202008, to N.S.).
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Chapter 4: The bm12 inducible model of systemic lupus erythematosus (SLE) in C57BL/6 mice*

*This is reprinted here with permission. The article was originally published in 2015: The bm12 Inducible Model of Systemic Lupus Erythematosus (SLE) in C57BL/6 Mice. Klarquist J and Janssen EM. J Vis Exp. 2015 Nov 1;(105).
SHORT ABSTRACT:
The transfer of bm12 lymphocytes into a C57BL/6 recipient is an established model of systemic lupus erythematosus. Here we describe how to initiate disease using this model and how to characterize T follicular helper cells, germinal center B cells and plasma cells by flow cytometry.

LONG ABSTRACT:
Systemic lupus erythematosus (SLE) is an autoimmune disease with diverse clinical and immunological manifestations. Several spontaneous and inducible animal models mirror common components of human disease, including the bm12 transfer model. Upon transfer of bm12 splenocytes or purified CD4 T cells, C57BL/6 mice rapidly develop large frequencies of T follicular helper cells (Tfh), germinal center (GC) B cells, and plasma cells followed by high levels of circulating anti-nuclear antibodies. Since this model utilizes mice on a pure C57BL/6 background, researchers can quickly and easily study disease progression in transgenic or knockout mouse strains in a relatively short period of time. Here we describe protocols for the induction of the model and the quantitation Tfh, GC B cells, and plasma cells by multi-color flow cytometry. Importantly, these protocols can also be used to characterize disease in most mouse models of SLE and identify Tfh, GC B cells, and plasma cells in other disease models.

INTRODUCTION:
Systemic lupus erythematosus (SLE) is a complex autoimmune disease characterized prototypically by anti-nuclear antibody (ANA) production and glomerulonephritis. Numerous other sequelae, including dermal, cardio-pulmonary, and hepatic lesions are associated with disease in some individuals. Prevalence estimates in the US vary widely, from 150,000-1,500,000, with particularly high incidence in women and minorities. Although the etiology of SLE has been difficult to discern, it is thought to arise from the interplay
of various genetic and environmental factors, which culminate in systemic autoimmunity.

Numerous animal models have been employed to study factors leading to disease onset and progression. Classic mouse models of SLE include genetically predisposed mouse strains including the NZB x NZW F1 model and its NZM derivatives, the MLR/lpr strain, and the BXSB/Yaa strain, and inducible systems, such as the pristane and chronic graft-versus-host disease (cGVHD) models. Early reports of autoantibody production in GVHD models used various mouse strains or hamster strains for parent into F1 transfers; more common methods used to study lupus-like disease currently include the DBA/2 parent→(C57BL/6 x DBA/2) F1, and the bm12 transfer model described here. Each model has its own caveats, but they generally share a common set of features that correlate with clinical features of human disease. The most often reported parameters in mouse models include splenomegaly, lymphadenopathy, nephritis, ANA production, and at the cellular level, the expansion of T follicular helper cells (Tfh), germinal center (GC) B cells, and plasma cells.

The inducible bm12 model is achieved by the adoptive transfer of lymphocytes from I-A^{bm12} B6(C)-H2-Ab1^{bm12}/KhEgJ (bm12) mice, a strain identical to C57BL/6 except for 3 amino acid substitutions on MHC class II, into I-A^{b} C57BL/6 (B6) mice. Alloactivation of donor CD4 T cells by recipient APCs leads to cGVHD with symptoms closely resembling SLE. Specifically, these include expansion of donor-derived Tfh, expansion of recipient-derived GC B cells and plasma cells, and production of ANAs including anti-dsDNA, anti-ssDNA, anti-chromatin, and anti-RBC antibodies. Over time, recipient mice develop glomerulonephritis associated with IgG deposits in the glomerular, interstitial, and vascular regions of the kidneys. We have recently shown that, similar to human disease, there is also a critical role for type I IFN in this model. Notably, the defining criteria for human SLE include the development of nephritis compatible with SLE in the presence of anti-dsDNA.
antibodies\textsuperscript{12}, both of which are prominent features of this mouse model.

There are several advantages of the bm12 model over the spontaneous models. Classic models that develop SLE-like signs spontaneously rely upon either hybrid mouse strains, inbred mouse strains not on the B6 background, or large genetic loci on the B6 background, which make crossing to knockout or otherwise genetically modified mice difficult and time consuming. With the bm12 inducible model, genetically modified mice can serve as either the donor or recipient, allowing more rapid identification of the cellular compartment in which particular genes may be important for disease. Furthermore, disease development in the bm12 model is much faster, requiring only 2 weeks until the appearance of ANAs, compared to several months for most spontaneous models. Moreover, in contrast to the spontaneous models that develop disease at different time points, the disease onset and progression in the bm12→B6 model is highly synchronized. This allows for the generation of appropriately sized cohorts that can be used for interventional or therapeutic strategies at any stage of disease development.

What follows is a detailed protocol for initiating SLE-like autoimmunity by the adoptive transfer of bm12 lymphocytes into C57BL/6 mice, or genetic variants on the B6 background. Additionally, we describe a flow cytometric staining protocol for enumerating Tfh, GC B cells, and plasma cells—cell types associated with human disease. Importantly, these protocols can also be used to characterize disease in most mouse models of SLE and identify Tfh, GC B cells and plasma cells in other disease models.

**PROTOCOL:**

Animal work was performed under specific pathogen-free conditions in accordance with
guidelines set by the Association for Assessment and Accreditation of Laboratory Animal Care International and our Institutional Animal Care and Use Committee (IACUC).

NOTE: Incorporate mice expressing a congenic marker such as CD45.1 on either donor or recipient animals if possible, because this allows for the monitoring of donor graft efficiency and specific expansion of the donor CD4 T cell population. If considering the use of otherwise genetically modified mice as donor or recipients, ensure the strain is properly backcrossed to the B6 background, or transferred cells may be rejected—this will be addressed in greater detail in the representative results and discussion sections.

NOTE: The following procedures detail volumes for harvesting 4 donor bm12 mice, which should yield enough cells to inject 12-16 mice. Six to twelve week-old bm12 mice yield roughly 100-140 million lymphocytes with approximately 25% CD4 T cells. If starting with different numbers of mice, or different mouse strains, scale volumes up or down accordingly. C57BL/6 mice generally give similar yields with closer to only 20% CD4 T cells.

NOTE: Perform all steps at room temperature and use room temperature media to avoid heat and cold shock, which can impair long-term lymphocyte viability. Perform tissue-harvesting and tissue-processing steps in a tissue culture hood using aseptic technique. All media in this protocol is IMDM with 10% heat-inactivated FBS, unless indicated otherwise, and will be simply referred to as “complete media.”

1. Novel method for genotyping bm12 mice

NOTE: A brief restriction digest-based protocol for genotyping is provided here, as a simple and inexpensive alternative to sequencing, which is currently the only published genotyping method for these mice.

1.1) Isolate genomic DNA from mouse tails. Please refer to a previous JoVE article for
detailed protocols on mouse tail clipping and the generation of cDNA from tail digestst

1.2) Perform a reverse-transcriptase polymerized chain reaction (PCR)\textsuperscript{14} the field of microbiology was transformed with the advent of Anton van Leeuwenhoek’s microscope, which allowed scientists to visualize prokaryotes for the first time. The development of the polymerase chain reaction (PCR to amplify a common 474 bp DNA fragment from MHC-II I-A\textsuperscript{b} and I-A\textsuperscript{bm12} using primers and thermocycling conditions listed in Table 1.

1.3) Perform restriction digest on \~7 μl of the PCR product using the enzyme Psul, or one of its isoschizomers (BstX2I, BstYI, MfII, or XhoII), which cuts wild type I-A\textsuperscript{b}, but not I-A\textsuperscript{bm12} mutant. Follow digest protocol provided by the manufacturer, which will specify a mixture of water, buffer and enzyme, and an incubation of 5 min. to several hours at 37 °C\textsuperscript{15}.

1.4) Load digest product and run on 1% polyacrylamide gel with ethidium bromide\textsuperscript{14} the field of microbiology was transformed with the advent of Anton van Leeuwenhoek’s microscope, which allowed scientists to visualize prokaryotes for the first time. The development of the polymerase chain reaction (PCR for 30-45 min. at 150V—though optimal voltage and time settings will vary depending on the PCR gel apparatus used. Visualize DNA bands with a UV illuminator. Representative results are shown in Fig. 1.

2. Harvesting donor cells

2.1) Sacrifice donor mice using Institutional Animal Care and Use Committee (IACUC)-ap

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**Figure 1. Representative bm12 genotyping results.**
To identify mice homozygous for I-Abm12/bm12, tail DNA was screened for bm12 by PCR/restriction digest genotyping (Step 1). Homozygous wild type (I-Ab/b) DNA yields two bands at 227 and 247 bp (visualized as one thick band at \~250 bp); homozygous bm12 (I-Abm12/bm12) DNA yields one band at 474 bp; and heterozygous (I-Abm12/b) DNA yields two bands at \~250 and 474 bp.
proved primary and secondary euthanasia methods. For example, euthanize mice by asphyxiation with CO₂ followed by cervical dislocation.

2.2) Using aseptic technique, harvest spleens and lymph nodes (superficial cervical, mandibular, brachial, axillary, mesenteric, and inguinal) into 15 ml tubes containing 10 ml complete media as in\textsuperscript{11-13}.

NOTE: Refer to previous reports for detailed lymph node dissection protocols\textsuperscript{16-18} we present methods for adoptive transfer of labeled T cells, isolation of lymph nodes, and imaging motility of CD4+ T cells in the explanted lymph node as first described in 2002 (2. This model is also successful using only mouse splenocytes for transfer, but the addition of lymph nodes significantly reduces the required number of donor mice.

2.3) Generate a single-cell suspension by mashing lymphoid tissues collected in step 2.2 through 70 μm cell strainers using the plunger from a syringe. For better yields, do not overload strainers—use ~6 strainers for every 4 mice processed, and rinse strainers frequently while processing. Combine tissues from 4 mice into two 50 ml conical tubes, then centrifuge cells for 5 minutes at 400 x g and decant supernatant.

2.4) Resuspend cells from both conical tubes in 50 ml complete media and transfer to new conical tube. Keep this tube at room temperature.

NOTE: Fat adheres to the sides of the tube, and if the tube is not replaced, final cellular yields can suffer.

3. Donor cell counting

NOTE: To preserve the highest viability, red blood cell (RBC) lysis of the entire sample is not recommended.

3.1) Mix single cell suspension from step 2.4 well, remove 1 ml and transfer it to a separate 50 ml conical. Set aside remaining, untouched cells (49 ml) at room temperature.
while counting.

3.2) Add 3 ml of an ammonium chloride-based red blood cell lysis buffer to the 1 ml donor cell sample. Mix gently for 1 min. by rocking, then fill to 50 ml with complete media, and centrifuge for 5 min. at 400 x g and decant supernatant.

3.3) Resuspend RBC-lysed cells in 10 ml complete media and count (e.g. with trypan blue using a hemocytometer). Multiply result by 49—this is the total number of cells remaining in the non-lysed sample that was set aside. Discard RBC-lysed cells.

4. Donor cell labeling and/or CD4 T cell purification

4.1) If desired, purify CD4 T cells at this stage, though this is not necessary. Additionally, if desired, label cells with CFSE, as in\textsuperscript{19}, or other cell tracking dyes, an example of which is shown in Fig. 4 of the representative results section.

NOTE: For CD4 T cell purification, negative magnetic selection is recommended, as it can achieve high purity and leaves cells untouched with high viability as described in\textsuperscript{20}. It is important that endotoxin-free buffers are used; therefore, instead of BSA, make separation buffer with 2% FBS and the recommended concentration of EDTA.

5. Injection of donor bm12 cells

5.1) After counting donor lymphocytes in step 3 (and purified or labeled as in step 4, as desired), centrifuge cells for 5 minutes at 400 x g. Decant supernatant and resuspend cells in PBS at 120 million lymphocytes per ml (or 30 million purified CD4 T cells per ml). Transfer cells to a sterile 5 ml round-bottom tube, or other sterile tube that easily accommodates a 1 ml syringe fitted with a 27.5 G x 13 mm needle.
5.2) Prior to injection, set aside a small sample of donor cells from step 5.1 at 4 °C for flow staining to determine the percentage of CD4 T cells within donor samples. Stain these samples as described in Step 8 using the minimal antibody panel (Table 2).

NOTE: If cells from different mouse strains are used as separate donors, this is an important consideration, and if CD4 T cell percentages vary substantially, purification may be required.

5.3) Mix cells gently, but thoroughly. This can be done by pipetting cells up and down using a 1 ml syringe without an attached needle. After mixing, draw cells into the 1 ml syringe. Attach needle after removing any air bubbles. Keeping the needle off while priming the syringe helps maintain cell viability.

5.4) Inject 250 μl per mouse (which is equal to 30 million lymphocytes, or 7.5 million purified CD4 T cells per mouse) intraperitoneally, as described in two-handed, and restraint with specially designed restraint objects will be illustrated. Often, another part of the research or testing use of animals is the effective administration of compounds to mice and rats. Although there are a large number of possible administration routes (limited only by the size and organs of the animal.

NOTE: In experiments shown here and in our prior work, each mouse is injected with 30 million total lymphocytes from bm12 donors, rather than the 100 million total splenocytes traditionally used. In unpublished data from our lab, no difference was observed in serum anti-dsDNA at day 14 following injections of 30 or 100 million lymphocytes per mouse. While this significantly reduces the number of mice needed for experiments, the development of nephritis with this number of cells has not been assessed.

7. Determine grafting efficiency
NOTE 1: This section will describe how to determine the degree of donor cell grafting in the recipient at day 3 in order to identify any mice which may have received suboptimal injections (e.g. the graft in one mouse is <10% of that seen in all other mice from the same group). These data can also help determine whether cells from a genetically modified mouse strain are rejected at later time points (for details, see representative results section, Fig. 6).

NOTE 2: This section is only possible if donors and recipients are from mice on different congenic backgrounds, e.g. when using CD45.1 bm12 donors and CD45.2 C57BL/6 recipients, or when donor cells are labeled with a cell tracking dye. Importantly, at 3 days post-injection, CD4 T cells have undergone minimal expansion (see representative results section, Fig. 4), so differences observed in the degree of grafting are due to variability in injections, not expansion.

6.1) Anesthetize mice with 4% isoflurane or other IACUC-approved method. Test rear foot reflexes to ensure mouse is properly anesthetized before proceeding to the blood draw.

6.2) Harvest 100-200 μl blood using an IACUC-approved method, such as retro-orbital puncture as in22. Collect blood into individual 0.5 ml microcentrifuge tubes containing an anti-coagulant, such as the plasma collection tubes referenced in the materials table, which contain lyophilized dipotassium EDTA. After blood collection, apply gentle pressure to the mouse eye using a sterile towel or gauze to ensure the bleeding stops, then place the mouse back in its home cage where it will remain until the final tissue harvest (Step 7).

NOTE: Do not leave mice unattended until mice have regained sufficient consciousness to maintain ventral recumbency, and do not return mice to the company of others until fully recovered.

6.3) Bring blood volume to 500 μl with 21 °C PBS and transfer to conical-bottom micro-
centrifuge tube, then slowly underlay 200 μl of 21 °C high density cell separation solution with a 200 μl pipet, being careful to minimize mixing between the two phases (see Materials Table for recommended solutions). Centrifuge cells at 700 x g for 20 min. (20-25 °C) with centrifuge brake set to low.

6.4) Remove top layer containing lymphocytes with a 1 ml pipet and transfer to a new microcentrifuge tube containing 800 μl cold complete media. Gently vortex to mix cells. Centrifuge cells at 700 x g for 5 min. (4 °C) and decant supernatant.

6.5) Resuspend cells in 200 μl complete media, transfer to a 96-well U-bottom plate, and stain for flow cytometry as described in Step 8 using a minimal antibody panel (Table 2) to determine the relative abundance of the CD4 T cell graft as a percentage of PBMCs.

7. Final tissue harvest

NOTE: The experiments described in the results section were harvested 14 days after injection of donor cells (or in some cases less time), as they focus on the initial development of Tfh cells and plasma cells; however, since this model is a chronic GVHD model of SLE, disease can be monitored at much later time points. The optimal timeframe will depend upon the research question posed in each individual experiment.

7.1) At a predetermined time point after injection of bm12 donor cells (step 5.4), sacrifice mice using an IACUC-approved method of euthanasia. Asphyxiation with CO₂ followed by exsanguination is recommended. Cervical dislocation can function as a secondary method of euthanasia, but this may reduce the blood draw yield.

7.2) Wet the abdomen of the mouse lightly with a spray bottle containing 70% ethanol. Make a small, superficial incision with surgical scissors approximately 1 cm above the genitalia. Draw back the skin of the abdomen toward the sternum, being careful to keep the peritoneal fascia intact.

NOTE: Diseased mice usually present with 0.5-3 ml ascites at day 14, which, although
it has not yet been well characterized, can be measured and analyzed as an additional
disease parameter.
7.3) Fit a 5 ml syringe with an 18-gauge needle. Insert the needle into the lower right
quadrant of the abdomen with the needle directed up toward the animal’s head and at
15-degree angle to the plane of the fascia. Position the needle tip near the cecum to help
prevent the needle from getting clogged with intestine while aspirating ascites.
7.4) Carefully rotate the mouse on its side, then slowly draw ascites into the syringe.
Once ascites has been recovered, remove the syringe and record the aspirate volume,
based upon the volumetric markings on the side of the syringe.
7.5) Discharge ascites into a 5 ml round-bottom tube and store on ice for later processing.
7.6) Harvest blood via draw from the inferior vena cava (IVC) essentially as in\textsuperscript{22}. Using dull
forceps, gently move intestines to the left side of the mouse, uncovering the IVC. Insert
a 27.5-gauge needle fitted to a 1 ml syringe into the IVC and slowly draw 400-500 μl of
blood.
7.7) To minimize hemolysis, slowly inject blood into a 0.5 ml microcentrifuge serum or
plasma collection tube. For later serum analysis of ANAs by ELISA, keep blood on ice.
7.8) Dissect and obtain additional relevant tissues (spleen and, if desired, lymph nodes
and kidneys, particularly if collecting at later time points and glomerulonephritis will be
scored). Remove spleen by gently placing the intestines back toward the right side of the
animal, and pulling on the pancreas, which is the spleen’s primary connective tissue.
7.9) Remove any pancreas remaining, then weigh spleens on a high precision balance
immediately after dissection, as gross splenomegaly is a commonly reported parameter
in mouse models of SLE. Place lymphoid tissues into individual 1.5 ml tubes filled with
1ml complete media on ice. Fix kidneys in 10% neutral buffered formalin or snap freeze
kidneys for later histology as in\textsuperscript{23}.
7.10) Centrifuge blood within 2 h of collection for 3 min. at 10,000 x g (4 °C). Remove se-
rum and store at -80 °C for later analysis of ANA by ELISA. Refer to previous reports for
detailed ANA ELISA protocols\textsuperscript{24,25}. Store serum in multiple 10-20 μl aliquots to minimize freeze/thaw cycles, and allow a greater number of future assays.

7.11) Centrifuge ascites 400 x g for 5 min. Remove the supernatant with a 1 ml pipet and aliquot into several 0.5 ml tubes. Freeze supernatant at -80 °C for later analyses of anti-nuclear antibodies (ANAs) or other soluble inflammatory mediators.

7.12) Resuspend cellular fraction in approximately 1 ml complete media and transfer 200 μl to a 96-well U-bottom plate for flow staining (as in Step 8).

7.13) Mash each spleen through 70 μm cell strainers into separate tubes and rinse with complete media. Resuspend splenocytes with 1 ml cold RBC lysis buffer for 1 min. and mix gently by rocking. Bring volume to 10 ml with cold complete media and centrifuge for 5 minutes at 400 x g and decant supernatant.

7.14) Resuspend cell pellet in 5 ml complete media and count using a hemocytometer. Adjust volume such that 200 μl of complete media contains 1-3 million cells. Begin staining for flow cytometry (Step 8) using an extended panel (Table 2) to quantify donor CD4 T cell and recipient B cell differentiation and expansion.

NOTE: Depending upon what laser, photomultiplier tube (PMT), and filter set combinations are available, separating the T and B cell analysis panel into multiple panels may be necessary.

8. Flow staining

8.1) Transfer 1-3 million splenocytes in 200 μl complete media into individual 5 ml round-bottom tubes, or into separate wells of a 96-well U-bottom plate.

NOTE: Using a 96-well plate is an efficient way to stain multiple samples, but take care to plate samples in every other well in order to prevent cross-contamination. One 96-well plate can accordingly hold 24 samples.
8.2) Centrifuge plate at 500 x g for 3 min., then flick supernatant from plate into appropriate (biohazard) container.

8.3) Resuspend the cell pellets with 100 μl of flow buffer (1% FBS in PBS) containing a fixable viability dye at the manufacturer’s recommended concentration and purified anti-CD16/anti-CD32 antibody cocktail at 1 μg/ml. Incubate for 10 minutes (20-25 °C), then add 100 μl cold complete media to quench viability dye.

NOTE: Splenocytes from diseased mice usually contain relatively high numbers of dead or dying cells; therefore, the inclusion of a viability dye is recommended to avoid non-specific antibody labeling of dying cells, resulting in cleaner, more reliable data. Similarly, the anti-CD16/anti-CD32 cocktail is included to block non-specific fluorescently-labeled antibody binding by Fc receptors.

8.4) Centrifuge plate at 500 x g for 3 min., then flick supernatant from plate into biohazard container. Resuspend cells in 200 μl flow buffer. Centrifuge plate at 500 x g for 3 min., then flick supernatant from plate into biohazard container.

8.5) Resuspend cells in 50 μl flow buffer containing antibody cocktail (Table 2). Incubate for 20 minutes (4 °C), then add 150 μl flow buffer. Repeat wash step 8.4.

8.6) Resuspend cells with 100 μl 2% paraformaldehyde in PBS. Incubate for 30 minutes (4 °C), then add 100 μl flow buffer. Centrifuge plate at 500 x g for 3 min., then flick supernatant from plate into biohazard container.

8.7) Resuspend in 200 μl flow buffer, transfer to flow tube inserts or standard flow tubes, add an additional 100-200 μl flow buffer, and store at 4 °C in the dark until acquiring on flow cytometer.

8.8) Acquire on a flow cytometer equipped with the appropriate lasers and PMTs for the chosen antibody panels within several days of staining. Record forward scatter width and/or height in addition to forward scatter area, side scatter area, and area of the fluorescent parameters utilized. For reliable results, acquire ≥1,000 donor cells in each WT sample, or an equivalent number of total lymphocytes in genetically modified or otherwise ma-
Manipulated mice that show minimal expansion of donor cells, where collection of so many events may not be feasible.

NOTE: Flow cytometry analyses are described in detail in the representative results section (Fig. 3-6).

**REPRESENTATIVE RESULTS:**

Diseased mice develop splenomegaly in as little as 14 days, exhibiting spleens 2-3 times the size of healthy mice in terms of mass and cellularity (Fig. 2).

![Figure 2. Spleen growth kinetics after injection of bm12 lymphocytes.](image)

Spleens were weighed on a high precision balance directly after excision (left). Live cell numbers were determined by counting with a hemocytometer using trypan blue to exclude dead cells (right). Results are depicted as mean ± SEM, where n = 9, 4, 3, and 6, respectively.

Splenocytes are sequentially gated on light scatter (FSC-A by SSC-A), elimination of doublets (FSC-W or -H by FSC-A), viable cells (low staining of viability dye), and CD4+TCRβ+ (Fig. 3A). Donor cells are distinguished from recipient cells based on CD45.1 and CD45.2 (Fig. 3B, bottom left). Donor cells predominantly adopt a T follicular helper (Tfh) cell phenotype, as characterized by the upregulation of PD-1, CXCR5, Bcl-6, and ICOS (Fig. 3B,C). A portion of the recipient CD4 T cell population also differentiates into Tfh (Fig. 3B, bottom right). After an initial die-off and/or migration of transferred cells, the expansion of donor-derived Tfh is logarithmic, reaching 10-20 million cells in the spleens of mice 14 days after injection (Fig. 3D).
CFSE-labeling of transferred lymphocytes demonstrated that donor CD4 T cells differentiate into Tfh early after activation; essentially all divided cells observed at days 3, 7, and 14 upregulated CXCR5 and PD-1 (Fig. 4). The proliferation peak profiles also suggest that a relatively low percentage of donor CD4 T cells underwent alloactivation and division. By day 14, most of the detectable donor cells are those that have divided beyond the maximum number of divisions measurable by CFSE.

Figure 3. Analysis of T follicular helper cell expansion in the bm12 model of SLE. CD45.1+ bm12 lymphocytes were transferred into C57BL/6 recipients and spleens were analyzed 14 days later. (A) Representative gating strategy showing “lymphocytes” (first panel), “single cells” (second panel), “live cells” (third panel), and “CD4 T cells” (fourth panel). (B) Donor cells are distinguished from recipient CD4 T cells by CD45.1 and CD45.2 staining and analyzed for expression of PD-1 and CXCR5. (C) Donor cells adopt Tfh phenotype, as indicated by the upregulation of several proteins commonly associated with Tfh. (D) Typical results showing the expansion of donor-derived Tfh (defined as CD4+CD45.1+PD-1+CXCR5+ live cells) at days 3, 7, and 14 post transfer. Results are depicted as mean ±SEM, where n = 5, 4, 3, and 6, respectively.
The expansion of donor-derived Tfh is accompanied by a corresponding accumulation of endogenous GC B cells and plasma cells (Fig. 5A). Plasma cell accumulation in the spleen is delayed compared to that of Tfh, exhibiting no increase over naïve animals on days 3 or 7 (Fig. 5B). Accordingly, anti-nuclear antibodies are not readily detectable prior to day 9 (data not shown), but can be reliably quantified on day 14.11

Figure 4. PD-1 and CXCR5 are upregulated on dividing cells. Bm12 cells were labeled with CFSE prior to injection into C57BL/6 recipients. Representative flow plots are shown for donor CD4 T cells at 3, 7, and 14 days after injection.

Figure 5. Expansion of splenic plasma cells and GC B cells. (A) Representative flow plots from naïve mouse spleens, or those from mice 14 days after CD45.1+ bm12 transfer. Plasma cells are defined as CD138+CD19low live cells (top panels). GC B cells are a subset of CD19+ B cells, which express GL-7 and Fas (bottom panels). (B) Quantitative data showing the accumulation of splenic plasma cells over time. Results are depicted as mean ± SEM, where n = 5, 4, 3, and 6, respectively.
Through the use of congenic markers, we have observed rejection of donor cells in multiple strains. While this is a common problem when mice are not sufficiently backcrossed to the C57BL/6 background, we also observed rejection when bm12 cells were transferred into B6.PL-Thy1a/CyJ (CD90.1) mice that are generally considered to be fully backcrossed. Therefore, mice are routinely screened at day ~3 by flow staining blood samples to assess the efficiency of the initial CD4 T cell graft; we know from CFSE proliferation experiments that grafted cells have expanded very little at this early time point. These results are then compared to results obtained from the day 14 harvest. In an example case of rejection, 30 million CD45.1+ bm12 lymphocytes were transferred into Cardif- mice which had been backcrossed to C57BL/6 mice for 12 generations. At day 5, all mice displayed equivalent grafting (2-3% of circulating CD4 T cells), but by day 14, bm12 donor cells were completely eliminated from the genetically modified recipient (Fig. 6).

Figure 6. Bm12 grafts are rejected by some genetically modified recipient mice. CD45.1+ bm12 lymphocytes were transferred into either genetically modified mice (top panels) or C57BL/6 (bottom panels) recipient mice. Mice were bled at 5 days post injection and assessed for graft efficiency. Splenocytes from the same mice were analyzed 14 days later.
DISCUSSION:
The bm12 inducible model is a relatively easy and efficient way to study the cellular and molecular processes of SLE. Chronic activation of adoptively transferred CD4 T cells directed against self antigens leads to the accumulation of Tfh, GC B cells, and plasma cells which can be measured by flow cytometry, as described here. Future studies using this model can quickly and easily interrogate the role of candidate genes and novel therapies in the autoimmune germinal center processes which resemble those occurring in patients with SLE, and ultimately govern the pathological accumulation of autoantibodies. Furthermore, the flow cytometric analysis described here can be used to study additional mouse models that involve the development of immunoglobulins including, but not limited to autoimmunity, infection, and allergy.

Like all animal models of human disease, this model also has its limitations. Given the speed with which disease develops and its magnitude, not all genes involved in the development of SLE are likely to be necessary for pathogenicity in this model. Additionally, care must be taken to rule out graft rejection when data suggest minimal expansion of Tfh in genetically modified recipients. Congenic markers should be used to confirm the presence of donor cells at harvest especially since recipient cells can also differentiate into Tfh (Fig. 3B), which could otherwise mask the absence of donor cells. Using congenic markers, we observed complete elimination of donor cells that evidently harbored rejection antigens by day 14 (Fig. 6). We have successfully used CD45.1+ bm12 and CD45.2+ bm12 mice as donors, and CD45.1+ BoyJ (B6.SJL-Ptprca Pepcβ/BoyJ) and CD45.2+ C57BL/6 mice as recipients (Fig. 3, 6, 7, and unpublished data). However, wild type congenic CD90.1+ cells from the B6.PL-Thy1a/CyJ mouse strain do not graft well, nor do CD90.2+ bm12 cells graft well in a CD90.1+ recipient (unpublished data), a phenomenon that is evidently not unique to this model26.
Either C57BL/6 or bm12 mice can serve as the donor or the recipient, as initially reported by Morris et al.\textsuperscript{9} However, in our lab we find the transfer of bm12 cells into B6 mice produces more consistent expansion of T cell and B cell populations at day 14. Furthermore, donor and recipient mice from different groups should be gender- and age-matched. Although experiments reported in the first description of the bm12 model found no significant difference in any disease parameter between male→male and female→female transfers\textsuperscript{9}, male→female transfers are not advised, as male antigen (H-Y) expressed by transferred CD4 T cells may induce graft rejection in female recipients\textsuperscript{27}.

When choosing antibodies and fluorochromes for congenic markers, it should be noted that donor cells become positive for the recipient congenic marker to varying degrees, such that a CD45.2\textsuperscript{-} gate would not constitute the entire CD45.1\textsuperscript{+} graft. The phenomenon is clearly illustrated in a comparison of CD45.2 expression by CD45.1\textsuperscript{+} naïve, CD45.1\textsuperscript{+} donor, and CD45.2\textsuperscript{+} recipient CD4 T cells (Fig. 7). Notably, donor cells also seem to upregulate expression of their own congenic marker, compared to naïve controls (Fig. 7). Similar results are also seen with CD45.2\textsuperscript{+} bm12 transfer into CD45.1\textsuperscript{+} BoyJ mice (data not shown). Presumably, acquisition of recipient CD45 results from trogocytosis\textsuperscript{28} by activated CD4 T cells following repeated interactions with recipient B cells. In fact, the bm12 model may prove a useful tool in studying the process of trogocytosis \textit{in vivo}.

![Figure 7. Donor cells acquire recipient CD45 congenic marker.](image-url) CD45.1\textsuperscript{+} bm12 lymphocytes were transferred into CD45.2\textsuperscript{+} C57BL/6 recipients. Donor, recipient, and naïve (CD45.1\textsuperscript{+}) CD4 T cells are assessed for CD45.1 and CD45.2 expression 14 days after transfer.
It has been shown that the transfer of purified bm12 CD4 T cells into C57BL/7 mice is sufficient to initiate disease\textsuperscript{29}; however, equivalent disease develops when whole lymphocytes are transferred, and purification is not necessarily required to study disease dependence upon T cell-intrinsic gene expression. Eisenberg and colleagues, clearly demonstrated that the antibody-producing cell in the bm12 model is almost exclusively of recipient origin\textsuperscript{30}. Furthermore, the requirement for recipient CD4 T cells\textsuperscript{10} is limited to the ‘nurturing’ of B cells during their development, which can be off-set by the addition of exogenous IL-4\textsuperscript{31}, although our data indicate that recipient T cells may participate somewhat in the germinal center response, as some of them do develop a Tfh phenotype (Fig. 3B, bottom right).

One critical decision that must be made for every bm12 experiment is when to harvest tissues for analyses. Of course the decision depends upon exactly what factors are important to the current study, which may vary. The experiments described here take up to 14 days, as they focus on the initial development of Tfh cells and plasma cells; however, since this model is a chronic GVHD model of SLE, disease can be monitored much longer. In fact, certain clinical features of SLE, including glomerulonephritis, develop later. Proteinuria has been detected as early as 2 weeks post-injection, but reaches peak levels at 4-8 weeks post-injection\textsuperscript{10,31}. Additionally, the flow cytometric analyses described here have been optimized for experiments lasting 2 weeks. We find a considerable number of GC B cells and plasma cells at this time point; although, given additional time, a large percentage of ANA-secreting plasma cells may reside in the bone marrow\textsuperscript{32}. Moreover, the plasma cells identified by this flow staining panel likely also include plasmablasts—in order to distinguish between these two cell types, additional antibodies would be required. Tfh expansion presumably reaches a peak at some point after the 14-day window on which we have focused. However, to our knowledge, no studies have reported bm12 donor cell number beyond 2 weeks, or used congenic markers to track adoptively transferred cells.
ACKNOWLEDGMENTS:
This work was supported in part by the Lupus Research Institute, NCI grant CA138617, NIDDK grant DK090978, Charlotte Schmidlapp Award (to E.M.J.), and the Albert J. Ryan Fellowship (to J.K.).

We are grateful for the support and instrumentation provided by the Research Flow Cytometry Core in the Division of Rheumatology at Cincinnati Children’s Hospital Medical Center, supported in part by NIH AR-47363, NIH DK78392 and NIH DK90971.

DISCLOSURES:
The authors have nothing to disclose.
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Chapter 5: STING-mediated DNA sensing promotes antitumor and autoimmune responses to dying cells*

*This is reprinted here with permission. The article was originally published 2014: STING-mediated DNA sensing promotes antitumor and autoimmune responses to dying cells. Klarquist J, Hennies CM, Lehn MA, Reboulet RA, Feau S, and Janssen EM. J Immunol. 2014 Dec 15;193(12):6124-34.
Abstract
Adaptive immune responses to antigens released by dying cells play a critical role in the development of autoimmunity, allograft rejection, and spontaneous as well as therapy-induced tumor rejection. Although cell death in these situations is considered sterile, various reports have implicated type I IFNs as drivers of the ensuing adaptive immune response to cell-associated antigens. However, the mechanisms that underpin this type I IFN production are poorly defined. Here we show that dendritic cells (DCs) can uptake and sense nuclear DNA-associated entities released by dying cells to induce type I IFN. Remarkably this molecular pathway requires STING but not TLR or NLR function and results in the activation of IRF3 in a TBK1-dependent manner. DCs are shown to depend on STING function in vivo to efficiently prime IFN-dependent CD8+ T cell responses to tumor antigens. Furthermore, loss of STING activity in DCs impairs the generation of follicular helper T (Tfh) and plasma cells as well as anti-nuclear antibodies in an inducible model of systemic lupus erythematosus (SLE). These findings suggest that the STING pathway could be manipulated to enable the rational design of immunotherapies that enhance or diminish anti-tumor and autoimmune responses, respectively.
**Introduction**

The immune system carefully balances its response to dead and dying cells in order to maintain homeostasis and prevent the development of autoimmunity. Although uptake and clearance of dying cells is generally considered a tolerogenic process, the existence of immunogenic cell death has been well described (1). Depending on the nature of the cell death, dying cells can emit damage-associated molecular patterns (DAMPs) (2) that act as danger signals and increase a dying cell’s immunogenicity. Many of these DAMPs seem to use the sensing and signaling pathways that are normally associated with the recognition and elimination of pathogens. Given the importance of immune responses to cell-associated antigens in autoimmunity, allograft rejection, and tumor rejection, the identification of these DAMPs, their cognate sensors, and their pro-inflammatory sequelae have become topics of intense research.

Ample studies have implicated type I IFNs in the development or progression of immune responses to self-antigens in autoimmune diseases such as rheumatoid arthritis (RA), type I diabetes mellitus (T1D), Sjögren’s syndrome and systemic lupus erythematosus (SLE) (3). However, type I IFNs were only recently identified as a crucial mediator in the priming of CD8+ T cells to cell-associated antigens in cancer and cancer treatments. Mice lacking type I IFN sensing—either by genetic IFN receptor (IFNAR) deletion or treatment with blocking Ab—develop more chemically-induced tumors and show poorer rejection of transplanted immunogenic tumors than WT mice, highlighting the requirement for type I IFN in spontaneous tumor rejection (4, 5). Additional studies showed that the spontaneous induction of tumor-specific CD8+ T cells in tumor-bearing mice was predominantly mediated by type I IFN sensing in dendritic cells (DC) (6, 7). A similar role for type I IFN was seen in therapy-induced tumor elimination. Burnette and Kang showed increased intratumoral production of type I IFN upon ablative radiotherapy or chemotherapy (8, 9). The ablative effect of the therapy was associated with enhanced (cross)priming capacity.
of tumor-infiltrating DCs and could be abolished by eliminating IFNAR from the hematopoietic compartment. Our previous work, using tumor cell therapy in vaccination and therapeutic settings, showed a comparable dependency of type I IFN in the induction of protective anti-tumor CD8$^+$ T cell responses (10-12).

While the general immunostimulatory effects of type I IFN on DCs are well studied, little is known on the cellular source of type I IFN, the type I IFN-inducing ligand, and the receptor/signaling pathways involved in its induction upon the sensing and clearance of dying cells. Our previous work indicated that DCs can produce type I IFN upon phagocytosis of dying cells (10-12). Importantly, DCs from MyD88$^{-/-}$/TRIF$^{lps/lps}$ double deficient mice showed normal type I IFN production upon phagocytosis of dying cells and type I IFN-dependent CD8$^+$ T cell priming to tumor-cell vaccines was comparable in WT and MyD88$^{-/-}$/TRIF$^{lps/lps}$ mice, indicating that the type I IFN induction requires an unidentified TLR-independent sensing pathway (11).

Using various murine cancer and tumor cell vaccination models and in vitro approaches, we show that the type I IFN production upon sensing of dying cells is not only TLR-independent, but also RLR-independent and requires stimulator of IFN genes (STING)-IRF3 mediated sensing of apoptotic cell-derived nuclear DNA structures by DCs. The ensuing type I IFN production enhances DC functionality in an autocrine manner, resulting in the increased clonal expansion, poly-functionality, and memory formation of tumor-specific CD8$^+$ T cells. Importantly, the role of the STING/IRF3/IFNAR nexus was not limited to CD8$^+$ T cell priming or tumor models; elimination of STING or IFNAR significantly impacted the development of CD4$^+$ T follicular helper cells, plasma cells, and anti-nuclear antibodies in an inducible model of SLE. Collectively, our results demonstrate that STING/IRF3 sensing of nuclear DNA-derived structures by DCs broadly drives the priming of adaptive immune responses to dying cells.
Materials and methods

Mice, cells lines, and peptides

Mice were maintained under specific pathogen-free conditions in accordance with guidelines by the Association for Assessment and Accreditation of Laboratory Animal Care International. C57BL/6J, B6.PL-Thy1a/CyJ (B6/CD90.1), B6.SJL.Ptpcr (B6/CD45.1), CD11c-DTR mice and B6(C)-H2-Ab1bm12/KhEgJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and H2-Kb−/− from Harlan (Indianapolis, IN). IFNAR−/−, MyD88−/−/TRIF−/−, CD11c-DTR-IFNAR−/−, and Act-mOVA/H2-Kb−/− mice were bred in our facility. IRF3−/− and IRF7−/− mice were a gift from Dr. K. Fitzgerald (U. Massachusetts, MA), STING−/− mice a gift from Dr. R. Vance (Berkeley, CA), and IPS-1−/− mice a gift from Dr. J. Tschopp (UNIL, Lausanne).

Mixed bone marrow chimeric mice were generated using donors on different congenic backgrounds in 1:1 ratios. All BM chimeric mice were rested for 12 weeks before experiments were started.

B16/F10, B16F10-OVA (B16-OVA), EL-4mOVA, MEC.B7.SigOVA, Tap sufficient and deficient MEFs expressing the human adenovirus type 5 early region 1 (Ad5E1-TAKO, I1.2), and ISRE-L929 IFN reporter cells have been described before (13-16). Peptides OVA257–264 (SIINFEKL), E1B192–200 (VNIRNCCYI), TRP-2180–188 (SVYDFFVWL) and LCMV GP33–41 (KAVYNFATC), were obtained from A&A Laboratories (San Diego, CA).

Cell isolations

T cells, B cells, Mph and DCs were sorted by flow cytometry using markers TCRb, CD4, CD8, CD11c, CD11b, CD19, CD45R, MHC II as described before (12). Purity of sorted cells was generally >98% and viability was >97% as determined by 7-AAD staining.

Erythrocytes, normoblasts, reticulocytes and red blood cells were generated using a mouse adapted protocol for the long-term ex vivo erythroid differentiation culture protocol described by Giarratana et al (17) and Konstantinidis et al (18). Briefly, low-density
bone marrow cells were cultured in erythroblast growth medium (StemPro-34 with 2.6% StemPro-34 supplement; Invitrogen), 20% BIT 9500 (StemCell Technologies), 900 ng/mL ferrous sulfate, 90 ng/mL ferrous nitrate, $10^{-6}$M hydrocortisone, penicillin/streptomycin, L-glutamine), in 3 subsequent phases. For the proliferative phase (days 1-5) cells were expanded with 100 ng/mL SCF, 5 ng/mL IL-3, and 2 IU/mL human erythropoietin (Amgen). In the differentiation step (days 6-7), the cells were supplemented with only erythropoietin in fibronectin coated plates. For enucleation (day 8-9) cells were grown without cytokines. Cells were sorted based on size gating and nucleotide staining using different combinations of Syto-16, Draq5, Mitotracker Red and MitotrackerGreen (Invitrogen).

**DC cytokine production, phagocytosis and T cell activation.**

Flow cytometry purified DCs were cultured in a 1:3 ratio with irradiated cells (1500-3000 Rad). Parallel experiments were performed with UV irradiation (120-240mJ/cm$^2$), Fas-cross linking (1-20ug/ml), or etoposide treatment (6hr, 1-10 mM) (11). When enucleated cells were compared to nucleated cells, all cells were gamma-irradiated and treated with thrombospondin-1 to upregulate phosphatidylserine on the membrane (19). After 20 hr type I IFN in the supernatant was determined by ISRE-L929 reporter assay that has detection limit of 0.3U/ml (13). For phagosomal acidification studies, diphenyleneiodonium (DPI, 10uM), ConB (1 nM), or chloroquine (50uM) were added at the start of the culture (12, 20). Parallel cultures using CpG and LPS were used as controls.

In vitro and ex vivo type I IFN analyses were performed by quantitative real-time PCR using SYBR Green and primers for $\beta$-actin (fw, TTGCTGACAGGATGCAGAAG; rev, GTACTTGCAGCCAGAGAG) and pan IFN-a (fw, TCTGATGCAGCAGGTGGG; rev AGGGCTCTCCAGACTTCTGCTG). Samples were treated with DNAse to eliminate genomic DNA contaminations. Gene expression was analyzed using the relative standard curve method and was normalized to Gapdh and $\beta$-actin expression.
For phagocytosis studies, DCs were incubated with CellTrace Violet-labeled irradiated splenocytes (Molecular Probes). After 3 and 16 hr, DCs were stained with Abs to CD11c, CD11b, CD8α, nuclear dye Draq5, and fixable live/dead staining and analyzed by ImageStream (Amnis, Seattle, WA). At least 10,000 live events were acquired and the number and size of phagocytosed particles were determined using the spot counting and spot size features after tight masking on the brightfield image to exclude membrane-associated extracellular particles for each condition as shown before (12, 21).

To determine T cell activating capacity, DCs were incubated with irradiated OVA-Kb-/- cells for 4 hr after which CFSE-labeled purified OT-1-CD45.1 CD8α+ T cells were added as described before (11). OT-1 cell proliferation and survival were determined after 70 hr by analysis of CFSE dilution together with staining for CD8α, Vα2, CD45.1 and 7-AAD.

**DC signaling studies**

Immune-coprecipitations: purified DCs were incubated with irradiated IRF3- or STING-deficient splenocytes. At different time points DCs were sorted, lysed, precipitated using agarose-bound antibodies to STING (3337, Cell Signaling) or IRF3 (D83B9, Cell Signaling) and probed for p-IRF3 (4D4G, Cell Signaling), TBK-1 (72B587, Novus Biologicals), p-TBK1 (D52C2, Cell Signaling), STAT6 (9362, Cell Signaling), and IPS1 (77275, Novus Biologicals).

In parallel, CellTrace Violet-labeled irradiated IRF3-/- splenocytes were cultured with WT and STING-/- DCs and analyzed by flow cytometry and Amnis Imagestream upon surface staining with CD11c-PacificBlue and intracellular staining with Draq5 and p-IRF3 (anti-pS386, 4D4G) combined with anti-Rabbit IgG Alexafluor 488. Correlations between intensity of phagocytosed material and p-IRF3 localization in the nucleus were determined using Imagestream by gating on CD11c+ single cells followed by tight masking on the cells to discriminate internalized from bound particles followed by tight masking on the
nucleus to determine colocalization of the p-IRF3 staining in the nucleus (12).

**In vivo models.**

*Immunizations:* mice were injected s.c. or i.p. with irradiated cells (OVA-Kb/-: splenocytes, $10^6-10^7$; B16-OVA, B16/F10 and 5E1-TAKO, 1-5x$10^6$). Alternatively, mice received i.v. 2x$10^6$ purified DCs that had been pulsed with peptide or exposed to irradiated cells in vitro (10-12, 21). For DC depletion studies CD11c-DTR mice and mixed bone marrow chimeric mice were treated 24 hr before and 24 hr after immunization with 4ng/g diphtheria toxin i.p.

*Tumor models:* For the cyroablation model mice were subcutaneously injected with 4x$10^5$ B16-OVA or B16/F10 in PBS. Ten days later tumors (7–10 mm) were cryoablated using Verucca-Freeze armed with a 6 mm probe (Brymill Cryogenic Systems) (22) for 3x25-second cycles. To test long-term tumor protection, the mice were challenged with 3x10^4 B16-OVA or B16/F10 40 days after the ablation of the primary tumor and monitored for tumor growth. In the EL-4-mOVA challenge model, mice were immunized with 10^7 irradiated OVA-Kb/-: splenocytes as described above and 40 days later challenged with 10^6 EL-4-mOVA cells s.c.

*SLE model (23):* mice of indicated genetic backgrounds received 3x10^6 B6(C)-H2-Ab1bm12/KhEgJ (bm12) splenocytes i.p.. Splenic B and T cell composition B was analyzed by flow every 2 weeks. Serum levels of anti-dsDNA IgG, IgG1 and IgG2a were determined by ELISA as previously described (24).

**T cell analysis**

Unless stated differently, analysis were performed 7 days after immunization. CD8+ T cells were enumerated in spleens and draining lymph nodes using OVA_{257-264}-Kb tetramers (Beckman Coulter) or E1B_{192-200}-Db decamers (Immudex) together with staining for CD8a, CD44 and 7-AAD. In parallel cytokine production and polyfunctionality was determined
directly ex vivo by intracellular cytokine staining after a 5 hr stimulation with cognate peptide in the presence of brefeldin A as described before (11, 16). Samples were collected on a LSRII flow cytometer with Diva software (BD Pharamingen), and data were analyzed with FlowJo software (Tree Star). In B16/F10 studies, responses to TRP2 \textsubscript{180-188} were determined by ELISPOT directly ex vivo. Capacity for secondary expansion in vitro was determined by stimulating the splenocytes or purified CD8\textsuperscript{+} T cells on irradiated MEC.B7.SigOVA (OVA-specific) or I1.2 cells (E1B specific) for 6 days and dividing the absolute number of antigen-specific CD8\textsuperscript{+} T cells at the beginning of the culture by the absolute number of antigen-specific CD8\textsuperscript{+} T cells at the end of the culture as described before (16, 25). In vivo secondary expansion was determined by re-injecting the mice with a 10-fold higher number of irradiated cells than used during immunization. Four days after the secondary challenge the frequency antigen-specific CD8\textsuperscript{+} T cells was compared to non-challenged immunized mice (16).

**Statistical analyses**

Data were analyzed using Prism software (GraphPad Software, Inc.). Unless stated otherwise, the data are expressed as means ± SEM. Survival responses were analyzed by Kaplan-Meier using a log-rank test. All other data were evaluated using a two-way ANOVA followed by a Dunnett’s test. A \( p \) value of <0.05 was considered statistically significant.
Results

Protective anti-tumor immunity requires type I IFN sensing by DCs

We and others recently showed that type I IFN sensing is critical for the induction of protective anti-tumor responses in various tumor models in both vaccination and therapeutic settings (6-8, 22). Cryoablation of B16-OVA tumors in WT mice resulted in effective priming of OVA257–264–specific CD8+ T cells that provided protective immunity upon subsequent B16-OVA challenge. In contrast, IFNAR−/− mice failed to induce adequate OVA257–264–specific CD8+ T responses and succumbed upon tumor rechallenge (figure 1a/b). Similarly, immunization with gamma-irradiated OVA-expressing Kb−/− splenocytes (OVA-Kb−/−) induced a significantly more robust OVA257–264–specific CD8+ T cell response in WT mice than in

FIGURE 1. Protective CD8+ T cell induction requires IFNAR on DCs. (A) WT and IFNAR2/2 mice were s.c. injected with B16-OVA tumor cells, and palpable tumors were cryoablated 10 d later. Cryoablated and naive mice were s.c. challenged with B16-OVA 40 d later and survival was monitored (n = 8–10/group). (B) OVA257–264–specific CD8+ T cell frequency in the spleen 7 d after cryoablation. (C) Mice were immunized with irradiated OVA-Kb2/2 or Kb2/2 splenocytes and 40 d later s.c. challenged with EL-4–mOVA cells s.c. (D) Frequency of splenic OVA257–264–specific CD8+ T cells 7 d after immunization as determined by intracellular cytokine staining for IFN-γ after peptide restimulation and by OVA257–264–Kb-tetramer staining. (E) Fold expansion of OVA257–264–specific CD8+ T cells upon stimulation with OVA257–264–expressing cells in vitro. Data of representative experiments (out of three to five) are shown (mean 6 SEM, n = 8–10/group, *p < 0.05).
IFNAR\textsuperscript{-/-} mice (figure 1.c/d). The OVA\textsubscript{257-264}-specific CD8\textsuperscript{+} T cells from WT mice but not IFNAR\textsuperscript{-/-} mice underwent expansion upon secondary encounter with antigen in vitro and in vivo and protected mice from EL-4-mOVA challenge in vivo (figure 1e, figure S1a). Importantly, this response was not restricted to OVA or the selected pathways of cell death. Similar results were found when the parental line B16/F10 was used and the response to self-antigen TRP-2 was probed (figure S1b/c). In addition, direct immunization with gamma-irradiated, UV-irradiated, Fas-crosslinked, or etoposide treated cells showed significantly decreased CD8\textsuperscript{+} T cell responses in IFNAR\textsuperscript{-/-} mice, illustrating a central role for type I IFN in CD8\textsuperscript{+} T cell priming to cell-associated antigens in various scenarios of cell death (figure 2).

As nearly all cells express IFNAR, we first used bone marrow (BM) chimeras to identify which cells required type I IFN sensing in the CD8\textsuperscript{+} T cell response to dying cells. BM chimeric mice (WT→IFNAR\textsuperscript{-/-}, WT→WT, IFNAR\textsuperscript{-/-}→WT, IFNAR\textsuperscript{-/-}→IFNAR\textsuperscript{-/-}) demonstrated that type I IFN needed to be sensed by the hematopoietic compartment (figure 3a/b).

**FIGURE 2. Impaired CD8\textsuperscript{+} T cell priming in IFNAR\textsuperscript{-/-} mice is independent of the method of cell death or cell type.** Frequency of splenic Ag-specific CD8\textsuperscript{+} T cells in WT and IFNAR\textsuperscript{-/-} mice 7 d after s.c. immunization with actmOVA-Kb\textsuperscript{-/-} splenocytes (which were gamma irradiated [1500 rad], UV irradiated [120 mJ/cm\textsuperscript{2}], or treated with FAS cross-linking Ab [Jo-1, 20 mg/ml; 4 h, 37\textdegree C]), 5E1-TAKO cells (3000 rad, 240 mJ/cm\textsuperscript{2}, or treated with etoposide [10 mM]), or B16-OVA or B16/F10 cells (3000 rad or 240 mJ/cm\textsuperscript{2}). Ag-specific T cell frequencies were determined by intracellular cytokine staining upon incubation with OVA\textsubscript{257-264}, E1B192–200, TRP\textsubscript{2180–188}, or control peptide GP\textsubscript{33–41} (white bar, control peptide; black bar, specific peptide). Data in all experiments are expressed as mean 6 SEM with n = 5. *p < 0.05.
Additional studies with WT/IFNAR\(^{-/-}\)→WT mixed BM chimeric mice indicated that the diminished CD8\(^+\) T cell response could not be attributed to the lack of IFNAR on the CD8\(^+\) T cells as IFNAR\(^{-/-}\) CD8\(^+\) T cells only showed a marginal decrease in clonal expansion (figure 3c). Given the important role of DCs in cross-presentation of cell-associated antigens, we next examined the role of type I IFN sensing on DCs. Mixed BM chimeras were generated in which IFNAR\(^{-/-}\) recipients received a combination of WT-CD11c-DTR

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**FIGURE 3. Selective requirement for type I IFN sensing in the DCs.** (A) Bone-marrow chimeric mice (WT→IFNAR\(^{-/-}\), WT→WT, IFNAR\(^{-/-}\)→WT, IFNAR\(^{-/-}\)→IFNAR\(^{-/-}\)) were immunized i.p. with irradiated 5E1-TAKO cells and the frequency of E1B\(_{192-200}\)-specific CD8\(^+\) T cells in the spleens was determined 7 d later (white bar, control peptide; black bar, E1B\(_{192-200}\) peptide). (B) The effect on memory formation using these chimeras was determined by measuring Ag-specific T cell expansion ex vivo. Secondary expansion of E1B\(_{192-200}\)-specific CD8\(^+\) T cells was calculated by dividing the absolute number of E1B\(_{192-200}\)-specific CD8\(^+\) T cells at the end of a 6 d culture by the absolute number at the start of the culture. (C) WT/CD90.1 mice were irradiated and reconstituted with WT, IFNAR\(^{-/-}\) or a 1:1 ratio of WT(CD45.1):IFNAR\(^{-/-}\)(CD45.2) bone-marrow. Mice were i.p. immunized with irradiated 5E1-TAKO cells and the frequency of splenic E1B\(_{192-200}\)-specific CD8\(^+\) T cells in each graft was determined 7 d later. (D) WTCD11cDTR:IFNAR\(^{-/-}\) and WT:IFNAR\(^{-/-}\)CD11c-DTR mixed bone-marrow chimeras were treated with DT or vehicle and immunized with irradiated 5E1-TAKO cells. The frequency of E1B\(_{192-200}\)-specific CD8\(^+\) T cells in the WT compartment was determined 7 d later. (E) Freshly isolated WT and IFNAR\(^{-/-}\) DCs were incubated with irradiated 5E1-TAKO cells, sorted and i.v. injected into WT and IFNAR\(^{-/-}\) mice. The frequency of splenic E1B\(_{192-200}\)-specific CD8\(^+\) T cells was determined 7 d later. Data of representative experiments (out of three to five) are shown (mean 6 SEM, n = 5/group, *p < 0.05).
and IFNAR<sup>-/-</sup>/CD45.1 BM or IFNAR<sup>-/-</sup>-CD11c-DTR and WT/CD45.1 BM. In this model the administration of DT yielded animals that contained either WT or IFNAR<sup>-/-</sup> DCs with an otherwise comparable composition of hematopoietic cells. Depletion of WT DCs significantly decreased the number and frequency of antigen-specific CD8<sup>+</sup> T cells. In contrast, depletion of IFNAR<sup>-/-</sup> DCs had no effect on the number or frequency of antigen specific CD8<sup>+</sup> T cells (figure 3d). Similar results were observed when purified WT or IFNAR<sup>-/-</sup> DCs were pulsed with irradiated cells and transferred into WT and IFNAR<sup>-/-</sup> recipients, illustrating the critical role of type I IFN sensing by DCs in CD8<sup>+</sup> T cell priming to cell-associated antigens (figure 3e).

**DCs predominate the type I IFN production elicited by dying cells**

Besides sensing type I IFNs in the context of CD8<sup>+</sup> T cell priming to dying cells, we reasoned that DCs may also be the main producers of type I IFN, thereby establishing a positive IFN-feedback loop. In vitro studies using purified cell populations indicated that type I IFN was produced by DCs upon incubation with dying cells, whereas only nominal IFN production was observed in cultures depleted of DCs (figure 4a/b/c) (11, 12). The in vitro

![Type I IFN production by DCs in vitro and in vivo.](https://example.com/figure4.png)

**FIGURE 4. Type I IFN production by DCs in vitro and in vivo.** Total and subset-depleted splenocytes (A) or purified cell populations (B) from spleens of naive WT mice were cultured with irradiated OVA-Kb<sup>-/-</sup> splenocytes, and type I IFN in the supernatant was determined 20 h later (black bars, irradiated cells; white bars, no cells). Data are expressed as mean 6 SEM with n = 4. (C) Splenocytes from CD11c-DTR mice (treated with PBS or DT 24 h prior) were isolated and cultured with irradiated OVA-Kb<sup>-/-</sup> splenocytes. Type I IFN in the supernatant was determined 20 h later. (D) CD11c-DTR mice were treated with vehicle or DT and 24 h later s.c. injected with irradiated cells. Type I IFNa mRNA in draining lymph nodes was assessed 6–8 h later. Representative data of one experiment (out of three to four) are shown (mean 6 SEM, n = 5, *p < 0.05).
data were supported by our observation that IFNa mRNA was readily detectable in the draining LN of control-treated but not DT-treated CD11c-DTR mice upon subcutaneous immunization with irradiated cells (figure 4d).

The STING/IRF3 pathway drives type I IFN in response to cell-associated antigen

Induction of type I IFNs is generally associated with innate sensing of pathogenic danger signals. To identify which innate sensing pathways facilitated the type I IFN production, primary DCs from WT, MyD88\(^{-/-}\)/Trif\(^{ps/p}\), IRF3\(^{-/-}\), IRF7\(^{-/-}\), IPS1\(^{-/-}\) (MAVS/Cardif), and STING\(^{-/-}\) mice deficient were tested for their type I IFN production upon culture with dying cells. Type I IFN production was similar in WT, MyD88\(^{-/-}\)/Trif\(^{ps/p}\), IPS1\(^{-/-}\), and IRF7\(^{-/-}\) DCs indicating that the IFN induction was TLR-\(^{-}\), RIG-I- and Mda5-independent. In contrast, significant reductions in type I IFN production were seen with DCs deficient in STING and

**FIGURE 5.** Type I IFN induction requires the STING/IRF3 pathway. (A) Type I IFN production by purified DCs from indicated strains upon 20-h culture with irradiated splenocytes. (B) Purified DCs from indicated strains were cultured at 4°C (white bars) and 37°C (black bars) with VT-labeled irradiated splenocytes. Uptake of VT materials was determined 6 h later by flow cytometry. Data are expressed as percentage of DCs that contain VT. Representative data of one experiment (out of three to four) are shown (mean 6 SEM, n = 4). (C and D) p-IRF3 kinetics in WT and STING\(^{-/-}\) DCs upon incubation with irradiated IRF3\(^{-/-}\)splenocytes. (E) ImageStream images of p-IRF3 nuclear translocation in WT DCs 8 h after incubation with VT-labeled irradiated IRF3\(^{-/-}\) cells. Original magnification 360. (F) ImageStream analysis of nuclear p-IRF3 intensity and intensity of cytosolic phagocytosed material in WT and STING\(^{-/-}\) DCs. Tight masking on the cytosol was done to discriminate between bound and internalized cellular material. At least three experiments were performed with 800–1000 analyzed cells/condition. *p , <0.05.
the transcription factor IRF3 even though their subset compositions, maturation status and phagocytic capacity were similar to WT DCs (figure 5a/b and not shown). Immunoprecipitation and western blotting studies indicated rapid IRF3 phosphorylation in WT DCs and a significant reduction and delay in IRF3 phosphorylation in STING+/− DCs upon exposure to dying cells (figure 5c/d). To determine whether the magnitude of the nuclear p-IRF3 signal was associated with the frequency and size of phagocytosed particles, WT and STING+/− DCs were incubated with CellTrace Violet (VT)-labeled, irradiated, IRF3−/− splenocytes and analyzed by conventional flow cytometry and imaging cytometry. WT DCs showed a strong correlation between nuclear p-IRF3 intensity and the amount of phagocytosed material. Although STING+/− DCs displayed similar uptake of VT material as WT DCs, they exhibited significantly reduced nuclear p-IRF3 staining, consistent with their limited IFN production (figure 5e/f, figure S2a). Using UV-irradiation, Fas-crosslinking, or etoposide to induce cell death yielded similar outcomes, indicating that the STING/IRF3 pathway was the dominant IFN-inducing pathway in various forms of sterile cell death (figure S2b).

**Nuclear DNA-derived structures induce type I IFN production by DCs**

We next set out to determine the ligand upstream of STING that was responsible for inducing type I IFN. STING facilitates immune responses to various nucleotide structures, including cytosolic dsDNA, and in some cases dsRNA (26-28). As dsRNA sensing utilizes the RIG-I/IPS-1 pathway and IPS1−/− DCs have normal type I IFN production, it is likely that the IFN-inducing species released by dying cells is a DNA- and not an RNA-based entity. Indeed, addition of DNAses, but not RNAses to WT DC/irradiated cell co-cultures significantly reduced type I IFN production without affecting uptake of cellular material or responses to non-nucleic TLR ligands (figure 6a, figure S2c/d). To more rigorously address the role of DNA complexes in the type I IFN induction, we exploited the process of erythropoiesis where red blood cell (RBC) precursors sequentially lose their nuclei,
mitochondria and ribosomes. Timed RBC cultures were sorted, irradiated and treated with thrombospondin-1 (irr/TSP) to induce comparable phosphatidylserine expression on the membrane and facilitate an “apoptotic phenotype” in the non-nucleated cells (figure 6b). ImageStream analysis showed comparable uptake of the irr/TSP cell subsets by DCs (figure 6c). Nucleated irr/TSP erythroblasts readily induced type I IFN in DCs while enucleated reticulocytes and RBC failed to do so (figure 6d). These data indicate that the IFN-inducing species is nuclear DNA-derived.

DC-intrinsic STING regulates CD8+ T cell responses to dying cells
Given the importance of STING-mediated DNA sensing in the type I IFN production, we next assessed the relative contribution of STING in the priming of CD8+ T cells to dying cell-associated antigens. WT, MyD88+/−Trif+/−/ips+/−, IPS−/−, and IRF7−/− mice showed comparable CD8+ T cell priming as determined by tetramer staining, intracellular cytokine staining,
and capacity for secondary expansion upon immunization with irradiated 5E1-TAKO cells (figure 7a/b). In contrast, IFNAR\(^{-/-}\), IRF3\(^{-/-}\) and STING\(^{-/-}\) mice showed significantly reduced CD8\(^{+}\) T cell priming (figure 7c/d). Moreover, the antigen-specific IFNAR\(^{-/-}\), IRF3\(^{-/-}\) and STING\(^{-/-}\) CD8\(^{+}\) T cells displayed less cytokine polyfunctionality and impaired capacity for secondary expansion (figure 7e). The defect in CD8\(^{+}\) T priming was fully DC-regulated as similar results on CD8\(^{+}\) T cell clonal burst, secondary expansion and cytokine poly-

![Figure 7](https://example.com/figure7.png)

**FIGURE 7.** STING regulates the CD8\(^{+}\) T cells responses to dying cells in vivo. (A) Mice of indicated strains were immunized with irradiated 5E1-TAKO cells, and the frequency of splenic E1B\(_{192-200}\)–specific CD8\(^{+}\) T cells was determined 7 d later (white bar, control peptide; black bar, E1B\(_{192-200}\) peptide). (B) Fold expansion of E1B\(_{192-200}\)–specific CD8\(^{+}\) T cells from indicated mouse strains upon culture with E1B\(_{192-200}\)–expressing feeder cells in vitro. (C) E1B\(_{192-200}\)–specific CD8\(^{+}\) T cell frequency in indicated mouse strains 7 d after immunization with irradiated 5E1-TAKO cells. (D) Secondary expansion of E1B\(_{192-200}\)–specific CD8\(^{+}\) T cells in vitro. (E) Ex vivo polyfunctionality of E1B\(_{192-200}\)–specific CD8\(^{+}\) T cells from (C) as determined by flow cytometry. (F) Frequency of E1B\(_{192-200}\)–specific CD8\(^{+}\) T cells in WT mice 7 d after transfer of indicated DCs pulsed with irradiated 5E1-TAKO cells in vitro. (G) Secondary expansion of E1B\(_{192-200}\)–specific CD8\(^{+}\) T cells primed by indicated DCs. (H) Ex vivo polyfunctionality of E1B\(_{192-200}\)–specific CD8\(^{+}\) T cells primed by indicated DCs. (I) DCs were purified from WT, IFNAR\(^{-/-}\) and STING\(^{-/-}\) mice and exposed to irradiated 5E1-TAKO cells or pulsed with 1 mM E1B\(_{192-200}\) peptide. DCs were repurified and 2 \(\times 10^5\) were injected into WT recipients. Seven days later, the frequency of splenic E1B\(_{192-200}\)–specific CD8\(^{+}\) T cells was determined (white bar, control peptide; black bar, E1B\(_{192-200}\) peptide). Representative data of one experiment (out of three to four) are shown (mean 6 SEM, n = 5–7, *p < 0.05).
functionality were seen when purified IFNAR−/−, IRF3−/− and STING−/− DCs were exposed to irradiated cells in vitro and transferred into WT recipients. (figure 7f/g/h). The priming defect of the IFNAR−/−, IRF3−/−, and STING−/− DCs could not be attributed to a decrease in overall DC functionality as peptide-pulsed IFNAR−/−, IRF3−/−, and STING−/− DCs induced comparable CD8+ T cell responses as WT DCs upon transfer in WT recipients (figure 7i). Together, these data demonstrate the requirement for STING, IRF3, and IFNAR in DCs in the cross-priming of CD8+ T cells to cell-associated antigens.

**STING regulates CD4+ T cell and B cell responses in the bm12 SLE model**

To assess whether STING has a broader role in the priming of adaptive immune responses to dying cells, we assessed the induction of CD4+ T cell and B cell responses in the bm12-cGVHD model where H-2b B6 hosts develop lupus-like disease upon transfer of B6.C-H2bm12 CD4+ T cells (29). Both IFNAR−/− and STING−/− mice developed considerably

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**FIGURE 8. STING regulates CD4+ T cell and B cell responses in the bm12 SLE model.** (A) Flow cytometric analysis of Tfh (gated on CD4+ T cells) and plasma cells (total spleen) in spleens of WT, STING−/−, and IFNAR−/− mice 14 d after i.p. transfer of live bm12 splenocytes. (B) Number of splenic Tfh and activated CD4+ T cells in the spleen of indicated mouse strains. (C) Number of plasma cells and activated B cells in the spleen of indicated mouse strains. Representative data of one experiment (out of three to five) are shown. (D) Anti-dsDNA IgG1 and IgG2a levels in indicated mouse strains (mean 6 SEM, n = 10–22, *p , 0.05).
less activated CD4⁺ T cells and T follicular helper cells than WT recipients upon transfer of bm12 CD4⁺ T cells (figure 8a/b). Moreover, both IFNAR⁻/⁻ and STING⁻/⁻ mice developed considerably less activated B cells, plasma cells and pathogenic anti-dsDNA IgG2a antibodies than WT recipients (figure 8a/c/d). Given that the transferred bm12-CD4⁺ T cells were IFNAR- and STING-sufficient, these data further support the role for antigen presenting cell-intrinsic STING and IFNAR in the induction of adaptive immune responses to dying cell-derived antigens.
Discussion

Type I IFNs have been implicated as the upstream events precipitating autoimmune disease and a prerequisite for effective anti-tumor radiotherapy. Here we identify DC sensing of cell-derived nuclear DNA entities via the STING/IRF3 pathway as a key component in the early type I IFN response to dying cells.

Dying cells can emit a plethora of structurally distinct DAMPs and it is likely that the molecular pathways involved in the sensing of these DAMPs are equally diverse. While many DAMPs can contribute to the final adaptive immune response to cell-associated antigens, our data identified type I IFN as the dominant pro-inflammatory factor and STING/IRF3 signaling as the principle pathway in the initiation of the immune responses to cell-associated antigens in our tumor models as well as the autoimmune responses in our SLE model. Although all nucleated cells can sense type I IFN and type I IFN has been shown to directly act on T cells, our data indicate that the early STING/IRF3-mediated type I IFN predominantly acts on DCs. Transfer of IFNAR\(^{-/-}\) DCs (pulsed with irradiated cells) into WT recipients resulted in similar deficiencies in CD8\(^+\) T cell expansion, functionality and memory formation as the direct immunization of IFNAR\(^{-/-}\) mice. Moreover, immunization of WT/IFNAR\(^{-/-}\) mixed bone marrow chimeric mice did not show any significant difference in CD8\(^+\) T cell clonal expansion or polyfunctionality between the WT and IFNAR\(^{-/-}\) grafts. Importantly, transfer of STING\(^{-/-}\) or IRF3\(^{-/-}\) DCs into WT recipients resulted in similar defects in CD8\(^+\) T cell responses as the transfer of IFNAR\(^{-/-}\) DCs. Likewise, STING\(^{-/-}\) recipients showed identical reduction in Tfh cell and plasma cell formation as IFNAR\(^{-/-}\) recipients in our SLE model. Together with the observation that STING\(^{-/-}\) and IRF3\(^{-/-}\) DC have significantly reduced type I IFN induction upon phagocytosis of dying cells, these data implicate that the STING/IRF3 pathway is the critical component in the type I IFN-dependent T cell priming to cell-associated antigens.
It has been suggested that different types of death may induce different DAMPs. We observed comparable type I IFN induction and STING/IRF3 engagement in DCs upon phagocytosis of cells treated with gamma irradiation, UV irradiation, Fas-crosslinking antibody, or etoposide, suggesting that these different types of cell death generated a similar nuclear DNA-derived DAMP (figure S2b, and not shown). It is also likely that similar nuclear DNA-associated DAMPs become available in vivo as type I IFN induction and type I IFN-dependent CD8\(^+\) T cell priming was readily observed in WT and MyD88/Trif deficient mice, but significantly reduced in STING\(^{-/-}\) and IRF3\(^{-/-}\) mice upon administration of dying cells (gamma, UV, or FAS-treated) or upon in vivo tumor cryo-ablation (11, 22).

Importantly, the crucial role for type I IFN in the priming of protective adaptive anti-tumor responses is not restricted to situations where massive tumor cell death occurs, as is the case for radiotherapy, chemotherapy, and cryoablative tumor therapies. Recent publications indicate that spontaneous and limited tumor cell death in tumor-bearing mice also resulted in type I IFN-dependent protective immune responses. Fuertes and Diamond showed spontaneous anti-tumor CD8\(^+\) T cell induction and tumor rejection in tumor-bearing mice that was critically dependent on type I IFN sensing by cross-priming DCs (6, 7). In this light, it is interesting to notice that STING\(^{-/-}\) mice—like IFNAR\(^{-/-}\) mice—develop significantly more lung metastases than WT mice upon i.v. injection of low numbers of untreated B16 melanoma cells, illustrating a role for the STING/IFNAR nexus in the anti-tumor response when cell death is limited (not depicted).

STING can facilitate innate responses to cytosolic bacterial cyclic dinucleotides (c-di-GMP) (30, 31), dsDNA and in some cases cytosolic dsRNA (26-28). Our data strongly suggest that the main type I IFN-inducing ligand is a nuclear DNA species. STING-mediated dsRNA sensing requires RNA with 5’-triphosphate groups in combination with the IPS-1/RIG-I pathway. The absence of IPS-1 recruitment to STING in WT DCs upon
phagocytosis of cellular materials as well as the normal type I IFN production and CD8+ T cell responses in IPS1-/- mice, effectively argue against a role for dsRNA sensing in the STING/IFN phenotype. Our hypothesis that the type I IFN inducing ligand is a DNA structure is strongly supported by our in vitro data that show significantly reduced type I IFN induction upon addition of DNAses to the dying cell/DC co-culture. Moreover, the use of enucleated cells dramatically reduced type I IFN production and cross-priming by WT DCs in vitro. Importantly, the latter experiment also suggested that mitochondrial or ribosomal nucleotide structures had no notable role in the type I IFN production as reticulocytes—enucleated but still containing mitochondria and ribosomes—failed to induce type I IFN.

Recent studies indicate direct binding of c-di-GMP and cyclic-GMP-AMP (cGAMP) to STING but have not provided evidence for direct dsDNA-STING interactions (32, 33) suggesting the involvement of upstream DNA sensors. Over the last few years several of candidate sensors have been identified, including cyclic GMP-AMP synthase (cGAS), which has been shown to signal through STING via the production of cGAMP(34-37). DExD/H-box protein family member DDX41 and IFI16 (p204) have also been implicated as possible DNA sensors acting through STING, but their precise molecular interactions have not been fully elucidated (38, 39). At present these candidate DNA sensors are studied in in vitro systems where the DNA is directly delivered into the cytosol via transfection, transduction or infection pathways. However, in order for the phagocytosed DNA-derived structures to be sensed by the STING pathway, either its key components should be recruited to the phagosome or the DNA-derived species should escape into the cytosol. Although STING can translocate from the ER to the Golgi and autophagosome-like compartments, we and others did not observe STING in phagosomes or phagolysosomes (not shown) (40-44). However, phagosomal DNA sensing via STING could still be possible by phagosomal recruitment of p204 that has reported migratory capacity or cGAS
that produces the highly mobile secondary messenger cGAMP (34, 36, 39). On the other hand, phagosomal escape is a well reported process in cross-presentation where protein structures escape into the cytosol to be processed for presentation in MHC class I (20, 45). The exact mechanism by which proteins escape into the cytosol is not known but it is strongly associated with alkalinization of the phagosome and prevention of phagosomal acidification (20, 45, 46). Consistent with the latter possibility, STING-mediated type I IFN production was strongly associated with inhibition of phagosomal acidification (45); in vitro treatments of DCs with agents that accelerated phagosomal acidification decreased type I IFN production while alkalinization or the delay of endosomal acidification significantly enhanced type I IFN production (figure S3). Moreover, we found that DC populations that have the greatest capacity for cross-presentation and slowest phagosomal acidification rate also produced the most type I IFN upon phagocytosis of dying cells (data not shown and (11, 12, 47)).

While the exact mechanism by which the nuclear DNA-derived structure activates the STING pathway needs further elucidation, our data strongly demonstrate its potent role in anti-tumor immunity and autoimmunity. Our observations are in line with the findings that mice lacking DNAse II, responsible for degradation of phagocytosed DNA, die of TLR-independent pro-inflammatory cytokine production unless the mice are crossed to the IF-NAR−/− or STING+/− background (48-51). Intriguingly, gain of function mutations in STING were recently associated with vasculopathy with onset in infancy (SAVI), a syndrome characterized by a severe cutaneous vasculopathy leading to extensive tissue loss and structural damage, with neonatal-onset systemic inflammation(52). Moreover, the deletion of TREX1 –DNAse III, a DNA exonuclease with functions in DNA degradation– was shown to trigger type I IFN-associated inflammatory responses in a cGAS-dependent manner(53, 54). Furthermore, mutations in TREX1 have been associated with a variety of (multi-organ) inflammatory syndromes, including SLE, Sőgrens Syndrome, chilblain
Together our data indicate that sensing of nuclear DNA-derived structures via the STING/IRF3 pathway in DCs is responsible for the early type I IFN induction upon phagocytosis of dying cells. This early type I IFN directly acts on the DCs and endows them with greater capacity to activate adaptive immune responses to cell-associated antigens. Given that type I IFNs have been implicated as the upstream events precipitating autoimmune disease and a prerequisite for effective anti-tumor therapy further characterization of the STING pathway as sensor of dying cell-derived DNA could enable the rational design of new therapies to enhance anti-tumor responses and interfere with the development and progression of autoimmune diseases.

**Acknowledgments**

We are grateful to Dr. H. Singh for comments and discussion on the manuscript; Dr. T. Kalfa for providing reagents and advice for the in vitro erythropoiesis experiments; A. White and M. DeLay for support with the Amnis Imagestream; and the Research Flow Cytometry Core at Cincinnati Children’s Hospital Medical Center.

This work was supported by the National Institutes of Health via National Cancer Institute grant CA138617 (E.M.J.) and National Institute of Diabetes and Digestive and Kidney Diseases grant DK090978 (J.K./E.M.J.) and the Charlotte Schmidlapp Award (E.M.J.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.
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Chapter 6: Type I interferon protects CD4 T cells from NK cell killing in a cGVHD model of SLE*

*The following manuscript is in preparation for submission.

Klarquist J, Hennies CM, Lehn MA, and Janssen EM.
Abstract

Myriad studies have linked type I interferon (IFN) to the pathogenesis of autoimmune diseases, including systemic lupus erythematosus (SLE). While increased levels of type I IFN are found in patients with SLE, and IFN blockade ameliorates disease in many mouse models of lupus, its precise roles in driving SLE pathogenesis remain largely unknown. Here, we dissected the role of type I IFN on disease development using the bm12 cGVHD model of SLE, where IA\(^b\) (C57BL/6) mice develop SLE-like disease upon transfer of IA\(^{bm12}\) (bm12) CD4 T cells. Our data show increased serum levels of type I IFN over time, concomitant with the development of T follicular helper cells (Tfh), germinal center (GC) B cells, plasmablasts, and anti-nuclear antibodies (ANA). Previously, we showed that these disease parameters were significantly reduced in IFN-α receptor-deficient (Ifnar\(^{-/-}\)) recipient mice, indicating an important role for type I IFN sensing by non-T cells. In this study, we assessed the effects of type I IFN sensing by CD4 T cells on the development of bm12 disease. Surprisingly, transfer of highly purified Ifnar\(^{-/-}\) bm12 CD4 T cells into WT C57BL/6 hosts resulted in poor expansion of the transferred T cells and limited development of GC B cells, plasmablasts, and ANA, indicating a need for IFN sensing by CD4 T cells. Transfer into perforin-deficient animals, or antibody-mediated natural killer (NK) cell depletion restored the expansion of Ifnar\(^{-/-}\) CD4 T cells and downstream sequelae, whereas the depletion of CD8 T cells had no effect on disease. These findings suggest a novel mechanism by which type I IFN contributes to autoimmune disease is by regulating the susceptibility of pathogenic CD4 T cells to NK cell killing, thus providing further rationale for the development of anti-IFNAR therapeutics for the treatment of SLE.
Introduction

The overproduction of type I IFN is a prominent feature associated with the development of SLE and has been implicated in the pathology of other autoimmune diseases including Sjögren’s syndrome, systemic sclerosis, adult-onset rheumatoid arthritis, and possibly type I diabetes mellitus\(^1,2\). A role for type I IFN in driving disease pathology has been demonstrated in several genetic and inducible murine models of SLE\(^3-6\). Moreover, the therapeutic use of IFN to treat patients with hepatitis C virus and cancer has been associated with the induction of SLE\(^7-9\), and the administration of IFN to lupus-prone mice induced earlier onset, lethal disease\(^10\). Furthermore, a type I IFN gene signature correlates with disease severity in SLE patients\(^11,12\). While these studies strongly implicate type I IFN in promoting SLE pathogenesis, relatively little is known about the precise functions of IFN in SLE.

Type I IFN comprises 13 IFN\(\alpha\) proteins, IFN\(\beta\), IFN\(\kappa\), and IFN\(\omega\), all of which signal through a common receptor, known as the interferon (alpha and beta) receptor, or IFNAR. IFNAR is expressed by essentially all nucleated cells and plays well described, key roles in antimicrobial defenses by conferring direct antiviral effects, inhibiting cellular growth, controlling apoptosis, and by promoting antimicrobial immune responses\(^13\). In SLE, one prevailing hypothesis is that by some combination of genetic and environmental factors, an accumulation of dying cells leads to increased exposure of self-RNA and self-DNA, which induces high levels of IFN production by plasmacytoid dendritic cells (DCs)\(^14-17\). Relatively few studies, however, have sought to characterize the mechanism of action of IFN specifically in SLE. Studies of SLE peripheral blood-derived DCs by Blanco et al. indicated a likely role of IFN sensing by DCs in SLE pathogenesis\(^18\). And, although the role for type I IFN sensing by B cells has not been specifically examined in the context SLE, it may also be important, given that type I IFN is known to promote B cell activation, class switching, and support the generation of antibody secreting plasma cells\(^19-21\).
CD4 T cells have been shown to provide vital B cell help during SLE development\textsuperscript{22–25}, and several studies have demonstrated that type I IFN sensing by CD4 T cells can dramatically enhance their priming in the context of certain viral infections and vaccines\textsuperscript{20,26,27}. Additionally, total disruption of type I IFN signaling nearly completely abrogated disease in several mouse models of SLE\textsuperscript{3–6}, whereas in our previous studies we observed only partial reduced disease reduction in $\text{Ifnar}$-deficient recipients of WT bm12 CD4 T cells. We therefore investigated the potential role for type I IFN sensing by CD4 T cells in the bm12 chronic graft-versus-host disease model of SLE, a well established murine model of SLE with clinical signs that correspond to those of SLE patients, including the development of ANA, lupus nephritis, and a recognized role for type I IFN\textsuperscript{28–30}. Here we show that type I IFN acts directly on CD4 T cells to dramatically increase the expansion of T follicular helper cells (Tfh), GC B cells, plasmablasts, activated B cells, and the development of ANA in the bm12 chronic graft-versus-host disease model of SLE. Surprisingly, we found that IFN-insensitive Tfh were selectively killed by NK cells, and the specific depletion of NK cells restored their expansion and allowed normal disease development. Our data provide evidence for novel roles of type I IFN and immunoregulatory NK cells in SLE, providing potential new targets for the development of future immunotherapies.
Results

IFN sensing by CD4 T cells is critical to the expansion of donor CD4 T cells and pathogenic B cell subsets in the bm12 cGVHD model of SLE

Previously, we found that upon the transfer of bm12 cells, development of SLE-like disease was dependent upon recipient expression of the type I IFN receptor (Ifnar). Recipient mice lacking Ifnar showed reduced expansion of Tfh, plasmablasts, and anti-nuclear antibodies (ANA). In order to better understand the function of type I IFN in this model, we investigated the kinetics of IFN production and the role of IFN sensing by transferred CD4 T cells.

Using a highly sensitive bioassay, we found that type I IFN increased over time in the serum of C57BL/6 mice injected with bm12 CD4 T cells. Moreover, we observed a corresponding increase in the serum levels of the type I IFN-inducible chemokines CXCL9 and CXCL10 (Fig 1a, and Fig S1a-c). Additional inflammatory cytokines including TNFα, IL-12, and the type II IFN, IFNγ were also induced, though these were present in relatively lower quantities and at generally delayed kinetics compared to the type I IFNs and the IFN-inducible chemokines (Fig 1a, and Fig S1d-g).

To explore additional possible roles for type I IFN in SLE, we next asked whether or not IFN sensing by CD4 T cells was important for driving disease development in the bm12 model. We generated CD45.1+ congenic bm12 mice lacking Ifnar, hereafter referred to as Ifnar−/− bm12 mice. Highly purified CD4 T cells from either wild type bm12 mice (also CD45.1+) or Ifnar−/− bm12 mice were adoptively transferred into C57BL/6 recipients, and after two weeks, mice were assessed for disease development. Surprisingly, we found a 3-fold greater accumulation of WT bm12 CD4 T cells compared to Ifnar−/− bm12 CD4 T cells (Fig. 1b,c). B cell responses in these mice were similarly affected. Specifically, we observed significant reductions in splenic plasmablasts (CD138+B220low), germinal cen-
Figure 1. Type I IFN sensing by CD4 T cells is critical for bm12 disease. C57BL/6 mice were injected with $8 \times 10^6$ purified WT bm12 CD4 T cells. At the indicated days after injection, serum type I IFN was analyzed by bioassay and additional cytokines and chemokines were analyzed by multiplex technology (a). In separate experiments, C57BL/6 mice were injected with $8 \times 10^6$ purified CD45.1$^+$ WT or Ifnar$^{-/-}$ bm12 CD4 T cells (n=11/group) and mice were sacrificed after 14 days (b-f). Representative flow cytometry plots depict the gating strategy for analysis of splenic bm12 CD4 T cells within C57BL/6 recipient mice (b). The absolute number of splenic bm12 CD4 T cells 14 days after transfer (c). Representative splenic B cell population gating (d). B cell populations were first gated on live cells, as in (b). Plasmablasts were then defined as CD138$^+$B220$^{low}$, GC B cells were B220$^+$CD138$^-$Fas$^+$GL-7$^+$, and activated B cells were B220$^+$CD138$^-$GL-7$^-$CD69$^+$. Splenomegaly was assessed by comparing spleen mass (f). Serum anti-dsDNA antibodies were assessed by ELISA. Data are combined from 3 representative experiments, depicting mean ± SEM, where $^*p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$ by the student’s t test.

WT and Ifnar$^{-/-}$ bm12 CD4 T cells both differentiate into T follicular helper cells

Next, we sought to determine whether type I IFN sensing controlled the fate of bm12 helper T cell differentiation. Previously, we showed that proliferating bm12 CD4 T cells adopted a T follicular helper cell (Tfh) phenotype as early as three days post transfer.
into C57BL/6 recipients. Here, both WT and Ifnar−/− bm12 CD4 T cells differentiated into Tfh, expressing high levels of PD-1, CXCR5, and the master regulator transcription factor, Bcl-6 (Fig. 2a). To specifically analyze those cells which were actively dividing, we also performed experiments where sorted bm12 CD4 T cells were labeled with a violet proliferation dye prior to transfer into recipient animals. After 2 weeks, essentially all of the violet dye-diluted, proliferating bm12 CD4 T cells were identified as Tfh, regardless of their genotype (Fig. 2b); this result was of particular importance, given recent contrasting reports on the effect of type I IFN on Tfh differentiation. Furthermore, mRNA levels were equivalently upregulated for the prototypical Tfh genes Pdcd1, Cxcr5, Bcl6, Icos, and Il21, whereas Ccr7 was downregulated in both WT and Ifnar−/− bm12 CD4 T cells (Fig. 2d). Taken together, these data indicate that type I IFN insensitivity did not block differentiation of the transferred Ifnar−/− cells into Tfh.
Type I IFN acts directly on CD4 T cells to improve their expansion

Because Tfh and GC B cell responses are reciprocally supportive of one another, we considered the possibility that type I IFN may have acted indirectly to promote the expansion of bm12 Tfh. That is, IFN sensing by bm12 CD4 T cells may have caused changes within the bm12 Tfh that better supported a germinal center B cell response. In turn, the boosted GC B cell response could have resulted in enhanced Tfh expansion. To determine whether type I IFN directly or indirectly enhanced CD4 T cell expansion, we transferred both WT and Ifnar−/− CD4 T cells into the same recipient mice, where they would have access to the same environment. Here, CD45.2 WT bm12 CD4 T cells were combined with CD45.1 Ifnar−/− bm12 CD4 T cells at a 1:1 ratio and transferred into CD45.1/2 recipient mice; for comparison, single transfers of only WT or Ifnar−/− cells were also performed using CD45.1/2 recipients (Fig. 3a). Splenic plasmablast, GC, and activated B cell populations were significantly greater in mice which received bm12 CD4 T cells from

Figure 3. Type I IFN acts directly on Tfh to enhance their accumulation. Eight million WT CD45.2 and Ifnar−/− bm12 CD4 T cells were transferred separately (a, left/middle) or together “Mix” (a, right) into WT CD45.1 recipient animals. After 14 days, responding B cells were enumerated (b) and bm12 cells from distinct donors were discriminated by congenic marker expression (c). The total numbers of bm12 Tfh were compared between recipients of either WT bm12, Ifnar−/− bm12, or a equal mixture of both (d). Data are from one representative of three independent experiments, depicting mean ± SEM, where *p<0.05, **p<0.01, and ***p<0.001 by the student’s t-test. A paired t-test was used to compare Tfh within the “Mix” in (d).
both WT and $Ifnar^{+/-}$ donors (mix) compared to those which received $Ifnar^{-/-}$ bm12 CD4 T cells alone, but were no different from those which received only WT bm12 CD4 T cells (Fig. 3b). In the mice which received cells from both donors, $Ifnar^{-/-}$ CD4 T cells had access to the same B cells as did their WT counterparts; yet, we still observed a significantly greater accumulation of WT cells over $Ifnar^{-/-}$ cells, demonstrating a direct effect of type I IFN on CD4 T cell accumulation (Fig. 3c-d).

IFN sensing does not prevent grafting

To determine whether type I IFN sensing improved the initial grafting of WT over $Ifnar^{-/-}$ CD4 T cells, which could have thereby significantly contributed to the enhanced accumulation of the WT Tfh, we transferred an equal mixture of CD45.2$^+$ CD90.2$^+$ WT and CD45.1$^+$ CD90.2$^+$ $Ifnar^{-/-}$ CD4 T cells into CD45.2$^+$ CD90.1$^+$ recipient mice. The cells used here were from mice which had not been crossed to the bm12 background, so they had proliferated only nominally, eliminating the potential confounding effects of divergent proliferation rates (data not shown). At one and two weeks post transfer, the CD4 T cells had not deviated from their initial 1:1 ratio in the spleen nor in the blood (Fig. S2), indicating that WT and $Ifnar^{-/-}$ CD4 T cells had grafted and persisted equally in this non-inflammatory setting.

Next, we confirmed there were no grafting disparities in the context of bm12 disease. CD4 T cells from WT or $Ifnar^{-/-}$ bm12 donors were labeled with a violet proliferation dye and injected into C57BL/6 hosts. The non-proliferated cells (proliferation dye-positive), which represent the majority of the transferred bm12 cells that do not react to recipient IA$^b$, were enumerated 2 weeks post transfer. Indeed, the number of non-proliferated WT and $Ifnar^{-/-}$ bm12 CD4 T cells were equal despite a reduced accumulation of proliferating cells (Fig. 4), indicating that the donors cells had also grafted equally in the context of the inflammation associated with bm12 disease.
IFN inhibits ongoing proliferation of mouse CD4 T cells in vitro

*In vivo* proliferation data can be skewed by silent clearance of dying cells or migration away from target organs; consequently, we examined the effect of IFN sensing on CD4 T cell proliferation *in vitro*. Highly purified CD4 T cells from WT and *Ifnar*<sup>-/-</sup> donors were combined at a 1:1 ratio in 96-well plates and stimulated with anti-CD3 and anti-CD28. WT and *Ifnar*<sup>-/-</sup> cells were distinguished from one another using congenic markers. Cells were cultured in the absence of exogenous IFN, or with the addition of IFNβ at 0, or 24 hours after culture initiation, and all cells were harvested after a total of 72 hours in culture. IFNβ inhibited the proliferation of activated WT, but not *Ifnar*<sup>-/-</sup> CD4 T cells (*Fig. 5*), and a comparable result was observed with IFNα (*Fig. S3*). These data demonstrated that the anti-proliferative effect of IFN involved direct signaling within the CD4 T cell, since the WT and *Ifnar*<sup>-/-</sup> cells were co-cultured, and IFN had no effect on *Ifnar*<sup>-/-</sup> proliferation. Furthermore, the ongoing proliferation of murine CD4 T cells was inhibited by type I IFN, as equivalent results were obtained from cultures where IFN was added immediately upon culture initiation and those in which IFN was added 24 hours later (*Fig. 5*). These data were seemingly at odds with the phenomenon observed *in vivo*, where IFN sensing allowed for the increased accumulation of WT CD4 T cells, we therefore shifted our focus toward the potential effect of type I IFN on the survival of these cells.
Ifnar<sup>−/−</sup> CD4 T cells show no cell-intrinsic survival disadvantage

Prior work on IFN has demonstrated a role for IFN in keeping activated T cells alive<sup>35</sup>. We considered that this mechanism may have contributed to the impaired accumulation of Ifnar<sup>−/−</sup> CD4 T cells in our bm12 model, so we performed experiments measuring the survival of our highly active bm12 CD4 T cells directly <i>ex vivo</i>. One week after transferring CFSE-labeled WT or Ifnar<sup>−/−</sup> bm12 CD4 T cells into C57BL/6 recipients, splenocytes were plated for 24 hours in medium containing serum, but without the addition of T cell activating factors. Survival was determined by comparing the number of live, proliferating bm12 cells directly <i>ex vivo</i>, to the number of live, proliferating bm12 cells remaining after 24 h. 

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**Figure 5. CD4 T cell proliferation is inhibited by type I IFN <i>in vitro</i>.** Highly purified CD4 T cells from WT CD45.2 and Ifnar<sup>−/−</sup> CD45.1 mice were labeled with a violet proliferation dye and combined at a 1:1 ratio in 24 well tissue culture plates. Cells were stimulated with plate-bound anti-CD3 and soluble CD28 in the presence or absence of recombinant IFNβ, which was administered at the initiation of the culture (top panels), or at 24 h after culture initiation (bottom panels). Data are from 1 representative of 3 independent experiments.

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**Figure 6. WT and Ifnar<sup>−/−</sup> bm12 CD4 T cells display equivalent survival and Bcl-2 family member expression <i>ex vivo</i>.** Bm12 cells were labeled with a violet proliferation dye prior to transfer into C57BL/6 hosts. Seven days after transfer, recipient splenocytes were plated in complete medium containing fetal bovine serum with or without 50 U/ml recombinant IFNβ. Survival of the violet-dye diluted, proliferating bm12 cells was determined by calculating the number of proliferating cells plated, dividing that number by the number of proliferating cells remaining after 24 h, and multiplying by 100% (a). Proliferating bm12 and non-proliferating bm12 cells were also assessed by flow cytometry directly <i>ex vivo</i> for pro- and anti-apoptotic Bcl-2 family member protein expression (b). Data are from 1 representative of 2 independent experiments, depicting mean ± SEM.
after 24 hours of *in vitro* culture. WT and *Ifnar*<sup>−/−</sup> bm12 CD4 T cells survived *ex vivo* culture equally in the absence of exogenous IFN. Although the addition of IFNβ slightly increased the survival of WT cells, this increase was not significant (*Fig. 6a*). Another method we employed to identify a possible survival disadvantage in *Ifnar*<sup>−/−</sup> cells was to analyze the expression of Bcl-2 family member proteins of proliferating bm12 cells directly *ex vivo*. Intracellular flow staining revealed an altered pattern of Bcl2 family member expression among proliferating (CFSE-negative) bm12 CD4 T cells compared to non-proliferating (CFSE-high) cells. Most notably, proliferating cells expressed high levels of the anti-apoptotic B!cl2A1 protein, which is suspected to play an important role in keeping activated CD4 T cells alive<sup>36,37</sup>; however, no differences were noted between WT and *Ifnar*<sup>−/−</sup> cells (*Fig. 6b*). Together, these data demonstrated that *Ifnar*<sup>−/−</sup> bm12 CD4 T had no intrinsic survival defect compared to their WT counterparts.

**IFN protects Tfh from perforin-mediated cell death**

We postulated that, since type I IFN imparted no obvious cell-intrinsic survival advantage, IFN may be protecting Tfh from cell-extrinsic pressure, namely, killing by NK cells or CD8 T cells. Therefore, we transferred WT or *Ifnar*<sup>−/−</sup> bm12 CD4 T cells into C57BL/6 or perforin-deficient (*Prf1*<sup>−/−</sup>) recipients. In C57BL/6 recipients, we again saw a 3-fold greater expansion of WT over *Ifnar*<sup>−/−</sup> bm12 Tfh. Transfer into *Prf1*<sup>−/−</sup> recipients, however, not only improved the expansion of *Ifnar*<sup>−/−</sup> cells, but it resulted in a significantly greater accumulation of *Ifnar*<sup>−/−</sup> Tfh than WT Tfh (*Fig. 7a*), perhaps now revealing an anti-proliferative effect of type I IFN *in vivo* similar to that which was observed *in vitro*. The enhanced expansion of *Ifnar*<sup>−/−</sup> Tfh in *Prf1*<sup>−/−</sup> animals dramatically augmented overall disease in these animals, including increases in plasmablasts, GC, and activated B cells, splenomegaly, and serum ANA (*Fig. 7b-d*).
IFN protects Tfh from NK cell killing in bm12 lupus-like disease

Since the two most obvious candidates for perforin-mediated cell killing are CD8 T cells and NK cells, we decided to deplete these two cell subsets in separate experiments. NK cells were specifically depleted by i.p. administration of anti-asialo GM1 antibodies prior to the transfer of bm12 CD4 T cells from WT or Ifnar-/- mice (ref38,39 and Fig. S4). NK cell depletion resulted in a significantly increased accumulation of Ifnar-/- bm12 Tfh compared to that in control, NK-sufficient animals (Fig. 8a). Although a subtle increase in WT bm12 Tfh was also seen upon NK cell depletion, this was not significant. As in experiments using Prf1-/- recipients, Ifnar-/- bm12 CD4 T cells transferred to NK-depleted recipients resulted in augmented signs of SLE-like disease, including activated B cell subsets, splenomegaly, and ANA (Fig. 8b-d). Highly efficient, specific depletion of CD8 T cells was achieved by i.p. injection of an anti-CD8β antibody (Fig. S5a); however, this had no effect on the expansion of Tfh (Fig. S5b). These data indicate that NK cells killed Ifnar-/- Tfh in a perforin-dependent manner, and that type I IFN sensing by WT Tfh protects them from NK killing and thereby promotes Tfh expansion and resultant disease in this cGVHD model of SLE.
IFN promotes development of plasmablasts and GC B cells, but B cells are not NK targets

A limited number of studies have examined the role of IFN sensing by B cells. The development of plasma cells was shown to be augmented by exogenous type I IFN\textsuperscript{19}, as was their ability to produce antibody\textsuperscript{20}. Isotype class switching was also found to be enhanced by exogenous IFN, but this appeared to function through DC IFN sensing\textsuperscript{21}. We hypothesized that the endogenous type I IFN produced in bm12 disease also enhanced the development of plasmablasts and GC B cells. We also wondered whether these activated B cell subsets may also be targets of NK cells in this disease setting. To test these hypotheses, we generated mixed bone marrow chimeras (Fig. 9a). Briefly, bone marrow from CD45.2\textsuperscript{+} WT and CD45.1\textsuperscript{+} Ifnar\textsuperscript{−/−} mice on a C57BL/6 background were mixed at a 1:1 ratio and transplanted into lethally irradiated CD45.2\textsuperscript{+} C57BL/6 recipients. After 12 weeks, half of the mixed bone marrow chimeric mice were depleted of NK cells using the same antibody treatment strategy described above, and half were not depleted. These mice were then given purified CD45.1\textsuperscript{+}/CD45.2\textsuperscript{+} WT bm12 CD4 T cells. After 2 weeks, the mice were analyzed for disease development. NK cell depletion had no significant im-
impact on splenic B cell populations or on any other parameter of disease development—in fact splenomegaly was slightly, but insignificantly reduced in mice receiving NK depleting antibody (Fig. 9b). We did, however, observe a significant impact of B cell genotype on the development of plasmablasts and GC B cells. Specifically, there was a significantly greater proportion of WT than Ifnar<sup>−/−</sup> plasmablasts and GC B cells, whereas their proportions were equal in the non-activated (CD138<sup>−</sup>GL7<sup>−</sup>CD69<sup>−</sup>B220<sup>+</sup>) B cell populations (Fig. 9c). These data confirm earlier reports that type I IFN can promote the development of plasmablasts, and go beyond these findings, demonstrating that this can occur in the context of endogenously produced type I IFN during the development of lupus-like autoimmunity. Furthermore, the data show that the development of GC B cells can also be augmented by the direct action of type I IFN.

Figure 9. B cell IFN-sensing augments plasmablast and GC B cell development. Depicted is the schema used for a mixed bone marrow chimera. Briefly, bone marrow from WT and Ifnar<sup>−/−</sup> mice was combined at a 1:1 ratio and transplanted into lethally irradiated C57BL/6 mice. Twelve weeks later, NK cells were depleted in half the mice using anti-asialo GM1 antibody. Then, all mice were given 8 million purified WT bm12 CD4 T cells. (a). Two weeks after transfer, mice were assessed for splenomegaly (b). The percentages of WT and Ifnar<sup>−/−</sup> cells were then determined within the resultant plasmablasts, GC B cells, and non-activated B cells (c). Data depict the mean ± SEM (n=4-6/group), where *p<0.05, **p<0.01, ****p<0.0001 by the student’s t-test.
Discussion

A connection between the overproduction of type I IFN and the development of SLE has been well-established, yet the mechanisms by which IFN promotes disease pathology have not been fully elucidated. In this study, we identified a key role for type I IFN in protecting CD4 T cells from NK cell-cytotoxicity in a murine model of SLE.

The potential for immunoregulation of T cell activity by NK cells has been uncovered in recent years. Mounting evidence has established a role for NK cells in limiting T cell responses toward lymphocytic choriomeningitis virus (LCMV) by direct killing of virus-specific T cells\textsuperscript{40–43}. Recently, NK cytotoxicity toward LCMV-specific T cells was shown to be inhibited by type I IFN\textsuperscript{44,45}. These reports were consistent with data published over four decades earlier, in which Hansson, Welsh, and colleagues showed that either LCMV infection, the induction of type I IFN by poly I:C, or pre-treatment with exogenous IFN reduced the sensitivity of normal mouse thymocytes to NK cells \textit{in vitro}\textsuperscript{46,47}. Here, we present the novel finding that type I IFN also inhibits a similar \textit{in vivo} regulation of activated CD4 T cells by NK cells in a sterile model of systemic autoimmune disease.

Taken together, these data suggest the intriguing possibility that peripheral tolerance may be maintained in part by the default NK killing of highly active T cells, and that activated T cells can be licensed by type I IFN during certain infections. Aberrant overproduction of type I IFN in a healthy individual could contribute to the inadvertent licensing of self-reactive T cells, and might thus be capable of initiating autoimmune disease. This may be one mechanism behind the induction of autoimmunity, including SLE, which occurs in some patients after the administration of type I IFN to treat conditions such as chronic hepatitis C viral infection\textsuperscript{7–9,48}. One question this raises is how T cells avoid NK killing during positive selection in the thymus. Positive selection involves moderately strong activation of T cells toward self-antigens, and \textit{bulk} thymocytes are indeed sensitive to killing by NK cells.
ex vivo\textsuperscript{46,47}. Type I IFNs, however, are massively upregulated in the thymus, particularly in the medulla, and the type I IFN-inducible protein MX1 is preferentially expressed on mature, single-positive thymocytes, which have recently undergone positive selection, suggesting they are especially sensitive to type I IFN, or that the signaling pathways downstream of IFN is enhanced in these cells\textsuperscript{49}. One function of type I IFN in the thymus might therefore be to limit NK killing of activated T cells during and after positive selection. Importantly, MX1 expression is limited to fetal and postnatal thymic tissues, and was not observed in secondary lymphatic tissues or in the peripheral blood of healthy adults, consistent with a role for type I IFN in licensing activated T cells in the periphery. Of course type I IFN sensing by T cells alone may not be necessary or sufficient for this type of licensing in all inflammatory settings—it has been reported to be critical for T cell responses in multiple additional viral infections and vaccination strategies\textsuperscript{20,26,50,}, but also dispensable in others\textsuperscript{27,51}, and not all productive inflammation involves type I IFN production.

The pleiotropic effects of type I IFN have been recognized for some time. It was therefore important to examine the possibility that the relatively poor expansion of \textit{Ifnar}\textsuperscript{-/-} bm12 CD4 T cells in our model was due to multiple direct effects of type I IFN. In fact, IFN likely also affected the proliferation of the transferred bm12 cells. Our \textit{in vivo} proliferation data showed that both WT and \textit{Ifnar}\textsuperscript{-/-} bm12 CD4 T cells proliferate many rounds very early after transfer (\textbf{Fig. 4a}), but these data could not conclusively assess the effect of IFN on proliferation since they were in an open system, where cell death or migration could confound the results. Thus, we monitored the effect of IFN on purified CD4 T cells \textit{in vitro}. Not surprisingly, given the growing consensus regarding the anti-proliferative effect of IFNs on multiple cell types including T cells\textsuperscript{27,52,53}, we also found that type I IFN inhibited the proliferation of activated WT, but not \textit{Ifnar}\textsuperscript{-/-} CD4 T cells (\textbf{Fig. 5}). Here, in contrast to results from one commonly referenced study using primary human T cells\textsuperscript{54}, the ongoing
proliferation of our murine CD4 T cells was inhibited by type I IFN. This is an important point; given the sustained high levels of type I IFN in SLE and other autoimmune diseases, it is vital to understand whether IFN might influence ongoing T cell proliferation, in addition to any effect of IFN might have had on the initial priming of auto-reactive T cells. This is particularly relevant since type I IFN receptor blockade is currently being pursued as a treatment strategy for SLE in clinical trials\textsuperscript{55}. In addition to our \textit{in vitro} data and that of others, we had some evidence that IFN exerted an anti-proliferative effect \textit{in vivo}. When the NK immunoregulatory pressure was eliminated by transferring cells into \textit{Prf1}-deficient animals, \textit{Ifnar}\textsuperscript{−/−} CD4 T cell response was not only restored, but these cells expanded significantly more than did WT CD4 T cells (\textbf{Fig. 7}).

One additional explanation as to why we observed significantly greater expansion of WT compared to \textit{Ifnar}\textsuperscript{−/−} CD4 T cells could have been that IFN caused changes within the bm12 Tfh which better supported a germinal center B cell response, considering Tfh and GC B cell responses are reciprocally supportive of one another\textsuperscript{34}. This was an intriguing possibility, especially since type I IFN has been shown to induce IL-21 production in resting and activated human T cells\textsuperscript{56}, and GC B cells rely upon IL-21 for directing their differentiation and proliferation\textsuperscript{57}. The results from our mixed transfer experiments (\textbf{Fig. 3}), however, demonstrated that \textit{Ifnar}\textsuperscript{−/−} cells exposed to the same environment as WT cells still accumulated less, fitting our ultimate conclusion, that the \textit{Ifnar}\textsuperscript{−/−} cells are chiefly more sensitive to NK cell attack.

Early work by Marrack et al. demonstrated that type I IFN directly enhanced the survival of activated CD8 T cells, and to a lesser extent activated CD4 T cells\textsuperscript{35}. These experiments were done with cells from C57BL/10SgSnJ and 129/SvEv animals, and the extent to which IFN improved the survival of activated T cells varied depending on the mouse strain used. When we cultured our activated CD4 T cells \textit{ex vivo} for 24 hours, we saw
little effect of IFN, although we did use a relatively low concentration of IFNβ compared to what was used in that study, and our cells were from mice on a C57BL/6 background, which was not included in that particular study. Importantly, the T cells studied in the Marrack paper were enriched using nylon wool columns, which efficiently depletes B cells and some myeloid cell populations, thus enriching samples for T cells, however this method also enriches for NK cells. Therefore, type I IFN may in fact have kept activated T cells alive in that study by protecting them from NK cells, in addition to or in the absence of conferring any cell-intrinsic survival advantage.

One of the recent studies on NK immunoregulation in the context of LCMV showed that type I IFN protected T cells by upregulating an as of yet unidentified Ncr1 ligand or ligands. In our sterile inflammatory setting, however, Ifnar−/−Tfh expanded just as poorly in Ncr1-deficient animals as they did in C57BL/6 animals (2.5 ± 0.6 million, versus 3.0 ± 1.3, p=0.77). Furthermore, we were unable to detect Ncr1 ligands upon staining with an Ncr1-hIgG fusion protein. We did consistently observe increases in the expression of several other NK cell receptor ligands in cells from NK-depleted mice, including Icam-1, Icam-2, Mult-1, H-2Db, and Cd48, although these changes were not unique to Ifnar−/− cells, and the variations were generally small (Fig. S6). Moreover, an unbiased comparison of RNA transcript levels in these mice by RNAseq primarily revealed differential expression of a number of IFN-inducible genes, with no known involvement in sensitivity to NK cell attack (Fig. S7 and supplemental data file 1). This suggests that either a complex combination of signals drives NK cell sensitivity in our model, or the main effector ligand or ligands are as of yet unknown. Multiple key differences exist between our studies and those which identified a role for IFN in modulating Ncr1 ligand expression—perhaps most importantly, the studies by Crouse et al. were done in the context of viral infection rather than the sterile inflammatory disease studied here, and they examined monoclonal, TCR-transgenic SMARTA and P14 T cells, whereas we assessed polyclonal CD4 T cells.
Either or both distinctions may have contributed to contrasting results regarding the key protein interactions leading to NK cell killing.

Our study provides a significant contribution toward understanding the ways type I IFN can promote autoimmunity. Here, we demonstrated that the direct action of type I IFN on CD4 T cells renders them less susceptible to NK cell killing in an inducible model of SLE. Additionally, we found that plasmablasts and GC B cells were not NK targets in bm12 disease, however, the development of these cells was augmented by the direct action of type I IFN. As we begin to more precisely appreciate the many functions of type I IFN in SLE, this may enable the rational design of more targeted therapies for SLE and other autoimmune diseases with pathological type I IFN production.
Materials and Methods

Mice and cell lines

Mice were maintained under specific pathogen-free conditions in accordance with guidelines by the Association for Assessment and Accreditation of Laboratory Animal Care International. C57BL/6J, B6.SJL-Ptpcr<sup>a</sup> (CD45.1), B6.PL-Thy1<sup>a</sup>/CyJ (CD90.1), B6.129S2-Ighm<sup>tm1Cgn</sup>/J (μMT), C57BL/6-Prf1<sup>tm1Sdz</sup>/J (Prf1<sup>-/-</sup>), and B6(C)-H2-Ab1<sup>bm12</sup>/KhEgJ (bm12) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Ifnar<sup>1/-</sup> mice were originally a gift from Dr. Jonathan Sprent and have since been bred in our facility. ISRE-L929 IFN reporter cells have been described previously<sup>59</sup>.

Mixed bone marrow (BM) chimeric mice were generated using lethally irradiated C57BL/6 mice. Mice received an initial dose of 700 RAD followed by an additional 475 RAD dose 3 hours later using a <sup>137</sup>Cs irradiator (J L Shepherd & Associates, Inc., San Fernando, CA). BM from WT and Ifnar<sup>1/-</sup> donors on different congenic backgrounds and were mixed at a 1:1 ratio prior to reconstitution, and 6 million total BM cells were transferred via tail vein injection. All BM chimeric mice were given 12 weeks for hematopoietic reconstitution before experiments were started.

CD4 T cell isolation

For in vivo and in vitro assays, CD4 T cells were sorted to >97% purity by negative selection using magnetic bead sorting technologies from Miltenyi Biotec (Bergisch Gladbach, Germany) and BioLegend (San Diego, CA). Post sort purity was determined by flow staining for live cells, CD45, CD3, and CD4.

bm12 model

The bm12 model experiments were performed essentially as previously described<sup>31</sup>. Briefly, 8 million donor bm12 CD4 T cells were injected intraperitoneally into recipient
mice of the specified strain. Fourteen days later, unless indicated otherwise, spleens were harvested, massed, and processed into single cell suspensions for counting and flow staining. Serum was harvested by retro-orbital bleeding prior to sacrificing mice, processed using serum separator tubes (BD Biosciences, San Jose, CA) and stored at −80°C for later cytokine and ANA analyses.

**Serum cytokine and ANA analyses**

Circulating cytokines were analyzed in the serum of mice by Luminex multiplex technology (Austin, TX). Serum type I IFN was measured using the highly sensitive ISRE-L929 bioassay. Serum levels of anti-dsDNA were determined using ELISA essentially as described previously.\(^6\)

**NK and CD8 T cell depletion**

NK cells were depleted via an intraperitoneal injection of 10 μl anti-asialo GM1 rabbit antiserum (Wako, Richmond, VA) at -1, 1, 5, and 10 days after bm12 cell transfer. CD8 T cells were depleted by an intraperitoneal injection of 25 μg anti-CD8β (clone YTS156.7.7) one day prior to bm12 transfer.

**Proliferation assays**

Spleens and lymph nodes were harvested from the indicated mouse strains, purified, and stained with 3 μM CellTrace Violet (Life Technologies, Carlsbad, CA) per the manufacturer’s protocol. Cells were then stimulated in 24 well polystyrene plates with 1 μg/ml plate-bound anti-CD3 (clone 17A2) and 1 μg/ml soluble anti-CD28 (clone 37.51), using LEAF\(^\text{TM}\) purified antibodies from BioLegend. Cells were stimulated for 72 hours in medium containing 10% fetal bovine serum with penicillin, streptomycin, and L-glutamine with or without 50 U/ml recombinant IFN beta or IFN alpha A (PBL Assay Science, Piscataway, NJ). The recombinant IFN was added immediately at culture initiation unless otherwise
indicated. For *in vivo* assays, bm12 CD4 T cells were purified as described earlier, then stained with 3 μM CellTrace Violet immediately prior to intraperitoneal injection into recipient animals.

**Flow cytometry**

Unless otherwise specified, cells were collected and immediately stained directly *ex vivo*. For the bm12 model, cells were stained essentially as described previously\(^{31}\). Intracellular Bcl-6 (clone K112-91, BD Biosciences) staining was performed using the FoxP3 Staining Buffer Set (eBioscience, San Diego, CA). Samples were collected on a Fortessa flow cytometer with FACSDiva software (BD Biosciences), and data were analyzed with FlowJo software (Tree Star, Ashland, OR).

**Flow cell sorting and RNAseq**

Flow cytometric flow sorting was used to purify bm12 CD4 T cells to >99%. Splenic single cell suspensions were stained with the Fixable Viability dye ef780 (eBioscience), CD4, B220, CD45.1 and CD45.2. Viable CD4\(^+\)B220\(^−\)CD45.1\(^+\) cells were sorted at 4°C using a MoFlo XPD cell sorter (Beckman Coulter, Brea, CA) and immediately processed with mirVANA lysis buffer (Life Technologies). RNA was extracted according to the mirVANA RNA extraction kit protocol. Subsequently, 300 ng of total RNA was purified by the NEBNext Poly(A) mRNA Magnetic Isolation Module (New England BioLabs, Ipswich, MA). The NEBNext Ultra Directional RNA Library Prep Kit was used for library preparation. Bioanalyzer (Agilent, Santa Clara, CA) analyses using a DNA high sensitivity chip were performed to determine quality and yield of the purified library. To study differential gene expression, individually indexed and compatible libraries were proportionally pooled (20-50 million reads per sample) for clustering in cBot system (Illumina, San Diego, CA). Libraries at the final concentration of 15 pM was clustered onto a single read (SR) flow cell using TruSeq SR Cluster kit v3, and sequenced for 50 bp using TruSeq SBS kit on
an Illumina HiSeq system. Sequence reads were then aligned to the genome by using a standard Illumina sequence analysis pipeline by The Laboratory for Statistical Genomics and Systems Biology at the University of Cincinnati.
Acknowledgements
We are grateful to Dr. Stephen Waggoner for providing reagents and discussion, to Jeff Bailey and Victoria Sumney in the Comprehensive Mouse and Cancer Core for help with bone marrow chimera experiments, to Alyssa Sproles for technical support with the Luminex assays, and to Monica DeLay and the CCHMC Flow Core for flow cytometry and cell sorting support.

Disclosures
The authors have no financial conflicts of interest to disclose.
Supplemental Figures

Figure S1. Cytokine and chemokine analysis in bm12 disease. WT bm12 cells were transferred into C57BL/6 recipient animals. Type I IFN was assessed by a highly sensitive bioassay (a). Multiplex technology was used to assess serum levels of CXCL9 (b), CXCL10 (c), TNF (d), IFN (e), and IL-12p40 (f). Data are depicted as mean ± SEM.
Figure S2. WT and Ifnar−/− CD4 T cells graft equally in non-inflammatory conditions. Purified CD4 T cells from WT CD45.2/CD90.2 and Ifnar−/− CD45.1/CD90.2 mice were co-transferred into WT CD45.2/CD90.1 recipients. Representative flow cytometry plots show how transferred CD4 T cells were distinguished from recipient splenocytes (a, left), and the ratio of WT to Ifnar−/− cells (a, right). At 0, 6, and 14 days after transfer, the proportion of WT and Ifnar−/− CD4 T cells were assessed as in (a), demonstrating equal grafting and persistence of these cells over time (b). Data depict mean ± SEM.
Figure S3. Both IFNα and IFNβ inhibit the proliferation of WT CD4 T cells in vitro. Highly purified CD4 T cells from WT or Ifnar−/− mice were labeled with a violet proliferation dye and stimulated with plate-bound anti-CD3 and soluble anti-CD28 in vitro for 72 hours in the presence or absence of 50 U/ml recombinant IFNα3 or IFNβ.
Figure S4. Anti-asialo antibody treatment specifically and efficiently depletes NK cells. Representative flow cytometry plots show the specific depletion of NKp46⁺ NK1.1⁺ NK cells in anti-asialo GM1-treated mice compared to control animals immediately prior to injection of bm12 cells (a). Splenic NK cells, CD4 T cells, and B cells from these mice were enumerated prior to injection with bm12 cells (b).
Figure S5. Anti-CD8β antibody treatment depletes CD8 T cells, but does not affect the expansion of bm12 Tfh. A representative histogram shows the efficient depletion of CD3+CD8α+ T cells in anti-CD8β-treated mice compared to control animals immediately prior to injection of bm12 cells (a). Splenic CD8+ T cells from these mice were enumerated prior to injection with bm12 cells (b). In a separate experiment using the same depletion strategy, purified bm12 CD4 T cells from WT or Ifnar−/− mice were transferred into C57BL/6 animals, and bm12 Tfh were assessed 14 days later (c).
Figure S6. WT and Ifnar−/− CD4 T cells express similar levels of several known NK activating and inhibitory proteins. The relative protein expression was calculated for bm12 CD4 T cells from NK-deficient recipients compared to NK-sufficient controls (a). Expression of Cdx48 was unique compared to other NK ligands—bm12 CD4 T cells exhibited a bimodal distribution, expressing either intermediate or high levels. The percentage of Cdx48hi expressing cells was compared between WT and Ifnar−/− bm12 CD4 T cells in control or NK-depleted hosts (b).
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Chapter 7: Dissection of metabolic activity in T follicular helper cells and B cells in systemic lupus erythematosus*

*The following manuscript is in preparation for submission.

Klarquist J1,*, Lehn MA1,*, Hennies CM1, Zhang X2, Ji H3, and Janssen EM1

1 Division of Immunobiology.
2 Division of Human Genetics
3 Division of Asthma Research
Cincinnati Children’s Hospital Medical Center and the University of Cincinnati College of Medicine, Cincinnati, OH, USA
* Authors contributed equally

Key words: metabolism, lupus
Abbreviations: GC, germinal center, 2-DG, 2-deoxyglucose, 2NBDG, fluorescent 2-deoxyglucose, FAO, fatty acid oxidation; OCR, oxygen consumption rate; ECAR, extracellular acidification rate.
Abstract

Systemic lupus erythematosus (SLE) is an autoimmune disease affecting multiple organ systems largely due to the production of autoantibodies. B cells and CD4 T cells play important roles in determining the strength and the nature of autoimmune pathologies in SLE patients. Recently, cellular metabolism has been identified as major regulator of immune cell function. We used an inducible model of SLE to determine the metabolic profiles of key CD4 T cell and B cell subsets, including T follicular helper cells (Tfh), germinal center (GC) B cells, and plasmablasts. We found all three cell subsets greatly increased their metabolic activity, shifting their metabolic programs toward one predominantly dependent upon aerobic glycolysis. Tfh, GC B cells, and plasmablasts also increased oxidative phosphorylation (OXPHOS) in a partially fatty acid oxidation (FAO)-dependent manner. The dependence of Tfh and B cells on glycolysis was so great, that its chemical inhibition via 2-deoxy-D-glucose (2-DG) administration was sufficient to completely prevent the development of lupus-like disease.
Introduction

Systemic lupus erythematosus is characterized by the production of myriad autoantibodies which drive immune complex-related inflammation in various tissues and organs. Breakdown of peripheral tolerance in both CD4 T cells and B cells is considered a major driver of disease. While B cells produce the autoantibodies, the CD4 T cells enhance the production of autoantibodies through their capacity to promote B cell differentiation, maturation and class-switching\(^1,2\). Consequently, therapeutic interference of both T cells and B cells and their functions is of high interest.

Metabolism has recently been identified as a key player in the regulation of homeostasis and the cellular function of lymphocytes. There are several metabolic pathways involved in the generation of cellular energy: glycolysis, which encompasses cells metabolizing glucose into pyruvate that can either be shuttled into the tricarboxylic acid (TCA) cycle (oxidative glycolysis) or converted outside of the mitochondria into lactate (aerobic glycolysis); fatty acid oxidation (FAO) and amino acid oxidation/glutaminolysis, the processes where metabolic products of fatty acids and glutamine feed into the TCA cycle for energy production via oxidative phosphorylation (OXPHOS).

There is significant metabolic heterogeneity in distinct lymphocyte subsets. Naïve T cells have low metabolic needs that are met through the oxidation of glucose derived pyruvate or fatty acids via OXPHOS\(^3\text{-}^6\). Upon activation T cells change their metabolic program to meet their energy needs for proliferation and development of specific effector functions. In vitro activated T cells rapidly increase glucose import for aerobic glycolysis and glutamine for OXPHOS while down regulating FAO\(^7,8\). However, evidence is accumulating that in vitro activated T cells may not correctly represent the metabolic profile of in vivo activated cells and that there may be large differences between T cells at the beginning and conclusion of an immune response as well as between acutely and chronically stimu-
lated T cells. It is currently thought that Th1, Th2, and Th17 CD4 T cells largely depend on aerobic glycolysis while Tregs depend on FAO\textsuperscript{9,10}. Other CD4 T cell subsets, including Tfh cells have not been well characterized. B cells also show metabolic reprogramming upon activation but seem to proportionally increase OXPHOS and aerobic glycolysis\textsuperscript{11,12}. While B cell activation in vitro has been studied, little is known on the metabolic needs of B cells in vivo during the generation of the GC B cell response and plasmablast/cell formation.

Studies to understand the cellular mechanism that drive SLE pathogenesis recently identified metabolic shifts in T cells from mice with features of SLE as well as patients with SLE. Fernandez et al showed elevated mTOR activity and mitochondrial membrane hyperpolarization in CD4 T cells from SLE patients\textsuperscript{13}. T cells from B6.sle1Sle2Sle3 and B6.lpr mice showed increased glycolysis and mitochondrial oxidative metabolism\textsuperscript{14,15}. As a consequence, treatment of B6.Sle1Sle2Sle3 and B6.MRL-Fas\textsuperscript{lpr} mice with either 2-DG (a competitive substrate for hexokinase, an inhibitor of glycolysis) or metformin (a mitochondrial electron transport chain complex I inhibitor) were sufficient to prevent autoimmune activation. Importantly, metformin plus 2-DG combination treatment of mice after establishment of disease significantly reduced ANA.

While these data are exciting and open a door to a new therapeutic approach, several questions remain to be answered. There is currently very little knowledge on the metabolic programs that regulate Tfh in vivo. The published studies used total CD4 T cells from the SLE models and in vitro activation of naïve T cells to assess the effects of the drug treatment. Although the studies claim that oxidation of glucose is particularly important, there are indicators that aerobic glycolysis could be a strong contributor to the disease development, and that oxidative phosphorylation of fatty acids may play a role as well. Moreover, little is known on the metabolic requirements of B cell responses during autoimmune manifestations. B cells do undergo a metabolic reprogramming upon activation
to increase glycolysis and lactate production and inhibition of this metabolic transition prevents antibody production. Inhibition of pathways that directly interfere with the B cell response would not only impact the development of the ANA, but also the expansion and maintenance of Tfh cells that is driven by GC B cells.

Here, we used an inducible model of SLE to dissect the metabolic profiles of Tfh cells, GC B cells and plasmablasts upon development of lupus-like disease. Our data show that Tfh have increased metabolic activity with a shift towards aerobic glycolysis, and increases in OXPHOS that are partially FAO-dependent. Aerobic glycolysis was the predominant metabolic pathway utilized by both GC B cells and plasmablasts. Consequently, inhibition of glycolysis, but not OXPHOS significantly reduced both Tfh and B cells responses. Furthermore, the B cell requirement for glycolysis was so strong, that GC B cells and plasmablasts did not develop, even when treatment was initiated after the development of a substantial Tfh population.
Results

Tfh cells display increased metabolic activity

To address the metabolic program in Tfh cells in SLE models, we used an inducible model of SLE where transfer of CD4 T cells from bm12 mice (on a CD45.1 background) into WT C57BL/6 recipients results in SLE-like disease\(^1\)\(^2\). In this model, transferred CD4 T cells rapidly expand and develop into Tfh, characterized by gene and protein expression of Bcl6, Cxcr5, PD-1, Icos, Cxcr3, and Il21 (fig.1a-c). Flow cytometric assessment of phosphorylated 4EBP1 and S6K, downstream targets of mTORC1—a key sensor of nutrients and regulator of metabolism—showed a significant increase in phosphorylation in both proteins in Tfh compared to naïve CD4 T cells (fig.1d,e). Importantly, a similar increase in p-S6K and p-4EBP1 expression was observed in Tfh cells obtained from B6.Nba2 mice that spontaneously developed ANA and proteinuria (fig.1f).

As this increase in mTOR activity suggested an increase in cellular metabolism, we next used Seahorse extracellular flux analysis to assess the level of OXPHOS and mitochondrial capacity in naïve CD4 T cells and Tfh in real-time. Tfh obtained from the bm12 model showed increased baseline oxygen consumption (OCR) levels compared to naïve CD4 T cells (fig.1g,h). Addition of a combination of rotenone and antimycin A, blockers of mitochondrial complex I and III, respectively, indicated that naïve CD4 T cells and Tfh cells had the same level of non-mitochondrial OCR. Importantly, blocking complex V with oligomycin A, thereby blocking conversion of ADP to ATP, significantly reduced the OCR to levels close to the level of non-mitochondrial OCR in both naïve CD4 T cells and Tfh. This suggests that the increase in baseline OCR in Tfh was used for increased ATP production. Interestingly, Tfh showed a significant increase in ECAR (extracellular acidification rate), suggesting increased use of glycolysis and production of lactate (fig.1i). Together these data indicated that Tfh were metabolically more active than naïve CD4 T cells and suggest that they are increasing both mitochondrial and non-mitochondrial pathways to generate energy.
Figure 1: Metabolic activity is increased in Tfh from murine models of SLE. Two weeks after transfer into C57BL/6 recipient mice, bm12 CD4 T cells were sorted based on CD45.1 expression. Flow cytometry plots of live splenocytes 1 day or 2 weeks after transfer are shown (A). qPCR was performed on purified cells for the Tfh-associated genes Cxcr5, PD-1, Bcl6, Icos, Cxcr3, and IL-21 (B). The levels of Tfh-associated proteins were determined by flow cytometry (C). Representative flow cytometry plots show p4EBP1 and pS6K protein expression (D). Summarized data show increased levels of p4EBP1 and pS6K in Tfh (E). Tfh isolated from B6.Nba2 mice showed similar increases in p4EBP1 and pS6K compared to controls (F). Mitochondrial stress tests demonstrated greater OCR associated with ATP production in bm12 Tfh compared to naive CD4 T cells (G). Basal respiration was determined by subtracting OCR after antimycin/rotenone treatment from baseline OCR; ATP-linked OCR was determined by subtracting the after oligomycin treatment from the baseline OCR; and the coupling efficiency was defined by the inverse ratio of these two values (H). Baseline ECAR levels were also compared between these cells (I).
**Glycolysis is increased in Tfh compared to naïve CD4 T cells**

Based on the dramatic increase in ECAR in the Tfh, we assessed whether Tfh increased their glycolysis to meet their metabolic needs. RNA-Seq and quantitative-RT-PCR (qPCR) showed that Tfh cells had increased gene expression of multiple genes associated with glycolysis compared to naïve CD4 T cells (fig 2.a-c). We next performed a glycolysis stress test where ECAR of naïve CD4 T cells and Tfh cells was first measured in the absence of pyruvate and glucose and subsequently upon addition of glucose to determine glycolysis (C). Glycolytic stress tests show that Tfh have increased glycolysis compared to naïve CD4 T cells (B). qPCR confirmed increases in several key genes controlling glycolysis (A). RNA-Seq analysis showed differential expression of these and additional related genes (B). qPCR confirmed increases in several key genes controlling glycolysis (C). Glycolytic stress tests show that Tfh have increased ECAR upon the addition of glucose, which was eliminated by 2-DG treatment (D). Glycolysis was determined by subtracting the baseline ECAR from the ECAR after glucose challenge; glycolytic capacity was determined by subtracting baseline ECAR from the ECAR after the addition of oligomycin; and glycolytic reserve was determined by subtracting the ECAR value after glucose challenge from the ECAR value after the addition of oligomycin (E). ATP production was determined in cells with and without the addition of exogenous glucose, and with and without 2-DG treatment (F).
the rate of glycolysis in the cells. Addition of oligomycin, which blocks mitochondrial ATP production and shifts energy production to glycolysis, was used to determine the maximal glycolytic capacity, while the addition of 2-deoxy-glucose (2-DG) was used to block all glycolysis (fig.2d). Compared to naïve T cells, Tfh displayed significantly higher levels of glycolysis (fig.2d). While the Tfh also display greater glycolytic capacity than naïve CD4 T cells, this glycolytic capacity was close to their baseline level of glycolysis, suggesting that Tfh cells function relatively close to their maximal glycolytic capacity (fig.2e). In line with these Seahorse data, assessment of ATP showed that ATP generation by Tfh cells was significantly reduced in the absence of glucose or upon treatment with hexokinase inhibitor 2-DG (fig.2f). Together these data indicate that Tfh use glycolysis for energy production at higher levels than naïve cells. Interestingly, CD4 T cells activated by CD3/CD28 in vitro showed comparable ECAR as Tfh, but significant higher OCR, implying that the existence of a metabolic program in Tfh that is distinct from a generally activated CD4 T cell (s.fig.1).

In line with these observations, Tfh cell expressed higher levels of glucose importer glut1 and showed higher uptake of fluorescent 2-DG (2-NBDG) than naïve CD4 T cells (fig.3.a-c). This was observed in both Tfh from the bm12-CD45.1 → C57BL/6 model as well as the spontaneous B6.Nba2 models. Similarly, when 2NBDG was administered i.v. in

![cumulative ECAR +/- 2DG and cumulative OCR +/- 2DG graphs]

**Supplementary Figure 1**: Distinct metabolic programs between Tfh and in vitro activated CD4 T cells. Extracellular flux assays using medium with added glucose show baseline ECAR levels and ECAR after the addition of 2-DG for sorted naïve CD4 T cells, bm12 Tfh, anti-CD3/anti-CD28 in vitro activated CD4 T cells. OCR before and after 2-DG treatment were also determined (B).
vivo, Tfh cells in both the inducible bm12-CD45.1→C57BL/6 model and the spontaneous B6.NBA2 model incorporated more 2NBDG than naïve CD4 T cells in the same mouse (fig.3.b,c). Similar to mouse Tfh cells, human peripheral Tfh cells (CD4+CXCR5+PD-1high) showed increased glut-1 expression and in vitro 2NBDG uptake compared to naïve CD4 T cells from the same donors (fig.3.d).

Fatty acid oxidation (FAO) is the predominant mitochondrial ATP producing pathway

As Tfh showed higher OCR/OXPHOS than naïve cells, we wondered which carbon source was needed to fuel this increased mitochondrial activity. Elimination of glycolysis did not alter OCR in Tfh, suggesting that the pyruvate generated in the glycolytic pathway did not feed into the TCA and contribute to the increased OCR (s.fig.1). We therefore analyzed the contribution of the other 2 main carbon sources for mitochondrial OXPHOS, glutami-
nolysis and fatty acid oxidation (FAO).

Compared to naïve CD4 T cells, Tfh showed a limited upregulation of genes associated with glutaminolysis (fig.4.a-c). Glutamine stress tests indicated that elimination of glutamine from the medium did not affect OCR by Tfh (fig.4.d). Similarly, administration of aminooxyacetate (AOA), a chemical inhibitor of glutamate-dependent transaminases that convert glutamate into α-ketoglutarate to cells cultured in the presence of glutamine did not change the OCR by Tfh (fig.4.e). Furthermore, ATP production by Tfh was not affected by the absence or presence of glutamine or AOA (fig.4.f).

While Tfh did not seem to depend on glutaminolysis, they seemed to partly depend on

Figure 4: Mitochondrial OXPHOS is not driven by glutaminolysis in Tfh. Diagram of key genes involved in glutaminolysis (A). RNA-Seq (B) and qPCR (C) analysis of these and additional related genes. OCR was measured in extracellular flux assays before and after the chemical inhibition of glutaminolysis by AOA using glutamine-supplemented media (D). OCR was also measured in cells before and after the addition of glutamine using otherwise glutamine-free media (E). ATP production was determined in cells with and without the addition of exogenous glutamine, and with and without AOA treatment (F).
fatty acid oxidation. Tfh showed modest, but significantly increased expression of various key genes in the FAO pathway (fig.5.a-c). Treatment of Tfh cells with etomoxir, an inhibitor of CPT1, the protein responsible for transporting fatty acids into the mitochondria, resulted in a partial inhibition of OCR (fig.5.d,e). Moreover, treatment with etomoxir significantly reduced ATP production, supporting the importance of fatty acids in T cell metabolism (fig.5.f). Intriguingly, blocking fatty acid oxidation in CD3/CD28 activated T cells resulted in a marked decrease in OCR, but a significant increase in ECAR, indicating that the cells increased the glycolytic pathway to meet their metabolic needs. In contrast, Tfh cells showed a marked decrease in OCR without increasing glycolysis (supplemental figure 2). This is in line with our earlier observation that Tfh function relatively close to their maximal glycolytic capacity.

Figure 5: FAO is critical for OXPHOS-dependent ATP production in Tfh. Diagram of genes involved in FAO (A). RNA-Seq analysis of these and additional related genes within Tfh and naïve CD4 T cells (B). qPCR was also performed on several genes controlling FAO, including the genes essential for long-chain fatty acid transport across the mitochondrial inner membrane, carnitine palmitoyltransferase I (Cpt1) and II (Cpt2) (C). OCR was measured at baseline and following chemical inhibition of Cpt1 by etomoxir (D-E). ATP production was also determined in Tfh before and after treatment with etomoxir (F).
Together these studies show that Tfh follow a specific metabolic program that is distinct from naïve or globally activated CD4 T cells that predominantly depends on glycolysis and to a lesser degree on fatty acid oxidation.

**GC B cell and plasmablast responses are associated with increased metabolic activity**

As SLE is a disease characterized by dysregulated B cell responses we next assessed metabolic programs of both germinal center B cells and plasmablasts. In the inducible SLE model, transfer of bm12 CD4 T cells results in a rapid increase GC B cells (B220+ Fas+GL7+) and plasmablasts (CD138+B220low), concomitant with the production of antibodies to various nuclear antigens, including dsDNA (fig.6.a,b). Flow cytometric analysis of GC B cells showed increased phosphorylation of S6K, while plasmablasts showed increased phosphorylated S6K and phosphorylated 4EBP1. Similar results were found in GC B cells and plasmablasts from B6.Nba2 mice with proteinuria (fig.6.c).

Real-time assessment of oxidative phosphorylation indicated that naïve B cells, GC B cells and plasmablasts have relatively comparable levels of OCR, ATP-dependent OCR, and coupling efficacy (fig.6.d,e). In contrast, GC B cells and plasmablasts displayed increased ECAR and ECAR/OCR rates compared to naïve B cells, indicating increased glycolytic activity in the GC B cells and plasmablasts.
Figure 6: Increased metabolic activity in key B cell subsets. Representative flow cytometry plots show B220+Fas+GL7+ GC B cells and CD138+B220+ plasmablasts 1 day and 14 days after transfer of bm12 CD4 T cells into C57BL/6 recipient mice (A). Serum levels of anti-dsDNA antibodies rise to detectable levels 14 days post transfer (B). p4EBP1 and pS6K expression was determined for naïve B cells, GC B cells, and plasmablasts from the bm12 model and from the B6.Nba2 model (C). Mitochondrial stress tests were performed using sorted populations (D). From these tests, basal OCR, ATP-dependent OCR, coupling efficiency, were determined as described earlier (E). Baseline ECAR and the ratio of ECAR to OCR was also determined (E).
Increased glycolysis in GC B cells and plasmablasts

Similar to Tfh, GC B cells and plasmablasts showed increased expression of genes associated with glycolysis (fig.7.a). Glycolysis stress tests indicated that both GC B cells and plasmablasts had a significantly increased level of glycolysis and glycolytic capacity compared to naïve B cells (fig.7.b-d). As with Tfh cells, both GC B cells and plasmablasts

Figure 7: Plasmablasts and GC B cells utilize glycolysis. qPCR analysis of glucose importer genes and other glycolysis pathway genes in sorted naïve B cells, GC B cells, and plasmablasts (A). Glycolysis stress tests were also performed on these cells (B). From these tests, glycolysis, glycolytic activity and glycolytic reserve were calculated as described earlier (C). Baseline ECAR and ECAR after the addition of 2-DG was determined for these cells as well as in vitro activated B cells (D). ATP production was determined in cells with and without the addition of exogenous glucose, and with and without 2-DG treatment (E).
had relatively small glycolytic reserves, indicating that they operated close to their maximal glycolytic capacity. The glycolytic pathway in the B cells was critically important for the generation of ATP as both the elimination of glucose or the inhibition of glycolysis with 2-DG significantly reduced ATP production (fig. 7.e).

Correlating with their baseline level of glycolysis, GC B cells and plasmablasts expressed high levels of Glut-1 and showed increased 2-NBDG uptake in vitro as well as in vivo (fig. 7.f,g). This correlative increase in glycolysis, Glut-1 expression and 2-NBDG was also observed in GC B cells and plasmablasts from B6.Nba2 mice with proteinuria (fig. 7.g).
Importantly, human peripheral plasmablasts (CD19⁺IgG⁻IgD⁻Cd27⁺CD38⁺) showed significantly higher levels of Glut-1 and 2-NBDG uptake compared to switched memory cells (CD19⁺IgD⁻IgG⁺Cd27⁺CD38⁻) and naïve B cells (CD19⁺IgD⁺IgG⁻Cd27⁻CD38⁻) from the same donor (fig.7.h).

**Limited dependence of B cell responses on FAO**

As with the Tfh cells, elimination of glycolysis did not affect OCR, suggesting that either glutaminolysis or FAO provided that carbon source for the mitochondrial ATP production. Analysis of gene expression of naïve B cells, GC B cells and plasmablasts indicated

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**Figure 8: B cell subsets in murine lupus do not utilize glutaminolysis.** Relative expression as determined by qPCR of genes associated with glutaminolysis in naïve, GC B cells, and plasmablasts from C57BL/6 mice 2 weeks after bm12 transfer (A). Baseline OCR and OCR after the addition of AOA, an inhibitor of glutaminolysis, or after the addition of exogenous glutamine was determined by extracellular flux assays (B). ATP production was also determined in cells with and without the addition of exogenous glutamine, and with and without AOA treatment (C).

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limited changes in genes associated with glutaminolysis (fig. 8.a). Seahorse analysis indicated that absence of glutamine or presence of glutaminolysis inhibitor AOA did not affect OCR or ECAR in any of the B cell subsets (fig. 8.b). In line with these observations, absence of glutamine or presence of AOA did not alter ATP production in the different B cells (fig. 8.c).

Interestingly, little changes in gene expression associated with fatty acid oxidation were observed (fig. 9.a). However, treatment of GC B cells and plasmablast with etomoxir resulted in a 30% reduction of OCR (fig. 9.b). Similarly, addition of etomoxir significantly reduced ATP production in the B cells (fig. 9.c), illustrating an important role for FAO in B cell metabolism.

**Figure 9: Limited role for FAO in plasmablasts and GC B cells.** qPCR analysis of genes associated with FAO in naïve B cells, GC B cells, and plasmablasts (A). OCR and ECAR levels were determined by extracellular flux assays at baseline and after the addition of the FAO inhibitor etomoxir (B). ATP production was also determined before and after etomoxir treatment (C).
Inhibition of glycolysis, but not FAO, blocks disease development in vivo

As Tfh cells, GC B cells, and plasmablasts in the bm12 model utilize both glycolysis and FAO for their energy production, we next determined the relative contribution of each pathway to disease development.

To address the contribution of FAO to disease, mice were treated with etomoxir for 2 weeks, starting the day after bm12 CD4 T cell transfer. As an additional control, a group of mice were treated with low doses of metformin that did not alter systemic glucose levels but reduced mitochondrial ATP production through the inhibition of mitochondrial complex 1. Mice treated with metformin did not show a significant change in the frequency or absolute number of Tfh cells, GC B cells, and plasmablasts compared to vehicle treated mice (fig.10.a,c). Treatment with etomoxir also did not affect the frequency or absolute number of Tfh cells, GC B cells, and plasmablasts, but resulted in a small reduction of ascites (fig.10.b).

Daily intraperitoneal treatment of mice with 2-DG significantly reduced splenomegaly, ascites, accumulation of Tfh, GC B cells, plasmablasts (fig.11.a-c). Interestingly, the Tfh
response was reduced by approximately 50%, the reduction in GC B cells and plasmablasts was close to 90%. This difference in impact of 2-DG treatment suggests that the development of GC B cell and plasmablast responses was more dependent on glycolysis than the development of the Tfh compartment.

In agreement with this observation, our in vivo administration of fluorescent 2-NBDG uncovered a significant difference in the uptake of glucose when these cell populations were compared in vivo (fig.11.d). Plasmablasts and Tfh expressed similar levels of Glut1, but plasmablasts were approximately 4-fold more efficient in the uptake of 2NBDG in vivo. GC B cells expressed 10-fold lower levels of Glut-1 than Tfh, but were still twice as efficient in the uptake of 2-NBDG in vivo (fig.11.d). Similar results were found in the spontaneous B6.Nba2 model with high glucose uptake in GC B cells and plasmablasts and significantly lower uptake in Tfh (supplemental fig. 3).
To further dissect the effect of glycolysis on Tfh and the B cells response, mice were either given 2-DG in the drinking water for the entire duration of the experiment (day 2-14) or only during the second week (day 8-14) and analyzed on day 7 and day 14. 2-DG treatment of mice for seven days did not affect the proliferation or accumulation of bm12 CD4 T cells compared to vehicle-treated mice (fig.12.a). Importantly, the percentage and level of expression of CXCR5 and PD-1 was comparable in the bm12 CD4 T cells between 2DG and vehicle treated animals, suggesting that the early development of Tfh cells was not impacted (data not shown). At this time no significant increase in GC B cells or plasmablasts over naïve mice was observed (fig.12.a).

Analysis on day 14 showed markedly reduced Tfh responses and the absence of GC B cell and plasmablast development in mice continuously treated with 2-DG (fig.12.b). Mice that were only treated for the second week of the model resulted in a partial reduction in Tfh compared to continuously treated mice. The frequency of Tfh in these mice was higher than the frequency observed at seven days after CD4 T cell transfer indicating that the Tfh continued to accumulate, even in the absence of glycolysis. However, 2-DG treatment for only the second half of the model still completely inhibited the expansion of GC B cells and plasmablasts, resulting in levels observed in naïve mice (fig.12.b). Together these data indicate that Tfh can develop in the absence of glycolysis, but that GC B cell and plasmablast responses are critically dependent on glycolysis.
Figure 12: The inhibition of glycolysis predominantly blocks plasmablast and GC B cell development. Mice were provided drinking water with or without 2-DG. After one week, bm12 Tfh, plasmablasts, and GC B cells were enumerated in mice given drinking water with 2-DG, control water, or mice which did not receive bm12 cells (naïve) (A). In parallel experiments, bm12 Tfh, GC B cells, and plasmablasts were determined after two weeks of treatment (B).
Discussion

Here we show that Tfh metabolism is elevated in Tfh cells from mice with inducible and spontaneous characteristics of SLE. The Tfh cells showed a strong shift to aerobic glycolysis and limited increase in OXPHOS. Similar results were found in GC B cells and plasmablasts. Consequently, treatment of mice with agents that inhibited OXPHOS (metformin) or FAO (etomoxir) had no effect on disease development while blocking of glycolysis at the level of the hexokinase step significantly reduced Tfh numbers and completely prevented the development of the B cell response. Together, these data suggest that aerobic glycolysis is the main driver of disease development and that OXPHOS using either fatty acids, glutamine, or pyruvate derived from glycolysis as carbon source is of lesser importance.

These exciting findings are somewhat in contrast with the observations made in two recent papers from Yin et al.\textsuperscript{14,15}. Both studies show that disease development in the spontaneous B6\textsuperscript{Sle1Sle2Sle3} model and the B6\textsuperscript{MRL-Faslpr} could be blocked with either the inhibition of glycolysis (2-DG), or the inhibition of complex I in the mitochondrial electron transport chain (metformin). A combination of both was required to prevent progression of ongoing disease. These studies concluded that oxidative glycolysis, but not aerobic glycolysis was the main driver of pathogenesis and that the effect resulted from an inhibition of the CD4 T cell response. Importantly, these studies do not take into account the metabolic requirements of B cells, key mediators of clinical disease manifestations in these animals.

There are several significant differences in the approach between their models and our models that could provide explanations for the divergent conclusions. Where the studies by Yin et al. used total CD4 T cells from sick mice as well as in vitro activated naïve T cells, we directly compared Tfh cells and naïve T cells. Our data indicate that Tfh cells have a very distinct metabolic profile that is significantly different from naïve T cells and
from in vitro activated T cells. Consequently, conclusions on the metabolic profile of cell populations relevant for disease development cannot be made in a mixed population, nor can they be reliably extrapolated from in vitro activated naïve CD4 T cells. Our data suggest that Tfh predominantly use aerobic glycolysis. The inhibition of glycolysis (at the HK level) completely blocked ECAR without affecting OCR. If oxidative glycolysis was critically important for Tfh, we would have expected to see a drop in OCR upon treatment with 2-DG. It is still possible that oxidative glycolysis does occur, and that the Tfh increase the oxidation of another carbon source (glutamine or FA), resulting in a seemingly unaltered OCR. Experiments blocking glycolysis in the absence of other carbon sources would clarify the relative contribution of oxidative glycolysis in these cells.

The Ying paper also incorporated the use of dichloroacetate (DCA) that inhibits the conversion of pyruvate into lactate. The authors concluded that this treatment did not alter disease as they did not see significant changes in their percentage of Tfh cells in the total CD4 T cell population and metabolic assessment of the total CD4 population. However, their data shows significant reduction in ANA levels, indicating an important role for the pyruvate-lactate conversion in disease development. Our preliminary data with DCA in the inducible SLE model were not significant due to the small sample size and large standard deviation. Further studies using DCA will need to be completed to determine whether DCA can affect Tfh development, or whether its effects may primarily affect the development of B cell subsets.

Another large difference between the Yin papers and our study is the choice of model. Whereas they used the spontaneous B6.Sle1Sle2Sle3 model and the B6.MRL-Fas<sup>pr</sup> model, we predominantly used the inducible bm12 model, which is a chronic GVH model of SLE. The accelerated development of disease in this model may shift the metabolic requirements to a somewhat more acute model that would favor aerobic glycolysis. A recent
paper by Nguyen et al., studying the metabolic programming of alloantigen-activated T cells after hematopoietic cell transplant (BM + T cells) in a lethally irradiated host, showed that T cells switched from FAO and oxidative glycolysis to a more aerobic glycolysis phenotype with increased dependency on glutaminolysis\(^\text{16}\). While this model shows several similarities with our model, it also adds the complexity of the absence of a normal immune system in the recipient, homeostatic expansion, as well as the inflammatory response after lethal irradiation. Interestingly, flow cytometric analysis of cells from the B6.Nba2 mice showed comparable changes in Glut-1 expression, 2-NBDG uptake and phosphorylation of 4EBP1 and S6K in Tfh cells, GC B cells, and plasmablasts to those seen in the inducible bm12 model, suggesting that many of the phenomena seen in the bm12 model also translate to the spontaneous model. However, additional studies using Tfh cells, GC B cells and plasmablasts from B6.Nba2 mice will be needed to confirm this assumption.

Through the isolated assessment of metabolic pathways in Tfh, we found that the expansion of Tfh was dependent upon similar metabolic program to other T helper subsets, including Th1, Th2, and Th17 cells. Namely, Tfh relied upon aerobic glycolysis and to some extent FAO; however, we were surprised to find that blocking glutaminolysis with AOA had no observable effect on the metabolic activity within Tfh. These results are suggestive that glutamine is not a primary player in intracellular recycling of amino acids that contribute to Tfh amino acid homeostasis. Additionally, although glutaminolysis can also replenish TCA cycle intermediates that are utilized for biosynthesis, Tfh may overcome this need through alternative mechanisms. With relatively high levels of glucose import, Tfh may shuttle glucose toward the pentose phosphate pathway (PPP), which is capable of amino acid and nucleotide biosynthesis. Further studies are required to examine the importance of the PPP in Tfh development.

Our metabolic assessments on purified cells indicated that both Tfh and B cells use FAO
to meet their metabolic needs. In vitro etomoxir treatment significantly reduced OXPHOS and ATP production in all tested cell subsets (without affecting viability). However, treatment with etomoxir in vivo did not alter the frequency or absolute number of Tfh cells, GC B cells or plasmablasts. Only a trend towards less ascites was observed (fig.10.b). It is possible that the Tfh cells, GC B cells, and plasmablasts do not require FAO or OXPHOS to properly expand and accumulate in vivo. However, it is also possible that these cells do require FAO, but that the effect of etomoxir is masked by an effect on a different cell population. Tregs have been shown to require FAO for their development and accumulation. It is indeed possible that treatment with etomoxir affects the Treg population in either number or function, thereby allowing the pathogenic response to increase. In this scenario the direct effect of etomoxir on the Tfh cells GC B cells and plasmablasts would be masked by the enhanced accumulation due to relaxation of the external regulation. Further analysis of the Tregs in this model could provide insight on the validity of this hypothesis.

Our in vivo glycolysis inhibition studies provided further insight into the metabolic requirements of the cell subsets we examined in vitro. Although Tfh clearly utilized glycolysis in our ex vivo assays, their in vivo dependence upon glycolysis was less clear. Tfh developed normally when glycolysis was blocked for the first 7 days after transfer, but when glycolysis was inhibited during days 8-14, their accumulation was constrained. This could be due to distinct temporal metabolic requirements, or it could be due to the close dependence of Tfh maintenance and expansion on the activity of GC B cells\textsuperscript{17}. GC B cells, which develop with delayed kinetics compared with Tfh\textsuperscript{18}, appeared extremely sensitive to the inhibition of glycolysis—their development was completely blocked by short- or long-term 2-DG administration. Thus, the inability of GC B cells to expand when glycolysis was blocked during days 8-14 may have indirectly stifled further Tfh expansion beyond what occurred in the first 7 days. Plasmablasts also failed to expand when mice were treated with 2-DG, indicating that the B cell compartment was particularly reliant upon glycolysis.
In B cells, a clear correlation between Glut1 expression, uptake of 2-NBDG, and the developing response was seen (fig.11.b). Naïve B cells had low levels of Glut-1 and 2-NBDG uptake, GC B cells had intermediate levels of Glut-1 and 2-NBDG, while plasmablasts expressed the highest levels of Glut-1 and 2-NBDG. Interestingly, studies in the aged B6.Nba2 mice indicated that Glut1 expression and 2-NBDG uptake was comparable in plasmablasts in the spleen and plasma cells in the bone marrow, suggesting that these later developmental stages have comparable glycolytic needs (data not shown).

Interestingly, Tfh did not fit the correlation of Glut1 expression and 2-NBDG uptake seen in B cells. Tfh expressed significantly higher levels of Glut-1 compared to GC B cells, although they imported less glucose. In fact, Tfh cells expressed similar levels of Glut-1 protein as plasmablast and plasma cells but internalized 3-fold less 2-NBDG. Where one could suggest the lower uptake in GC B cells and Tfh cells resulted from higher competition and perhaps lower levels of 2-NBDG in the GC compared to the extrafollicular plasmablasts, this does not explain the differences between the GC B cells and Tfh cells. GC B cells expressed 10-fold lower levels of Glut-1 than Tfh, but still imported twice as much 2-NBDG. A simple explanation would be that glucose transporters other than Glut-1 contribute to the glucose uptake in GC B cells. Currently, 13 glucose transporters have been identified, with many of them based on sequence homology and with little functional data available. There is speculation that each glucose transporter isoform could play a specific role in glucose metabolism that is determined by its pattern of tissue expression, substrate specificity, transport kinetics, and regulated expression under different physiological conditions. Further characterization of these importers and their expression may provide a better explanation for how these cell subsets meet their distinct glycolytic needs.

Together, our data show that Tfh, GC B cells, and plasmablasts from mice and humans
utilize aerobic glycolysis to meet their metabolic needs. Our findings that disrupting glycolysis via 2-DG in the bm12 model blocked disease development and inhibited the expansion of these key cell subsets has important implications for pathology of SLE. Finally, these studies uncover a potential new avenue for targeting Tfh, GC B cells, and plasmablasts in SLE as well as during other harmful or otherwise unwanted inflammatory immune responses.
Materials and Methods

Mice and SLE models

Mice were maintained under specific pathogen-free conditions in accordance with guidelines by the Association for Assessment and Accreditation of Laboratory Animal Care International. B6(C)-H2-Ab1\textsuperscript{bm12}/KhEgJ mice were purchased from The Jackson Laboratory and crossed to a B6.SJL.P\textit{tpcr} (B6/CD45.1) background (bm12-CD45.1). C57BL/6J mice were bred in our facility. B6.NZB\textsuperscript{(D1Mit47-D1Mit209)/Bkot} mice (B6.Nba2) that contain the NZB-derived lupus susceptibility QTL (\textit{Nba2}) and spontaneously develop antinuclear antibodies and proteinuria were a gift from Dr. Divaker Chaubey, (UC Cincinnati, Cincinnati) and were aged in-house.

For the inducible SLE-like model, 8 x 10\textsuperscript{6} negatively selected CD4\textsuperscript{T} cells (Miltenyi, Bio-Legend) from Bm12-CD45.1 mice were i.p. transferred into B6 WT recipients\textsuperscript{19–21}. T and B cell responses in the spleen and bone marrow were analyzed at 14 days after transfer unless stated otherwise\textsuperscript{18}. Serum levels of anti-dsDNA IgG, IgG1 and IgG2a were determined by ELISA as previously described\textsuperscript{22}.

Interference with metabolism in vivo was achieved by daily intraperitoneal injections of 2-DG (1 mg/g), metformin (0.1 mg/g) or etomoxir (15 ug/g), or administration of 2-DG in the drinking water (10 mg/ml) for the duration described in the results section. In vivo glucose uptake studies were performed by intravenous administration of 200 ug 2-NBDG followed with tissue harvest 15 minutes later.

Human donors

Blood samples from 3 healthy de-identified donors was procured via the Cell Processing Core at CCHMC. PBMC were isolated by density-gradient-separation and cultured with 100 uM 2-NBDG for 20 minutes (Life Technologies) before staining for flow cytometric
analysis. Human T cells were analyzed using antibodies to CD4, CD3, CXCR5, PD-1, (BioLegend) Glut1 (R&D Systems), together with a fixable viability dye (Life Technologies). Human B cells panels included CD19, IgD, IgG, CD27, CD38, HLA-DR (BD Pharmingen), Glut-1 and fixable viability staining. For analysis purposes cells CD19+IgD+IgG- CD27- were considered to be naïve B cells, CD19+IgD-IgG+ CD27+ CD38- switched memory B cells, and CD19+IgD-IgG+ CD27+ CD38+ plasmablasts. Samples were collected on a LSRII flow cytometer with Diva software (BD Pharmingen), and data were analyzed with FlowJo software (Tree Star).

**Flow cytometry and cell sorting**

Assessment of mouse T cells was performed using combinations of the following antibodies CD4, CD45.1, CD45.2, CXCR5, PD-1, CD69, Icos, Bcl-6, pS6K, p4EBP1, (BioLegend) Glut-1, together with a fixable viability staining. Murine B cell panels included combinations of antibodies to CD19, B220, CD138, GL7, CD95, CD69 (BioLegend), pS6K, p4EBP1 (Cell Signaling), Glut-1, together with a fixable viability staining. Samples were collected on a LSRII flow cytometer with Diva software (BD Pharmingen), and data were analyzed with FlowJo software (Tree Star).

T cell sorting of bm12 Cd45.1 T cells was performed by Moflo (Beckman Coulter) based on expression of CD45.1, PD-1 and CXCR5 and viability dye. B cells were sorted based on their expression of B220, CD19, CD138, GL7 and viability for metabolism experiments and gene expression. Naïve T cells and B cells were isolated based on their absence of CD69 and expression of CD62L and were used as controls. In general, purity was greater than >99% and viability >95%.

**Gene expression and RNAseq**

Purified bm12 CD4 T cells were processed with mirVANA lysis buffer (Life Technologies). RNA was extracted according to the mirVANA RNA extraction kit protocol. Subsequently,
300 ng of total RNA was purified by the NEBNext Poly(A) mRNA Magnetic Isolation Module (New England BioLabs, Ipswich, MA). The NEBNext Ultra Directional RNA Library Prep Kit was used for library preparation. Bioanalyzer (Agilent, Santa Clara, CA) analyses using a DNA high sensitivity chip were performed to determine quality and yield of the purified library. To study differential gene expression, individually indexed and compatible libraries were proportionally pooled (20-50 million reads per sample) for clustering in cBot system (Illumina, San Diego, CA). Libraries at the final concentration of 15 pM was clustered onto a single read (SR) flow cell using TruSeq SR Cluster kit v3, and sequenced for 50 bp using TruSeq SBS kit on an Illumina HiSeq system. Sequence reads were then aligned to the genome by using a standard Illumina sequence analysis pipeline by The Laboratory for Statistical Genomics and Systems Biology at the University of Cincinnati. Genes of interest were confirmed by standard quantitative PCR using SyBRGreen and L32 as an internal control as described before.

**Metabolic assays**

**In vitro glucose uptake:** for in vitro glucose uptake, cells were cultured for 30 minutes with 100 uM 2-NBDG at 37°C, 5% CO2 before staining with the indicated antibodies and subsequent flow cytometric analysis.

**Real-time analysis of oxidative phosphorylation (Seahorse XF96):** Tfh cells, GC B cells, and plasmablasts were purified from spleens from mice in the inducible bm12 SLE model. Naïve CD4 T cells and B cells as well as in vitro activated CD4 T cells (CD3/CD28, 1 ug/ml, 48 hr) and in vitro activated B cells (CD40 (1 ug/ml) + IgM (10 ug/ml), 48 hr) were used as controls. Purified cells (5 × 10⁵) were plated in XF-96 extracellular flux analysis plates (Seahorse Bioscience) in XF running buffer adapted for the different assays as described below. The oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) were determined after correction for protein content in each well.

To assess overall metabolic health, cells were plated in XF media (supplemented with
10 mM dextrose, 2 mM L-glutamax) and cells were sequentially challenged with 10 μM oligomycin, 4 μM FCCP, and 1 μM rotenone plus 10 μM antimycin A (Sigma-Aldrich). For the glycolysis stress test cells were plated in XF media without dextrose or pyruvate and sequentially treated with glucose (final conc. 10mM), oligomycin (final conc. 10 uM), and glycolysis inhibitor 2DG (final conc. 50 mM). For the glutaminolysis stress test cells were plated with XF media without glutamine and treated with glutamine (final conc. 2 mM) followed by glutaminolysis inhibitor AOA (final conc. 100 uM). Fatty acid oxidation was assessed by culturing cells in XF medium (with glucose and glutamine) and blocking the mitochondrial fatty acid importer CTP1 by etomoxir (final conc. 200uM).

**ATP production**: Intracellular ATP levels were assessed in indicated purified cell populations with indicated treatments (20 min, concentrations listed above) by Adenosine 5'-tri-phosphate (ATP) bioluminescent assay kit (Sigma-Aldrich).

**Statistical analyses**

Data were analyzed using Prism software (GraphPad Software, Inc.). Unless stated otherwise, the data are expressed as means ± SEM. All data were evaluated using a student’s t test (for comparisons of two groups), or a one-way ANOVA (for comparisons of three or more groups) followed by a Dunnett’s test. A p value of <0.05 was considered statistically significant.
References


Chapter 8. Summary and Discussion
Overall Summary

Immune responses to dying cells are kept in check by multiple layers of immune regulation to avoid autoimmunity. Nevertheless, immune responses to dying cells can and do occur. In some cases these responses prevent the outgrowth of neoplasms, whereas in other cases, they contribute to autoimmune disease. The goal of the studies conducted and reported in this dissertation was to elucidate molecular mechanisms responsible for driving sterile inflammation and immune responses to self antigens. Further insight into these pathways could allow for the development of novel interventions for transplantation, cancers, and autoimmune diseases. Type I IFN has been identified as a central mediator of several sterile inflammatory processes. Spontaneous immune responses to tumor cells\textsuperscript{1,2}, immune responses to dying tumor cells following ablative therapies\textsuperscript{3,4}, and several autoimmune diseases, especially SLE\textsuperscript{5–7}, all rely upon IFN signaling; however, relatively little is known concerning the precise functions of IFN in the development of these immune responses. Moreover, the pleiotropic and sometimes opposing effects of type I IFN make determining its functions in disease difficult\textsuperscript{8}. We have employed reductionist approaches to decipher specific roles of IFN signaling in sterile inflammation, despite its complexity. Additionally, our studies have defined the individual metabolic needs of the T and B cell subsets as being particularly important for driving autoimmunity.

The studies reported in this dissertation reveal several novel mechanisms driving sterile inflammatory processes, with a special emphasis on defining the roles of type I IFN (Figure 1), and the metabolic reprogramming required for the development of key cells associated with humoral autoimmunity. Specifically, the major findings and conclusions of this dissertation are:
Chapter 5a: Successful tumor ablative therapies, including radiation and cryotherapy, rely upon STING-mediated type I IFN production, and the CD8 T cell responses it supports.

i. Although essentially all nucleated cells express type I IFN, DC sensing of type I IFN is necessary to drive antitumor CD8 T cell responses following ablative therapy (Figures 3 and 7F-I).

ii. Not only do DCs need to sense type I IFN for optimal T cell priming, DCs are the chief producer of type I IFN in response to dying tumor cells which have been killed by multiple agents, including gamma radiation, ultraviolet radiation, or the DNA-damaging chemotherapeutic etoposide (Figure 4 and data not shown).

iii. Type I IFN induction requires the cytosolic signaling intermediate (and DNA sensor in its own right) STING, as well as IRF3, but not TLRs or RLRs (Figures 5 and 7).

iv. The STING/IRF3 axis is activated in response to nuclear DNA, but not RNA or mitochondrial DNA (Figure 6).

Chapter 5b: The bm12 cGHVD model of lupus relies upon recipient STING and IFNAR expression.

i. Recipient STING and IFNAR are critical to the expansion of bm12 Tfh cells and activated CD4 T cells (Figure 8b).

ii. Recipient STING and IFNAR augment the development of GC B cells, plasmablasts, and ANA in bm12 disease (Figure 8b).

Chapter 6: Type I IFN sensing by CD4 T cells is essential for the expansion of Tfh and ensuing disease in the bm12 model of SLE.

i. In an apparent paradox to the well-studied anti-proliferative effects of IFN,
Ifnar-deficient bm12 CD4 T cell expansion is minimal compared to that of WT controls, despite normal differentiation into Tfh (Figures 1 and 2).

ii. The effect of IFN on Tfh is direct: co-transfer of WT and Ifnar<sup>−/−</sup> bm12 CD4 T cells did not restore the expansion of Ifnar<sup>−/−</sup> Tfh (Figure 3).

iii. IFN exerts opposing functions: it simultaneously inhibits CD4 T cell proliferation and protects Tfh from being killed by NK cells (Figures 5, S3, and 8).

iv. NK killing of Tfh is perforin-dependent. In the absence of perforin, Ifnar<sup>−/−</sup> Tfh expand to even greater numbers than WT Tfh (Figure 7).

v. The generation of GC B cells and plasmablasts is IFN-dependent, but independent of NK cell regulatory pressure (Figure S6).

Chapter 7: Blocking glycolysis in the bm12 transfer model completely abrogates lupus-like disease: Metabolic profiles of Tfh, GC B cells, and plasmablasts show that each is heavily dependent upon glycolysis.

i. RNAseq analysis on naïve bm12 CD4 T cells and bm12 Tfh (bm12 CD4 T cells two weeks after transfer into C57BL/6 mice) revealed a large number of differentially expressed genes involved in glycolysis, FAO, and to a lesser extent glutaminolysis. Careful examination of metabolic pathways revealed that Tfh from the bm12 and B6.Nba2 SLE models met their energetic needs through glycolysis and FAO, but not glutaminolysis (Figures 1-5).

ii. Parallel studies in GC B cells and plasmablasts from bm12 and B6.Nba2 mice showed that these activated B cell subsets were more heavily dependent upon glycolysis, with less FAO, and no utilization of glutaminolysis (Figures 7-9).

iii. Increased glucose transport was also observed in SLE patients’ circulating follicular helper-like T cells and B cells with phenotypic
markers of plasmablasts (Figures 3 and 7).

iv. *In vivo* disruption of glycolysis using 2-deoxy-D-glucose (2-DG) nearly completely inhibited disease development in the bm12 model. Of note, Tfh numbers were reduced by half, whereas GC B cell and plasmablast numbers were reduced more than 5-fold compared to controls, underscoring the relative importance of glycolysis to the development of these cells (Figures 11 and 12).

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**Figure 1. Working model: the critical roles for type I IFN in antitumor immunity and bm12 lupus-like disease.** Red lines indicate the multiple effects of type I IFN demonstrated by the work presented herein. We demonstrated that the STING/IRF3 pathway is activated upon the uptake of dying cell material by DCs. Furthermore, STING activation is dependent upon nuclear DNA and leads to the production of type I IFN by DCs. Subsequently, type I IFN signaling within DCs, but not CD8 T cells, promotes the productive priming of CD8 T cells toward cell-associated antigens (Chapter 5). We also showed that type I IFN sensing by CD4 T cells and B cells was important for the development of autoimmunity in the bm12 cGVHD model of lupus. The expansion of CD4+ Tfh cells was limited by NK cell killing, but the direct action of type I IFN on Tfh prevented this killing, allowing better expansion and advanced disease progression. B cells were not NK cell targets, however plasmablast and GC B cell expansion was also augmented by type I IFN through as of yet unknown molecular mechanisms (Chapter 6). We also examined the metabolic program of Tfh, plasmablast and GC B cells in bm12 disease. The green text depicts the relative dependence of these cell types on glycolysis or fatty acid beta-oxidation (FAO), as indicated by font size. None of these cell types were found to utilize glutaminolysis for their energetic needs. All utilized glycolysis, and to a lesser extent, oxidative phosphorylation downstream of FAO (Chapter 7).
Discussion

Despite years of intensive research, the current clinical strategies to boost anticancer immune responses still fail to produce durable protective immunity. Uncovering the molecular pathways that underlie immune responses to dying tumor cells may have significant impact on future treatments; many of the pitfalls of current strategies may be avoided by exploiting the existing mechanisms that promote these immune responses. Yet as we advance our understanding of the sterile inflammation that gives rise to immune responses toward cell-associated antigens, we must also examine whether any of these processes also contribute to autoimmune disease. Further mechanistic knowledge may also greatly benefit treatments for autoimmune diseases, not only increasing their efficacy, but increasing specificity, which may also reduce the complications associated with generalized immunosuppression. The sections that follow will discuss broad implications of the work contained within in the body of this dissertation, and will pose questions to be addressed by future studies.

In the Chapter 5, we found that nuclear DNA sensing drove STING-mediated type I IFN production, thereby enhancing immune responses to tumor cell-associated antigens. We identified DCs as the producers of IFN and the cell type whose sensing of IFN promoted antitumor CTL function. These findings have important implications for future work. Given the amount of effort put into the development of DC-based cancer vaccines and the relatively meager clinical results of those efforts, some authors have suggested that macrophages\textsuperscript{9} or B cells\textsuperscript{10} may be viable alternatives to DCs as professional APCs capable of initiating antitumor responses. Our data strongly suggest that DCs are the predominant cell type that productively primes antitumor CTLs naturally, and underscore the importance of DC exposure to type I IFN during CTL priming. Moreover, our data showing that
nuclear DNA structures induce type I IFN via the signaling adapter STING also provide a novel signaling pathway that might be therapeutically enhanced during priming. Current DC-based vaccine studies have targeted Toll-like receptors as adjuvants which can induce the production of type I IFN\textsuperscript{11}; however, activating STING may represent an alternative approach with a more nuanced signal that provides more effective and longer lasting protection. We found that STING also promoted autoimmunity in the bm12 cGHVD model of SLE. Thus, treatments specifically inhibiting the STING pathway may provide a way to reduce autoimmunity without compromising the ability to fight infections, a common side effect to the generalized immunosuppressive approaches currently employed\textsuperscript{12,13}.

The precise ligand or ligands which activate STING in our model systems are still unknown, as are the receptor(s) upstream of STING. Do dying cells activate STING through endogenous cyclic di-nucleotides produced by cyclic GMP-AMP synthase (cGAS)? Or do upstream cytosolic DNA sensors, such as DDX41, IFI16, or possibly DAI activate STING signaling? No known DNA sensor upstream of STING has been implicated by genome-wide association or sequencing studies, although rare mutations in the cytosolic exonuclease TREX\textsuperscript{14–17} are believed to dramatically increase the risk for SLE. Furthermore, a loss-of-function variant of MAVS, the signaling intermediate downstream of the cytosolic RNA sensors RIG-I and MDA5, has been associated with a subset of lupus patients with a unique clinical phenotype, including reduced circulating type I IFN levels compared with controls\textsuperscript{18}. As further progress is made in defining the candidate receptors upstream of STING, their involvement in SLE can be interrogated. And, greater insight into the ligands and receptors upstream of STING could provide an even more specific and effective target for future anticancer immunotherapies and treatments for autoimmune disease.

After showing a role for STING in the bm12 inducible model of SLE, and that disease was
partially dependent upon type I IFN sensing by recipient cells, we saw an opportunity to easily test whether the transferred bm12 CD4 T cells also depended upon IFN for disease induction. To our surprise, we found that disease was even more dependent upon CD4 T cell sensing of type I IFN than IFN sensing by any other cell type in the model. We went on to discover that the type I IFN produced in this system protected bm12 Tfh from being killed by NK cells. These findings raise a number of intriguing questions. One of the central questions that emerges is how does type I IFN protect Tfh from NK attack? NK regulation of T cell responses during infection has been the subject of other recent studies\(^{19-22}\), and some evidence has suggested that IFN signaling reduces the expression of an as of yet undetermined NKp46 ligand\(^{22}\). However, in our studies, we did not detect NKp46 ligand expression, nor did NKp46 deficient NK cells exhibit reduced killing of IFN-insensitive Tfh.

Whether the protective effect of IFN on CD4 T cells is multivariate or context-dependent is yet to be determined and remains an interesting question for future studies. We employed RNAseq as an unbiased approach to determine differentially expressed RNA transcripts, with the hope of discovering differentially expressed NK ligands in our WT and \(\text{Ifnar}^{-/-}\) Tfh. Unfortunately, analyses of these data did not provide any indication as to how IFN protected the Tfh from NK cells. Future studies utilizing single cell RNAseq would allow for a more precise analysis, where subsets of especially stressed cells may emerge, and may show differential expression of certain NK ligands. As the science of proteomics develops, this may be an even more appropriate method to answer this question, since it has been well established that RNA transcript levels do not always reflect differences in protein levels, and it is protein, not RNA, which largely mediates cellular functions.

We also found that type I IFN sensing by B cells is crucial to the development of GC B cells and plasma cells in bm12 disease. This expands upon previous reports that show
that the addition of exogenous type I IFN can enhance the ex vivo differentiation of B cells toward a plasma cell phenotype\textsuperscript{23,24}, and that using IFN as an adjuvant during chicken gamma globulin vaccination increases antibody output\textsuperscript{24,25}. Interestingly, the mechanisms by which type I IFN affects B cells appear to differ from those by which it affects T cells. Whereas IFN signaling protects T cells from NK cell attack, B cells were not subject to NK cell regulatory pressure in bm12 disease, and thus the effects of IFN signaling within B cells were completely independent of NK cells. Moreover, in the absence of perforin-mediated destruction, \textit{Ifnar}\textsuperscript{-/-} Tfh cells expanded significantly better than WT Tfh, indicating IFN exhibited an anti-proliferative effect \textit{in vivo}. Despite a vast body of literature describing the anti-proliferative effects of type I IFN on various cell types (nicely reviewed by Bekisz et al.\textsuperscript{26}), no such anti-proliferative effect has been reported \textit{in vitro} or \textit{in vivo} for B cells, and in fact here we observe the opposite effect of IFN on GC B cells and plasmablasts. In fact, previous studies using transgenic V\textit{l}10 transgenic B cells also showed poorer expansion of \textit{Ifnar}\textsuperscript{-/-}-deficient compared to WT plasmablasts\textsuperscript{24}. One possibility, however unlikely, is that GC B cell and plasmablast expansion is controlled by an Asialo-GM1-negative cell, perhaps even CD8 T cells; in fact, CXCR5-expressing, GC-homing “follicular” CD8 T cells have recently been described, although their function remains unknown\textsuperscript{27}. Future studies looking at the mechanism by which IFN sensing promotes B cell expansion should include perforin-deficient hosts, especially considering this method was more effective than NK depletion at restoring Tfh expansion in our model.

Our studies were limited to the early immune responses following the transfer of bm12 CD4 T cells into C57BL/6 mice—one exciting avenue for future studies would be to look at the longer-term effects of IFN-sensing by B cells. With longer term studies we could ask how IFN sensing by B cells affects the development of bone marrow-resident long-lived antibody-secreting plasma cells. This question could easily be answered by examining CD138-expressing, B220-negative plasma cells in the bone marrow at later time points
after bm12 transfer. One tool which would make these studies more practical and perhaps more physiologically relevant than mixed bone marrow chimeras, which we have used in all of our B cell experiments to date, would be mice with Ifnar−/− B cells, generated by flanking the Ifnar gene with loxP sites, and crossing them to CD19-Cre mice. One of our collaborators, Senad Divanovic, is currently generating these mice, and we are planning to perform future experiments with this tool. To determine when IFN sensing is required during disease development, an inducible Cre system could be employed. Or, to look more precisely at when IFN sensing is required during B cell development, Cy1-Cre or AID-Cre could be utilized. AID (Aicda) is expressed mainly by GC B cells during CSR and SHM. Similarly, Cy1 (Ighg1) is expressed upon CSR and would be found in a subset of GC B cells and plasma cells, mostly those that express IgG1. To further delineate the requirements of IFN sensing by GC B cells, plasmablasts, and plasma cells, GL7-Cre or CD138-Cre could be used. Although GL7 is also expressed on Tfh, in our bm12 model this would not be a significant confounder, since the Tfh responsible for interacting with these B cells would be the transferred bm12 cells, which would not harbor the GL7-Cre gene.

Another interesting question we could ask with longer term studies is how IFN sensing by B cells affects their memory potential. Answering this question would require even more sophisticated tools, and would probably best be tested using a transgenic B cell in another model system. Since the B cell responses we observe in the bm12 model are toward self antigens, these cells would have continual access to their cognate antigen. A transgenic system, however, would allow cells to be primed in the presence of a model antigen, rested, and then re-challenged again after several months. SWHEL mice would be an excellent model as they are transgenic for a HEL-specific BCR that is capable of CSR. A similar IFNAR and Cre system to those proposed above could be used to determine the role of IFN sensing in the development of memory. The initial priming could
be done via subcutaneous challenge with soluble HEL and adjuvant. Memory could then be determined 3-6 months later after HEL-rechallenge. Approximately 2 weeks after re-challenge, single cell suspensions from bone marrow and spleen could be subjected to IgG ELISpots, and memory potential would be determined by the comparing the number of HEL-specific antibody secreting cells.

Taken together, we found that type I IFN independently increases the expansion of several key disease-causing adaptive immune cell populations—namely, Tfh, GC B cells, and plasmablasts. Importantly, each of these cell types have been implicated in the pathology of human SLE29–31. Thus, these data have significant implications for the potential of IFN or IFNAR neutralization in lupus patients, providing a mechanism as to why this strategy may be successful. Recent attempts at clinical IFN blockade via neutralizing antibodies produced poor results32,33, although the inability of these antibodies to neutralize all IFN subtypes may easily explain their failure. Early results from a phase 2 clinical trial using an anti-IFNAR antibody, however, showed that patients given the antibody, anifrolumab, had significantly reduced disease activity across multiple clinical parameters34. This treatment is currently being further investigated in a phase 3 trial.

One important consideration for the development of IFNAR neutralization therapies is how its neutralization may affect NK cell activity. Type I IFN signaling has been shown to stimulate the production of IFNγ by NK cells, and NK cells from Ifnar-deficient mice exhibit substantial reductions in their ability to kill certain target cells compared to those from WT mice35,36. This activation of NK cells by type I IFN has been shown to be critical to the protective functions of NK cells in the context of infection35 and antitumor immunity36. Moreover, our findings indicate that active NK cells may also help limit Tfh cells that are participating in autoimmune germinal center reactions. Thus, removing one primary mechanism by which NK cells become activated may have a two-fold negative impact on
the health of patients—potentially increasing their susceptibility to infection and cancer, and removing a regulatory control of autoimmune Tfh cells. Indeed, higher incidences of influenza and herpes zoster infections were reported in patients receiving anifrolumab compared with controls in the phase 2 study of the anti-IFNAR antibody.

We propose that a combination therapy, including neutralization of type I IFN or its receptor, and a means of boosting NK cell activity may result in a more effective treatment with fewer side effects. Of course, non-specific activation of NK cells using, for example, intravenous IL-2, IL-12, or IL-18 would not be advised, given the ability of those cytokines to activate the cells involved in the autoimmune pathology. Instead, we propose a highly targeted approach to activate NK cells either through agonists to the NK cell activating receptor NKp46 (NCR1), or by adoptive transfer of autologous ex vivo activated NK cells. Of course the practicality of the latter approach would be limited by the ability to expand NK cells from each individual undergoing treatment, but this technique has already been pioneered and is being optimized by ongoing translational research in the field of anticancer immunotherapy. In fact, ex vivo expansion and adoptive transfer of NK cells may prove the best way of enhancing NK cell function, because despite the fact that NK cells from the periphery of SLE patients show phenotypic markers of activation, including higher percentages of CD69+ and IFN-γ+ cells, they also exhibit lower cytotoxicity, and are found in significantly reduced numbers compared to controls. Thus, expanding newly generated NK cells in vitro and subsequent adoptive transfer would more likely enhance NK cell activity in vivo.

Our data within the bm12 model suggests activated NK cells would target activated Tfh in autoimmune GC reactions, but would not target B cell populations. Thus, activating NK cells is not expected to affect the reactivation of autoimmune memory B cells, but it may help prevent the generation of new autoimmune B cells by indirectly limiting GC
reactions. Furthermore, transfer studies in a mouse model similar to the bm12 model showed the transfer of IL-2-activated autologous NK cells significantly limited autoantibody production. Moreover, studies in the streptozotocin-induced model of type I diabetes (T1D) showed that the transfer of IL-18-activated NK cells delayed and partially prevented the onset of diabetes. These studies provide substantial rationale for the potential efficacy of activated NK cell transfers in SLE, but they also highlight the possibility of a broader role for NK cell immunoregulation in additional autoimmune diseases. Actually, a type I IFN gene signature precedes the onset of T1D, and the treatment of viral hepatitis with type I IFN has been shown to induce T1D, although the role for IFN in T1D pathogenesis has not been elucidated. It would be interesting to deplete NK cells in the context of a T1D model to determine whether endogenous NK cells exert any pressure on T cells within that disease.

Our investigations into cellular metabolism, presented in the final research chapter of this dissertation, began with the hypothesis that type I IFN may exert some of its effects on Tfh cells by changing their metabolic program. Specifically, we posited that type I IFN may prevent metabolic stress, and thereby enhance the survival of activated CD4 T cells, by reducing proliferation rates and by controlling expression of c-Myc and mTOR, both of which are known to play pivotal roles in directing cellular metabolism. In fact, we did observe effects of IFN on the metabolic program of in vitro activated (anti-CD3, anti-CD28), purified CD4 T cells (Figure 2, unpublished data). We found that Ifnar-deficient CD4 T cells had a lower respiratory reserve than WT cells, and elevated glycolytic rates. These data fit the hypothesis that IFN-insensitive cells undergo greater metabolic stress than do their WT, IFN-sensing counterparts. It is interesting to speculate whether the apparent metabolic stress that IFN-insensitive Tfh are subject to contributes to their susceptibility to NK cell killing, by either expression of activating NK ligands, or a reduction of inhibitory ligands. These results were fascinating, and merit further investigation.
At the same time we made these observations, we investigated the metabolic program of Tfh, since it has not yet been described. Given the massive expansion of Tfh in the bm12 model, and the relative ease by which these cells can be sorted (they are the only cells which express CD45.1 in a CD45.2 host), we had a unique opportunity to analyze large numbers of Tfh. We found that Tfh utilize glycolysis and fatty acid oxidation, and went on to profile the metabolic programs of GC B cell and plasmablasts, which relied almost exclusively on glycolysis. This culminated in a compelling study, where we found that inhibiting glycolysis \textit{in vivo} using 2-DG completely ameliorated bm12 disease. This was particularly exciting, as the same chemical used in our studies to inhibit glycolysis

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**Figure 2.** Type I IFN alters CD4 T cell mitochondrial respiratory reserve and reduces aerobic glycolysis. (A) Schematic depicting mitochondrial function as defined by a mitochondrial stress test, in which cells are subjected to oligomycin, FCCP, and finally antimycin + rotenone A. (B) One representative experiment demonstrating the effects of IFN signaling on mitochondrial function after 24 h of stimulation. (C-D) Summarized OCR (C) and ECAR (D) data from 3 experiments where values are relative to WT, which was defined as 1.0. (E) 2-NBDG uptake (gMFI) by CD4 T cells 24 h after stimulation.
has been successfully tested for safety in a phase I clinical cancer trial\textsuperscript{54}, suggesting it has potential for therapeutic use in patients with autoimmune disease.

In our studies that examined glucose transporter levels and glucose uptake, an interesting divergence between our B cell subsets and T cell subsets emerged. As B cells became activated and differentiated into GC B cells or plasmablasts, we noted a stepwise increase in the uptake of glucose, as represented by 2-NBDG fluorescence, that was logarithmically proportionate to an increase in the glucose transporter, Glut1 (Slc2a1). With T cells, however, we observed an equivalent increase in Glut1 expression as seen in plasmablasts, but only a marginal increase glucose uptake. This could reflect a number of distinct mechanistic possibilities—perhaps, simply, plasmablasts utilize additional glucose transporters to obtain such high levels of glucose uptake, whereas Tfh rely chiefly upon Glut1 for glucose uptake. Alternatively, perhaps plasmablasts have much more glucose available to them due to their extrafollicular localization within secondary lymphoid organs\textsuperscript{55}, such that an equivalent glucose transporter level would allow much more glucose uptake. However, factors other than localization must also be contributing to this phenomenon, since GC B cells and Tfh both localize to the germinal centers, where they rapidly proliferate, and their glucose uptake:Glut1 ratios differ drastically.

An additional question our metabolic data raises is why glucose is evidently so much more important to plasmablasts than the other cell types participating in this autoimmune reaction. As it is with T cells, it may be that mature, activated B cells require a shift to glycolysis for their effector functions rather than for their differentiation and proliferation\textsuperscript{56,57}. Indeed there is some evidence to suggest that B cell utilization of glycolysis is essential for antibody production\textsuperscript{50}. Future studies could test the roles of glycolysis in specific B cell subsets by generating CD138-Cre or GL7-Cre mice and crossing them to Glut1\textsuperscript{fl/fl} mice, given the high specificity of these phenotypic markers to plasmablasts and GC
B cells, respectively. Follow up studies should also more closely examine the potential divergent use of additional glucose transporters, many of which have been detected on lymphocytes\textsuperscript{58–61}.

In summary, our use of mouse modeling has led to significant advancement in our understanding of T cell and B cell biology during sterile inflammation, particularly in the context of anticancer immunity and autoimmunity. Carefully controlled cellular and molecular studies have elucidated previously unappreciated roles for type I IFN in directing T cell and B cell differentiation, proliferation, and function. These studies have identified novel targets for potential use in cancer immunotherapies and treatments for autoimmune disease and raise many interesting questions to be addressed in future studies. We have revealed STING as an important mediator of type I IFN production in cancer treatments as well as in a model of cGVHD-driven autoimmune disease. We also uncovered multiple functions of type I IFN on DCs, CD4 T cells, and B cells, which if specifically targeted could allow for more nuanced, tailored therapies. We also found a potential link between type I IFN and cellular metabolism, though these studies need further investigation. Finally, our work examining the cellular metabolism of Tfh and autoimmune B cells suggests promising opportunities for using inhibitors of glycolysis in the treatment of autoimmune diseases.
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April 19, 2016

Jared Klarquist  
Cincinnati Children's Hospital Medical Center  
240 Albert Sabin Way  
Cincinnati, OH 45229  
United States  
Email: klarquist@gmail.com

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Melanoma-infiltrating dendritic cells
Limitations and opportunities of mouse models

Jared S. Klarquist and Edith M. Janssen*

Division of Cellular and Molecular Immunology; Cincinnati Children’s Hospital Research Foundation; University of Cincinnati College of Medicine; Cincinnati, OH USA

Keywords: dendritic cells, melanoma, mouse model

The infiltration of melanoma lesions by dendritic cells (DCs) has been suggested to play a tumorigenic role due to the capacity of DCs to induce tumor tolerance and promote angiogenesis as well as metastasis. However, it has also been shown that tumor-infiltrating DCs (TIDCs) induce antitumor responses and hence may be targeted in cost-effective therapeutic approaches to obtain patient-specific DCs that present relevant tumor antigens, without the need for ex vivo DC expansion or tumor antigen identification. Unfortunately, little is known about the composition, nature and function of TIDCs found in human melanoma. The development of mouse melanoma models has greatly contributed to the molecular understanding of melanoma immunology in mice, but many questions on TIDCs remain unanswered. Here, we discuss current knowledge about melanoma TIDCs in various mouse models with regard to their translational potential and clinical relevance.

Introduction

The incidence of cutaneous malignant melanoma has been steadily increasing over the last decades. While complete surgical excision yields high 5-year survival for patients with localized tumors exhibiting a depth < 0.75 mm, the outcome is poor for patients with a greater depth of invasion or bearing metastases. The development of novel therapeutic approaches is therefore of great importance. Interestingly, melanomas are relatively immunogenic tumors and sensitive to cytotoxic T lymphocyte (CTL)-mediated lysis. As dendritic cells (DCs) are the main antigen-presenting cell (APC) population capable of inducing CTLs, DC transfer, DC targeting and in situ DC induction, recruitment and/or activation have been explored as promising immunotherapeutic strategies against melanoma. The topical or intratumoral administration of DC-activating agents—including interferon α (IFNα), bacillus Calmette-Guérin (BCG), or purified Toll-like receptor (TLR) ligands such as imiquimod—are recommended as treatment options for patients with in-transit melanoma metastasis.1,2,3 While this approach is relatively successful against cutaneous metastases, efficacy is limited for subcutaneous metastases. An improved understanding of the type, nature and functionality of TIDCs could lead to novel and more effective therapeutic approaches. To circumvent ethical issues and TIDC availability constraints associated with human research, various animal models for melanoma have been established in organisms including Xiphophorus, Danio rerio, guinea pigs, opossum and small rodents, all of which have unique advantages and disadvantages. The relevance of the model under examination depends on the questions to be answered and how closely the model mimics the histological, immunological and metastatic pattern observed in humans.4 To date, most work is performed in mice due to the availability of genetically modified animals, insights into mouse immunology, pathology and physiology and the plethora of mouse-specific research tools.

Here, we will briefly review the current knowledge of TIDCs obtained in the most common mouse melanoma models and the insight they have provided into the human disease.

Selection of Mouse Model for Melanoma

Melanoma models are generally divided into 3 different groups based on research focus: xenograft models, which allow for the study of tumor cell behavior; transplantation models, to study melanoma immunology; and genetically modified animal models, which focus on melanomagenesis. Pure chemical carcinogen-induced melanoma models have decreased in popularity as they have relatively low relevance to human disease and therefore will not be discussed further in this article.

Xenograft models consist of orthotopic or ectopic transplantation of human cancer cells or solid tumors into immunocompromised mice. The primary advantage of these models is the preservation of human cancer cell behavior, including metastatic potential and tissue preference. However, the absence of a functional immune system does not allow for the study of the interactions between tumors and immune cell subsets. While DC function is relatively normal in some immunocompromised mouse strains, various others—including those with a NOD.

Abbreviations: APC, antigen presenting cell; CCR7, chemokine receptor 7; cDC, conventional DC; CTL, cytotoxic T lymphocyte; DC, dendritic cell; dDC, dermal DC; IFN, interferon; GEMM, genetically engineered mouse model; LC, Langerhans cell; LXRα, liver X receptor alpha; NLR, NOD-like receptor; pDC, plasmacytoid DC; SOCS3, suppressor of cytokine signaling 3; TIDC, tumor-infiltrating DC; TLR, Toll-like receptor; VEGF, vascular endothelial growth factor

*Correspondence to: Edith M. Janssen; Email: edith.janssen@cchmc.org
Submitted: 09/12/12; Revised: 10/24/12; Accepted: 10/24/12
http://dx.doi.org/10.4161/onci.22660

Division of Cellular and Molecular Immunology; Cincinnati Children’s Hospital Research Foundation; University of Cincinnati College of Medicine; Cincinnati, OH USA

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Cg-Prkdceil2rg background—exhibit defective DC development and function. In addition, human tumor-derived mediators might affect the recruitment, retention, development and function of mouse DCs in a different fashion than their mouse homologs. The more recent development of human melanoma models in humanized mice has not only circumvented these issues and provides an intriguing platform for clinically relevant TIDC studies.

Syngenic transplantation models have been around since the identification of the Cloudman S91 melanoma in BDA/2 mice, Harding-Passey melanoma in BALB/c × DBA/2F1 mice and B16 melanoma in C57BL/6 mice. B16 is currently the most widely used melanoma model and has the advantages that it expresses at least 5 homologs of the best characterized human melanoma antigens (gp100/pmel17, MART-1/MelanA, tyrosinase, TRP-1/gp75 and TRP-2/DCT), it is immunogenic and it displays metastatic behavior. The main drawback of this model is the rapid growth of the primary tumor, resulting in problems related to vascularization, necrosis and swift mortality that preclude the assessment of prolonged tumor burden on TIDC behavior. Nevertheless, most TIDC studies have been performed in B16 melanoma models.

Genetically engineered models (GEMM). The identification of genetic and epigenetic abnormalities in human melanomas has led to the development of genetically engineered mice with a heritable predisposition to the development of melanoma. The tissue-specific expression of oncogenes including Ret, mutant forms of (N/K/H)-Ras and Braf and Hgf, coupled or not to backcrossing in susceptible genetic backgrounds (Jak6/Arf, Tp53, Cdkn2a, Cdkn2d, Cdkn2a-1, Cdk4R24C/R24C, etc.) has yielded melanoma models with different latency, penetrance and metastatic potential (reviewed in refs. 18, 19). Although the distribution of melanocytes differs between mice and humans, these models have great clinical relevance as they are based on genes known to be involved in the genesis and progression of human melanoma, and can be easily combined with relevant environmental triggers such as UV irradiation, to accelerate melanoma incidence. Only recently the field has begun to use these models for TIDC studies.

Dendritic Cells

DCs are a heterogeneous population in terms of origin, morphology, phenotype and function. DCs are derived from common myeloid and lymphoid precursors and rely heavily on FLT3 ligand (FLT3L) and/or granulocyte macrophage colony-stimulating factor (GM-CSF) for their development. DCs express ligand (FLT3L) and/or granulocyte macrophage colony-stimulating factor (GM-CSF) for their development. DCs express a broad range of soluble pro- and anti-inflammatory mediators, including multiple cytokines and chemokines. T cells interacting with DCs via cognate TCR-peptide-MHC complexes will undergo apoptosis, anergy, or develop a regulatory phenotype if the balance of co-stimulation is tilted on the negative side. Conversely, if positive signals surpass an intrinsic threshold, T cells will undergo proliferation, differentiation and acquire effector functions. Immature DCs display great phagocytic functions, relatively poor antigen-presenting capacity and low levels of positive co-stimulatory molecules. Upon activation via innate receptors such as TLRs or NOD-like receptors (NLRs), pro-inflammatory cytokines or cross-linking of CD40, DCs mature, reduce their phagocytic potential, increase antigen-presenting capacity, upregulate co-stimulatory molecules, change their cytokine production profile (qualitatively and quantitatively) and migrate to draining (lymphoid) areas, where they interact with T cells.

Cells with DC characteristics have been repeatedly described in human melanoma samples. Depending on study, markers used, localization and maturation status, DC infiltration has been linked to a positive or negative prognostic outcome. The discrepancy in outcomes can be attributed to differences in clinical stages, the use of primary vs. metastatic lesions, as well as use of markers that are relatively non-specific or restrictive to a subpopulation of DCs.

Studies in several other tumor systems indicate that malignant cells inhibit dendroptosis, decelerate DC differentiation and maturation, induce functional DC deficiencies and accelerate cell death in DCs or their precursors. The maintenance of an immature phenotype or the promotion of a tolerogenic one could lead to anergy/deletion of tumor-specific T cells and the induction of cells with immunosuppressive functions such as FOXP3+ regulatory T cells (Tregs). An inhibited DC differentiation might also contribute to the accumulation of myeloid-derived suppressor cells, as the latter generate from precursors that under physiological conditions would differentiate into DCs, macrophages and neutrophils. In addition, immature DCs and pre-DCs have been suggested to promote angiogenesis through the secretion of growth factors (i.e., vascular endothelial growth factor, VEGF) that directly act on the endothelium, or the production of mediators that enhance the sensitivity of endothelial cells to growth factors. Some studies suggest that DC precursors might even undergo endothelial transdifferentiation or provide a scaffold for subsequent lining by endothelial cells.

Murine and Human DC Populations

Recent genomic and proteomic approaches have discovered significant similarities between human and mouse DC populations, thereby strengthening the relevance of TIDC research in mouse melanoma models. While several aspects of localization, surface marker and TLR expression, phagocytic potential and antigen presenting capacity are relatively comparable between some mouse and human DC subsets, these are not perfect matches and in some cases the equivalent populations are absent. We will briefly describe the mouse and human DC populations in the following sections.

Mouse DC populations. Under steady-state conditions, mouse DCs express Cdl1c as well as MHC Class II molecules and can be subdivided into plasmacytoid DC (pDCs) and conventional DCs (cDCs). pDCs express intermediate levels of Cdl1c as well as high levels of Cd45ra (B220), Pdca1 (Cd317), Tlr1, Tlr2, Tlr4, Tlr7 and Tlr9, and play an important role in infection due to their capacity to produce large amounts of Type I IFNs. Antigen presentation by pDCs is thought to be relatively...
levels of CD205 and the DC immunoreceptor (DCIR). Both DCIR and CD205 are associated with antigen uptake and induction of antigen-specific T-cell responses. LCs express mRNA coding for TLR1, TLR2, TLR5, TLR6 and TLR9 (but not for TLR4, TLR7 and TLR8). The number of dDCs populations described in humans has recently been expanded. The major dDC population is BDCA-1+, and most of these cells express CD11c while only about 50% of the BDCA-1+ population express CD1a. CD1c BDCA-3+ dDCs represent about 10% of all CD11c+ dDCs and demonstrate superior cross-presentation of soluble antigens as compared with other DC populations. Most dDCs express mRNA coding for TLR1, TLR2, TLR4, TLR8 and TLR10 but the exact distribution of these TLRs is not known for specific DC subsets.

Human melanoma TIDCs. Melanoma-infiltrating DCs have been found in primary and metastatic lesions and encompass a broad spectrum of DC-like cells, including CD207+ LCs, pDCs and CD1a+ DCs (Table 1). Due to differences in patient material, the relatively low frequency of TIDCs, the use of ambiguous analytical markers, and approaches that limit the number of available analytical markers, there is little consensus on the exact composition of the TIDC population. However, there is a general agreement on the fact that the frequency of TIDCs is higher in the peritumoral area than within neoplastic lesions and that TIDCs with the most mature phenotype (DC-LAMP+CD83+fascin+) tend to reside in the peritumoral area. It is thought that immature DCs enter tumors via the vasculature and follow further differentiation and activation—migrate toward the tumor edge. There, DCs either form T-cell clusters or migrate toward the draining lymph node, where they interact with T cells. The relationship between the presence and location of different TIDC subsets and clinical outcome remains a puzzle, as it not only depends on the type of TIDCs, but also on their activity as well as on functional interactions with other cells, all aspects that remain poorly understood.

Mouse melanoma TIDCs. While mouse models have the advantage of providing abundant tumor material, which allows for an easy selection of tumors at different developmental stages, there is surprisingly little consensus in the field about mouse TIDC frequency, composition and function. Some of these discrepancies result from the use of different model systems or genetic backgrounds. When we compared two xenograft models, 3 syngeneic transplantation models and 2 GEMMs, we observed significant differences in TIDC frequency (data not shown) and composition between models (Fig. 1A). The highest frequency of TIDCs was seen in syngeneic transplant models, while GEMMs exhibited significantly less TIDCs. However, GEMMs showed a greater diversity of TIDCs, with marked infiltration by pDCs, LCs and dDCs. Xenografts showed the least diverse variety, completely lacking LCs and dDCs, while in syngeneic transplant models an occasional dDC (CD207+EpCAM+) subset was found. Although a full comparison is hard to make as not all studies used the same set of markers, a review of the current literature on mouse melanoma revealed similar findings in different model systems (Table 2).
Table 1. Human melanoma TIDC

<table>
<thead>
<tr>
<th>Study</th>
<th>DC marker</th>
<th>DC specifics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Garcia-Plata</td>
<td>S100, CD1a, HLA-DR</td>
<td>S100+CD1a+ (LC) increased in peritumoral infiltrate compared with overlying epidermis. HLA-DR levels variable.</td>
</tr>
<tr>
<td>Movassagh</td>
<td>CD123, DC-LAMP, fascin, CD1a, CD207</td>
<td>CD1a+ and CD207+ cells in epidermis of regressing lesion infiltration; fascin+/DC-LAMP+ cells accumulation around microvessels within tumor area (tumor regression)</td>
</tr>
<tr>
<td>Salio</td>
<td>CD123, BDCA2, CLA</td>
<td>Observed in majority of melanomas; numbers higher in infiltrating and metastatic samples. Numbers increase with severity of disease</td>
</tr>
<tr>
<td>Vermi</td>
<td>CD1a, CD123, CD207, DC-Sign DC-LAMP, MR</td>
<td>Increase in dermal myeloid and pDC compared with healthy skin. Intratumoral immature: MR+/DC-Sign+/CD1a- and CD1a+/CD207- cells Peritumoral immature: CD1a+/CD207+; MR+/DC-Sign+/CD1a-; CD1a+/CD207+; CD123+/BDCA-2+; Peritumoral mature: CD83+DC-LAMP+</td>
</tr>
<tr>
<td>Ladanyi</td>
<td>CD1a, DC-LAMP</td>
<td>CD1a+ in melanoma cell nests and stroma, DC-LAMP+ in peritumoral area: inverse correlation CD1a+ and DC-LAMP+ cells with melanoma thickness</td>
</tr>
<tr>
<td>Simonetti</td>
<td>CD83, CD207</td>
<td>Inverse correlation langerin+ cells with tumor depth; lower density of CD83+ DC in thick melanomas</td>
</tr>
<tr>
<td>Charles</td>
<td>BDCA-2</td>
<td>Observed in 37% of cases. Located close to the tumor within the peritumoral leukocyte infiltrate, representing 2–5% of these cells</td>
</tr>
<tr>
<td>Jensen</td>
<td>CD123, DC-LAMP</td>
<td>CD123 infiltration: tumor stroma (~30%), tumor nest (~15%) of samples DC-LAMP+ infiltration: tumor stroma (~30%), peritumoral (~50%) of samples</td>
</tr>
<tr>
<td>Erdag</td>
<td>DC-LAMP, CD163neg</td>
<td>&gt; 1% of CD45 cells: Metastasis to LN contain higher number of LAMP+ cells compared with metastasis to skin/soft tissue peritoneum, small intestine</td>
</tr>
<tr>
<td>Martinet</td>
<td>DC-LAMP, fascin</td>
<td>DC-LAMP+ cells frequently associated with tumor HEV; Density of DC-LAMP+ cells correlates with density of tumor HEV</td>
</tr>
</tbody>
</table>

The differences in the composition of TIDCs across models and species highlight the importance of model validation for each type of study. While all models have significantly contributed to the current understanding of melanoma immunology, pre-clinical DC targeting studies would benefit from models that more accurately resemble the TIDC composition seen in patients.

Mouse TIDC activation status. As in human melanoma, mature mouse TIDCs tend to reside in the peritumoral areas and total TIDCs seem to increase upon disease progression (Fig. 1B and C). Most studies assessing mouse TIDC activation and maturation status were based on the flowcytometric analysis of CD11c+ cells from the entire tumor. Consequently, most reports show a biphasic distribution of the maturation markers CD40/CD80 and CD86,78,79,80 The differential analysis of the peritumoral and intratumoral zones of B16/F10 melanomas replicate histological observations, showing significantly more mature TIDCs in the peritumoral area as compared with the intratumoral one (Fig. 1D). It is thought that the tumor environment promotes the recruitment of DC precursors and immature DCs, but little is known on the ability of melanomas to support in situ DC differentiation.78,79,80,81,82 Diao, et al. showed that adoptively transferred immediate DC precursors (Lin−CD11c+MHCII+ cells) are recruited to B16/F10 tumors, where they proliferate and differentiate into cells with T-cell priming capacity in vitro, suggesting at least a partial acquisition of DC-like functions.77 On the other hand, in vivo data from Fainaru, et al. demonstrate that the recruitment of immature DCs promotes angiogenesis and tumor growth by enhancing endothelial cell migration and the subsequent formation of vascular networks.78 Moreover, the depletion of CD11c+ cells in CD11c-diphtheria toxin receptor (DTR) transgenic mice has been shown to significantly reduce the tumor mass of intraperitoneally injected B16/F10 melanoma cells.78 While other models suggest a role for an endothelial-like differentiation of DC precursors, VEGFA, β defensin, basic fibroblast growth factor (bFGF) and transformin growth factor β1 (TGFβ1) in this process, the mechanism underpinning DC-supported vasculogenesis in melanoma has not been clearly established.79,80

Mouse TIDC functionality. In order to operate as bona fide APCs, DCs need to acquire antigens through one of the phagocytic pathways, process and present them and communicate with T cells locally or upon migration to draining areas. Studies injecting beads into tumors revealed that a sizable fraction of TIDCs acquire one or more beads, indicating that that particulate uptake mechanisms is relatively intact.71,73 However, Gerner et al. showed that TIDCs manifest a defect in the uptake of intratumorally injected proteins as compared with dDCs from healthy tissue.73 Separating peritumoral and intratumoral TIDCs, we found that the in vitro uptake of proteins and apoptotic cell material was higher for peritumoral, as compared with intratumoral, TIDCs (Fig. 2A). Similar observations were made when peritumoral and intratumoral TIDCs were analyzed 4 h after the intratumoral injection of proteins in vivo. Interestingly, the co-administration of lipopolyascharide (LPS) appears to decrease the phagocytic uptake by peritumoral TIDCs, but not by their intratumoral counterparts (Fig. 2B). Most studies reveal a decreased CD4+ and CD8+ T-cell activating capacity of TIDCs isolated from antigen-expressing tumors or upon antigen pulsing in vivo.70,73,74 However, other studies indicate potent T-cell priming capacity of TIDCs, both in vitro or in vivo.71,77,81 This discrepancy can be partly explained...
by the fact that these studies differed relative to TIDC composition, TIDC localization, TIDC maturation state, TIDC isolation methods and in vitro functional assessment protocols. By separating TIDCs based on GR1 expression, Diao et al. showed that GR1+ expressing TIDCs produce more interleukin (IL-10) and exhibit lower CD8+ and CD4+ T-cell priming capacity than GR1− TIDCs when loaded with antigens in vitro. In addition, CD8+ T cells primed by GR1+ TIDCs demonstrated significantly reduced cytokine production compared with CD8+ T cells primed by GR1− TIDCs. Gerner, et al. suggested that the decreased TIDC capacity for CD4+ T-cell activation results predominantly from reduced antigen uptake as they found antigen processing and presentation to be unaltered. To further dissect the antigen presenting and T-cell priming/activating potential of TIDCs, we isolated peritumoral and intratumoral TIDCs from ovalbumin (OVA)-expressing B16 tumor-bearing mice and cultured them with an OVA257–264-specific reporter cell line (B3Z) and CFSE-labeled OVA257–264-specific OT-1 T cells. We included brefeldin A in the isolation procedure to prevent the turnover of MHC-I-peptide complexes while preserving the TIDC maturation state. Importantly, significant antigen presentation was observed only when brefeldin A was present during
the isolation period, illustrating the importance of optimizing and standardizing TIDC isolation protocols. The total TIDC fraction poorly activated B3Z cells (Fig. 2C), suggesting a low frequency of OVA257–264-MHC complexes. Consequently, total TIDC-mediated OT-1 T-cell activation and proliferation, as determined by CD69 upregulation and CFSE dilution assays, was relatively poor (Fig. 2C and E). However, peritumoral TIDCs displayed a comparatively higher frequency of OVA257–264-MHC complexes and activated (while inducing the proliferation) of a sizable fraction of OT-1 T cells (Fig. 2C–E). Intratumoral TIDCs exhibited less OVA257–264-MHC complexes and activated OT-1 T cells without inducing proliferation. This lack of proliferation could be restored by the addition of IL-2 but not upon the blockade of IL-10 or TGFβ, suggesting the induction of T-cell nonresponsiveness. Importantly, the treatment of peritumoral TIDCs with TLR4 or TLR9 ligands significantly increased their potential to induce T-cell proliferation, while the same treatment did not improve the functionality of intratumoral TIDCs (data not shown). Altogether, these observations show that differences in isolation protocols, TIDC subsets, and functional assays significantly complicate the comparison between studies and the extrapolative value of their findings. While many studies indicate a decrease in the maturation and functionality of melanoma TIDCs, the mechanisms that underpin such changes in APC functions are still unclear. Increased expression of immunosuppressive cytokines and membrane-associated molecules by TIDCs has been implicated in TIDC dysfunction.84–88 Other models suggest that tumor-derived cytokines or a reduction in the sensitivity of TIDCs to innate signals prevents maturation, migration and thereby impair TIDC function.72,83 Other models suggest that the mechanisms of TIDC dysfunction.72,83 Other models suggest that the mechanisms of TIDC dysfunction could be restored by the addition of IL-2 but not upon the blockade of IL-10 or TGFβ, suggesting the induction of T-cell nonresponsiveness. Importantly, the treatment of peritumoral TIDCs with TLR4 or TLR9 ligands significantly increased their potential to induce T-cell proliferation, while the same treatment did not improve the functionality of intratumoral TIDCs (data not shown). Altogether, these observations show that differences in isolation protocols, TIDC subsets, and functional assays significantly complicate the comparison between studies and the extrapolative value of their findings.

Table 2. Mouse melanoma TIDC characteristics

<table>
<thead>
<tr>
<th>Model</th>
<th>DC marker</th>
<th>Frequency</th>
<th>DC subpopulation</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scid-MV3 xenograft</td>
<td>BSA-I binding</td>
<td>~35/5 high power fields</td>
<td>-</td>
<td>Partially activated; reduced capacity for protein uptake and subsequent MHC II presentation; less sensitive to TLR stim</td>
</tr>
<tr>
<td>B16/F10. s.c.23</td>
<td>CD11c, MHCIi</td>
<td>-</td>
<td>All CD11b+; further negative for EpCAM, PDCA-1, CD4, CD8α</td>
<td>GR1+ less mature populations, fails to stimulate MLR, produce more IL-10; protein pulsed Gr1+ DC poorly activate OVA-specific CD4 and CD8 T cells in vivo</td>
</tr>
<tr>
<td>B16/F10. s.c.75</td>
<td>CD11c, MHCIi</td>
<td>-</td>
<td>All CD11b+; most F4/80+ ~23.5%, GR1+; few pDC</td>
<td>Pre-DC (Lin-CD11c+MHCIi-Fit3+) cells are recruited in the tumor, differentiated and activated CD8 T cells in vitro upon peptide pulsing</td>
</tr>
<tr>
<td>B16/F10 sec.c.77</td>
<td>CD11c, MHCIi</td>
<td>~30% of TIL</td>
<td>Mostly CD11b+; ~5% pDC, hardly CD207+</td>
<td>Immature phenotype; fail to activate OVA-specific CD4 and CD8 T cells ex vivo</td>
</tr>
<tr>
<td>B16/F10 sec.c.50</td>
<td>CD11c</td>
<td>~20% of TIL</td>
<td>~33% CD11b-CD8αDC, rest CD11b+CD8αDC</td>
<td>Partially mature; no in vitro activation of OVA-specific CD4 and poor activation of CD8 T cells</td>
</tr>
<tr>
<td>B16/F10 sec.c.73,81</td>
<td>CD11c, MHCIi</td>
<td>0.13 ± 0.07% of total cells</td>
<td>~3% pDC, ~2.25% CD8αDC, &gt; 95% non-pDC non CD8α</td>
<td>Decreased number compared with skin; immature phenotype, particle uptake in vivo normal; protective upon transfer.</td>
</tr>
<tr>
<td>K17–35</td>
<td>CD11c, MHCIi</td>
<td>4.0 ± 0.22% of total cells</td>
<td>~15% pDC, ~12% CD8αDC</td>
<td>Increased number compared with skin; immature phenotype; particle uptake in vivo normal</td>
</tr>
<tr>
<td>Tyr-N-Ras50,S4, DMBA/C12,07</td>
<td>CD11c, MHCIi</td>
<td>0.02 ± 0.004 of total cells</td>
<td>~58% pDC, ~40% non pDC non CD8αDC</td>
<td>Decreased number compared with skin; immature phenotype</td>
</tr>
<tr>
<td>MT/ret</td>
<td>CD11c, MHCIi</td>
<td>3–10% of TIL</td>
<td>-</td>
<td>Increasingly immature phenotype upon melanoma progression</td>
</tr>
</tbody>
</table>
to target TIDCs or support TIDC functions, it is likely that they only partly activate TIDCs, as (1) some specifically targeted DC populations are absent or poorly represented in the tumor, and (2) some specifically targeted receptors are poorly expressed by TIDCs or rendered non-functional by the tumor microenvironment. In these cases, it is more likely that other cells in the tumor environment are stimulated to promote a DC activating/restoring microenvironment.

In order to improve the clinical relevance and translational potential of mouse melanoma models for the design, optimization, and identification of novel therapeutic interventions that target TIDCs we will have to overcome several hurdles. An improved identification and characterization of human TIDCs will be critical to identify and validate the best mouse models for each type of study. Eventually, the panel of DC specific markers used in human and mouse studies will have to be standardized, even as investigators continue to discover new markers and DC populations.32 Furthermore, the optimization and standardization of protocols for TIDC isolation and functional assessments disciplines beyond classical immunology will have to be incorporated into the experimental approaches.

Scientific and Therapeutic Considerations

Mouse models have been extensively used to test topical therapeutic therapies. Comparable to human melanoma, the injection of GM-CSF, IFNα, imiquimod, or BCG has been shown to result in various degrees of therapeutic success in mice.53–96 In many of these approaches, either increased numbers of DCs or enhanced DC maturation was observed in the tumor or tumor-draining lymph node.53–96 In addition, other purified TLR ligands including poly(I:C), CpG oligonucleotides, LPS, alone or coupled to additional immunomodulatory therapies have been used successfully.97–99 The intratumoral administration of crude bacterial products, cytokines and stimulatory molecules delivered by viral vectors, microspheres or nanoparticles is well established in mouse models but has not been translated to the human system.3,100,101 While all these therapeutic approaches were suggested to target TIDCs or support TIDC functions, it is likely that they only partly activate TIDCs, as (1) some specifically targeted DC populations are absent or poorly represented in the tumor, and (2) some specifically targeted receptors are poorly expressed by TIDCs or rendered non-functional by the tumor microenvironment. In these cases, it is more likely that other cells in the tumor environment are stimulated to promote a DC activating/restoring microenvironment.

In order to improve the clinical relevance and translational potential of mouse melanoma models for the design, optimization, and identification of novel therapeutic interventions that target TIDCs we will have to overcome several hurdles. An improved identification and characterization of human TIDCs will be critical to identify and validate the best mouse models for each type of study. Eventually, the panel of DC specific markers used in human and mouse studies will have to be standardized, even as investigators continue to discover new markers and DC populations.32 Furthermore, the optimization and standardization of protocols for TIDC isolation and functional assessments
will be essential for allowing study-to-study comparisons and the extrapolation of data across species as well as laboratories. This said, a great gain might be made by an increased collaboration between different research disciplines. This could result, for instance, in the generation of better mouse models, such as humanized mice for xenograft transplantation studies and GEMMs with TIDC patterns that resemble human TIDC profiles at different stages of disease, as well as new analytical platforms for extended TIDC analyses.

Although it is unlikely that mouse melanoma models will ever completely recapitulate the complexity of human melanoma in clinical situations, so far we have only scratched the surface of the true potential of mouse models for the analysis of TIDC dysfunction and the development of therapeutic interventions. Combining and integrating current models, standardizing analytical methods and expanding the disciplines of research will be instrumental for significantly improving the clinical relevance of mouse models and the identification of novel therapeutic targets.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
This work is supported by NIH grant CA138617 (NCI) and AI079545 (NIAID) to E.M.J.

Ethical Statement
All animal experiments were performed in strict accordance with animal protocols approved by the Institutional IACUC at CCHMC and LIAI that operate according to the guidelines by the Association for Assessment and Accreditation of Laboratory Animal Care International and the recommendations in the Care and Use of Laboratory Animals of the National Institute of Health.

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4. Molenkamp BG, Sluijter BJ, van Leeuwen PA, Santegoets SJ, Meijer S, Wijnands PG, et al. Local administration of PF-3512676 CpG-B instigates clinical and immune responses against melanoma in clinical situations, so far we have only scratched the surface of the true potential of mouse models for the analysis of TIDC dysfunction and the development of therapeutic interventions. Combining and integrating current models, standardizing analytical methods and expanding the disciplines of research will be instrumental for significantly improving the

現在は、細胞内への感染に対する免疫の反応を改善する体系の発見が、新しいマウスモデルの利用を可能にしています。これらのモデルを組み合わせると、より効率的な免疫応答が形成され、癌細胞への対抗が強化されます。したがって、新しい治療法が見つかることも期待されています。


Review Article

Dendritic Cells in Systemic Lupus Erythematosus: From Pathogenic Players to Therapeutic Tools

Jared Klarquist,1 Zhenyuan Zhou,2 Nan Shen,2,3 and Edith M. Janssen1

1Division of Immunobiology, Cincinnati Children’s Hospital Medical Center and the University of Cincinnati College of Medicine, Cincinnati, OH 45229, USA
2Shanghai Institute of Rheumatology, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China
3Center for Autoimmune Genomics and Etiology (CAGE), Cincinnati Children’s Hospital Medical Center, Cincinnati, OH 45229, USA

Correspondence should be addressed to Edith M. Janssen; edith.janssen@cchmc.org

Received 9 November 2015; Accepted 13 March 2016

Academic Editor: Carolina T. Piñeiro

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Systemic lupus erythematosus (SLE) is a multifactorial systemic autoimmune disease with a wide variety of presenting features. SLE is believed to result from dysregulated immune responses, loss of tolerance of CD4 T cells and B cells to ubiquitous self-antigens, and the subsequent production of anti-nuclear and other autoreactive antibodies. Recent research has associated lupus development with changes in the dendritic cell (DC) compartment, including altered DC subset frequency and localization, overactivation of mDCs and pDCs, and functional defects in DCs. Here we discuss the current knowledge on the role of DC dysfunction in SLE pathogenesis, with the focus on DCs as targets for interventional therapies.

1. Introduction

Systemic lupus erythematosus is a chronic autoimmune inflammatory disease that affects multiple organ systems, prototypically characterized by high levels of circulating autoantibodies and glomerulonephritis. Clinical symptoms also encompass musculoskeletal, dermatological, neuropsychiatric, pulmonary, gastrointestinal, cardiac, vascular, endocrine, and hematologic manifestations. The reported incidence of SLE nearly tripled over the last 40 years due to improved detection of mild disease [1], but SLE prevalence estimates still vary considerably, ranging from 10 to 150 cases per 100,000, depending on geography, race, and gender [2–5]. In the United States, the prevalence of SLE is higher among Asians, African Americans, African Caribbeans, and Hispanic Americans compared with Caucasians [6–9]. Similarly, in European countries SLE prevalence is higher among people of Asian and African descent [5–9]. Interestingly, SLE is reported infrequently in Africa [10]. Mortality rates are relatively low, at 10–50 per 10,000,000 of the general population and show correlation with renal and cardiovascular manifestations as well as infection [11]. Importantly, patients commonly experience profound fatigue and joint pain and a decreased quality of life [12–15].

The precise etiology of SLE remains unclear and likely varies, considering its diverse clinical manifestations. Nevertheless, SLE is believed to result from dysregulated immune responses, loss of tolerance of CD4 T cells and B cells to ubiquitous self-antigens, and the subsequent production of anti-nuclear and other autoreactive antibodies. This dysregulation is associated with high serum levels of type I IFN, observed in greater than 70% of patients [16, 17]. Current “standard of care” treatments encompass high-dose corticosteroids, anti-malarials, and immunosuppressive drugs that are associated with significant adverse side effects. As these treatments suppress symptoms and do not cure the disease, new therapies are needed.

Contemporary treatment strategies have been shifting emphasis toward the identification of immunological processes, both soluble and cellular, in order to redirect aberrant immune responses. Dendritic cells have recently been recognized as important players in the induction and progression of autoimmune diseases, including SLE [18]. Human and mouse studies have associated lupus development with
altered DC subset frequency and localization, overactivation of mDCs or pDCs, and functional defects in DCs [19, 20]. However, full dissection of the relative contribution of the causes and the consequences of the dysfunctionality in the different DC subpopulations is needed to understand the processes that govern SLE development, progression, remission, and relapses, in order to design interventional treatments that have the potential to redirect the immune system and eventually lead to a cure for this disease.

2. DC Populations in Humans

DCs are a heterogeneous population of professional antigen presenting cells, which bridge innate and adaptive immunity. In the absence of exogenous triggers, DCs contribute to the clearance of dying cells and the maintenance of tolerance. During infection, or in the context of autoimmunity, however, DCs play a pivotal role in the activation of CD4 and CD8 T cells. DCs were initially identified by Ralph Steinman and lack typical lineage markers for T cells (CD3), B cells (CD20), and NK cells (CD56) while expressing high levels of MHC class II [35, 36]. Within this population comparative studies have identified a small number of subsets that have homologues in several mammalian species [37, 38].

2.1. Myeloid DCs: BDCA1+ DCs and BDCA3+ DCs. Myeloid DCs are considered "conventional" or "classical" DCs and are characterized by expression of CD11c and CD11b and lack of CD14 and CD16. Within this population we currently distinguish two populations based on the expression of the markers CD11c/BDCA1 and BDCA3/CD141 [39]. The BDCA1+ DCs are the major myeloid DC population and are found in blood, lymphoid organs, and most tissues. BDCA1+ DCs express a wide variety of pattern recognition receptors including TLR1–8, lectins, and cytokines, allowing them responsiveness to a diverse array of environmental cues. BDCA1+ DCs are strong stimulators of naïve CD4 T cell responses, which can be shaped differently depending on which innate stimuli are present [37]. The BDCA3+ DCs make up >10% of the mDCs and have been found in lymphoid and nonlymphoid tissues as well as blood and bone marrow. BDCA3+ DCs express high levels of TLR3, XCR1, and CLEC9 and have been shown to display an increased capacity to phagocytose dying cells and cross-present cell-associated antigens to CD8 T cells compared to other DCs subsets [34, 40, 41].

2.2. Plasmacytoid DCs. pDCs lack the classic mDC markers CD11b and CD11c and express high levels of CD123, CD303 (BDCA2), and CD304 (BDCA4). pDCs are known for their capacity to produce vast amounts of type I IFNs in response to viruses and/or virus-derived nucleic acids predominantly via engagement of TLR7 and TLR8. pDCs have been shown to prime CD4 T cells and cross-prime CD8 T cells, especially in the context of infection [42]. Several studies implicate pDCs in the induction and maintenance of tolerance through the induction of regulatory T cells (Tregs) [43–45].

2.3. Monocyte-Associated DCs. There are currently several populations of DCs that are thought to develop from monocytes rather than common DC precursors. These cells display a variety of phenotypes and functions, but there is no consensus on their exact classification or their role in vivo.

CD14+ DCs are observed in several nonlymphoid tissues, including the skin. These cells express CD11c but lack BDCA1 or BDCA3. The CD14+ DCs express low levels of costimulatory molecules or chemokine receptors that promote migration. While these cells have been suggested to be poor at stimulating naïve T cells, they have been found to support the formation of T follicular helper cells and to provide direct help to B cells [46–49]. Inflammatory DCs (iDCs) have been suggested to originate from classic CD14+ blood monocytes under inflammatory conditions. These cells may express some of the myeloid DC markers and seem prone to produce proinflammatory cytokines. In vitro studies suggest that different types of inflammatory stimuli give rise to populations with distinct proinflammatory phenotypes. TNFα/iNOS expressing inflammatory DCs have been found in skin lesions of patients with psoriasis and atopic dermatitis [50, 51]. SlanDCs encompass a subset of monocytes with high expression of MHC class II, CD16, and 6-sulpho LacNac (slan). SlanDCs were shown to express TRL7 and TLR8 and to produce IL-12, IL-23, and TNF, preferentially promoting Th1 and Th17 cell differentiation. This population has been isolated from the inflamed skin of psoriatic patients and SLE patients with cutaneous lupus, the colon, and draining lymph nodes of patients with inflammatory bowel diseases, as well as CSF samples and inflammatory brain lesions of patients with MS [52–55]. Interestingly, SlanDC infiltration in tumors is associated with tolerance and poor prognosis, indicating either diversity within the slanDC population or heterogeneity in its function.

2.4. Tissue DCs. Nonlymphoid tissue resident DCs are present in most tissues in steady state and have been associated initially with induction of tolerance to self-antigens [36–38, 56–58]. These cells migrate at a very low rate to the draining LN under steady state conditions but show significant increased migration under inflammatory conditions. Several studies have identified networks of tissue resident DCs in the skin, lung, gut, and liver [59, 60]. Each of these networks consists of several subpopulations with different capacities for phagocytosis, antigen processing and presentation, migration, and the type of immune response they promote. Due to accessibility, skin DCs, especially Langerhans cells (LC), have been the most studied tissue-DC in the context of SLE.

2.5. DC Activation of T Cells. One of the defining features of DCs is the expression of class I and class II major histocompatibility proteins and the processing and presentation of peptide antigens to T cells. DCs predominantly present self-antigens in low quantities resulting in immunologic tolerance. Once activated, however, DCs mature in a process that usually involves migration to a draining lymph node and the priming of T cells [61–63]. The factors governing the functional result of T cell priming are multifactorial,
including the relative concentration of surface peptide/MHC, costimulatory molecule expression, and cytokine release. Ultimately, the combination of these signals will result in either T cell anergy, deletion, or activation, proliferation, and differentiation [64–66].

A wide variety of cell surface costimulatory proteins expressed by DCs can signal both activation (41-BB, CD40, CD70, CD80, CD83, CD86, GITRL, ICOSL, LTBR, and OX40L) and inhibition (PD1L, PD1L2) of an engaged T cell (reviewed in [67, 68]). In addition, secretion of pro- and anti-inflammatory cytokines by DCs contributes to the outcome of T cell priming. DCs can produce a wide variety of cytokines; which cytokines are produced depends upon environmental signals as well as upon the DC subtype. Cytokine production is driven by input from paracrine and autocrine cytokine signaling, as well as input from innate pattern recognition receptors (PRRs) including toll-like receptors (TLRs). The combination of these signals not only influences whether a T cell becomes activated, but also plays a key role in directing T cell differentiation toward various effector fates.

3. Role of DCs in SLE
Development and Progression

Although it is not certain how immunological tolerance is broken in SLE, DCs are thought to play key roles [30]. Perhaps the most prominent model proposes that the initial injury is due to a build-up of dying cells, a result of either dysregulated apoptosis or insufficient clearance of dying cells by DCs and other phagocytes [22, 23, 69]. Indeed, high levels of apoptotic cells are found in SLE patient serum, germinal centers, and inflamed tissues, such as the skin and kidney [24, 27]. Mounting evidence indicates that self-RNA and self-DNA from these dying cells induce the unremitting output of type I IFN by pDCs [21] via engagement of TLR9 or TLR7 [31, 70] and potentially via other cytosolic nucleotide sensing pathways such as RIG-I/IPS1 and STING (TMEM173) [28, 71, 72]. Type I IFNs produced by DCs promote their own activation and maturation in an autocrine manner, including increased IFN output and increased surface expression of CD80, CD86, and MHC class II, making them better at activating T cells [21, 25, 26, 73]. Furthermore, type I IFNs directly promote B cell activation, antibody production, and T cell survival and expansion [29, 32, 33]. Altogether, these data suggest that DCs are key players in SLE pathogenesis and point to DCs as promising therapeutic targets.

4. DC Abnormalities in SLE Patients

Several reports indicate that the frequency, composition, and phenotype of DCs in SLE patients differ from those of healthy individuals (see Tables 1 and 2). However, it is difficult to compare results between laboratories, given differences in disease activity and manifestations, the effect of various drug treatments on DC development and phenotype, and the variations in analytical parameters.

<table>
<thead>
<tr>
<th>Markers used to identify subset</th>
<th>Reference</th>
<th>Frequency</th>
<th>Phenotype</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDCA2+ CD123+</td>
<td>Tucci et al. [82]</td>
<td>↓ in blood, correlated with LN and ↑ in kidney (more than other DC subsets)</td>
<td>DCs in kidney were immature (DC-LAMP−), localized to tubulointerstitium, in clusters, and lacked dendrites</td>
<td></td>
</tr>
<tr>
<td>BDCA2+ (blood) and BDCA4+ (kidney)</td>
<td>Fiore et al. [78]</td>
<td>↓ in blood in active disease and ↑ in kidney (more than other DC subsets)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BDCA2+ Lin− HLA-DR+</td>
<td>Migita et al. [77]</td>
<td>↓ in blood</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD123high CD11c− CD16− HLA-DR+</td>
<td>Henriques et al. [80]</td>
<td>↓ in blood</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BDCA2+ CD123high</td>
<td>Kwok et al. [90]</td>
<td>Normal in blood</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BDCA2+ BDCA4+ CD123+</td>
<td>Jin et al. [79]</td>
<td>↑ in blood per total PBMC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BDCA2+ CD11c+</td>
<td>Gerl et al. [81]</td>
<td>na</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1: pDCs in SLE.
Table 2: DCs in SLE.

<table>
<thead>
<tr>
<th>Markers used to identify subset</th>
<th>Reference</th>
<th>Frequency</th>
<th>Phenotype</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDCA1+</td>
<td>Fiore et al. [78]</td>
<td>↓ blood in active disease and ↑ kidney in active disease</td>
<td>DCs in kidney were immature (DC-LAMP−), localized to tubulointerstitium</td>
<td></td>
</tr>
<tr>
<td>BDCA3+</td>
<td>Fiore et al. [78]</td>
<td>↓↓ blood and ↑↑ kidney inactive disease</td>
<td>DCs in kidney were immature (DC-LAMP−), localized to tubulointerstitium, with elongated processes</td>
<td></td>
</tr>
<tr>
<td>BDCA1+ CD11c+ BDCA4− CD19+</td>
<td>Jin et al. [91]</td>
<td>↓ in blood per total PBMC</td>
<td>↓ CD3, especially in active disease, normal HLA-DR, CD86, and CCR7</td>
<td></td>
</tr>
<tr>
<td>HLA-DR+ Lin− CD4+</td>
<td>Scheinecker et al. [76]</td>
<td>↓ in blood</td>
<td>↓ CD40+, B7+, and CD11c+</td>
<td>↓ T cell proliferation in MLR</td>
</tr>
<tr>
<td>BDCA1+ CD11c+</td>
<td>Tucci et al. [82]</td>
<td>Normal in blood, relatively few in kidney</td>
<td>Normal T cell proliferation in MLR, moDCs fail to increase costimulatory molecule expression upon activation</td>
<td></td>
</tr>
<tr>
<td>CD11c+ Lin−</td>
<td>Crispín et al. [83]</td>
<td>↑ in blood (though not significant)</td>
<td>↑ CD86+, CD80+, normal HLA-DR+, and CD40+</td>
<td></td>
</tr>
<tr>
<td>CD11c high CD14−</td>
<td>Gerl et al. [81]</td>
<td>na</td>
<td>↑ CD86, BAFF, normal HLA-DR, CD83, CD40, CCR7, CCR1, and CCR5 and ↓ CMKLR1</td>
<td></td>
</tr>
<tr>
<td>Adherent, monocyte-derived DCs (MDDCs)</td>
<td>Ding et al. [93]</td>
<td>na</td>
<td>↑ CD86, CD80, HLA-DR, and CD11a and ↓ CD83 after 5–7 d culture</td>
<td>↑ T cell proliferation in MLR</td>
</tr>
<tr>
<td>CD14+ sorted, monocyte-derived DCs (MDDCs)</td>
<td>Köller et al. [92]</td>
<td>na</td>
<td>↑ HLA-DR after 8–10 d culture, normal CD86, CD83, CD80, CD40, CD54, and CD33</td>
<td>↑ antigen-specific T cell proliferation and normal MLR</td>
</tr>
<tr>
<td>M-DC8 (slanDCs)</td>
<td>Hänsel et al. [53]</td>
<td>↑ in skin of patients with cutaneous LE and “strong inflammation” SLE</td>
<td>In situ TNF production in cutaneous LE</td>
<td>↑ TNFα production by healthy donor slanDCs in response to SLE serum compared with control serum</td>
</tr>
</tbody>
</table>

Studios have shown reduced [74–81], normal [80, 82], and increased [83] levels of CD11c+ mDC frequencies in PBMC from lupus patients compared to healthy controls. Similarly, pDC levels were found to be unaffected, reduced [74–78, 84, 85], or increased [79, 86]. Decreased frequencies of pDCs or mDCs were most often associated with active disease and to a lesser degree with nonactive disease [75]. Interestingly, studies showing peripheral pDCs decreased observed a concomitant infiltration of pDCs in nephritic kidneys, suggesting that active pDCs may have migrated to the sites of inflammation [78, 82]. Similarly, Fiore et al. showed that besides pDCs, BDCA1+ DCs and BDCA3+ DCs were increased in the renal tubulointerstitium of patients with lupus nephritis [78]. Increased numbers of pDCs and inflammatory/slantDCs are also found in cutaneous lesions of lupus patients, further suggesting migration of DCs to target organs [87, 88]. It is likely that DCs that reside in or have been recruited into the affected tissues will display different characteristics than those circulating in the periphery. Consequently, these populations should be included in further assessments in order to understand their contribution to disease pathogenesis and allow for a rational design of DC-targeting therapeutics.

5. SLE-Associated Dysfunction in Primary DCs

The few published maturation and functionality studies with primary human DCs have given conflicting results. Earlier reports indicated that DCs from SLE patients have normal or even reduced levels of costimulatory molecules and are poor stimulators of allogeneic T cells in mixed lymphocyte reactions. Scheinecker et al. reported that in SLE patients B7+ and CD40+ DCs were reduced and that DC-enriched APC from SLE patients displayed a diminished T cell-stimulatory capacity in both the allogeneic and the antigen-specific MLR, as compared with healthy individuals [76]. On the other hand, Mozaffarian et al. showed increased CD80/CD86 and
reduced PDL-1 expression on mDC during disease flares and an upregulation of PDL-1 during remission [89]. Similarly, Gerl et al. [81] published that monocytes and mDCs from SLE patients expressed higher levels of CD86 and BAFF, but not CD83 and CD40. Upon further assessment of their migratory capacity, they found that pDCs and mDCs from SLE patients had normal expression of CCR1, CCR5, and CCR7 but reduced expression of the chemokine receptor ChemR23 (CMKLRI). However, pDCs from the SLE patients showed an increased basal and CCL19-specific migration in vitro.

Assessment of peripheral monocytes, total DCs, BDCA1+ DCs, and CD14lowCD16+ DCs by Henriques et al. showed that a higher percentage of SLE monocytes and CD14lowCD16+ DCs produced proinflammatory cytokines as well as a higher amount of cytokines produced per cell, particularly in active disease. Data from Kwok et al. [90] seemed to indicate that type I IFN production by pDC upon TLR9 engagement was diminished in SLE patients, leading them to hypothesize that the persistent presence of endogenous IFNα-inducing factors induces TLR tolerance in pDCs of SLE patients, resulting in impaired production of IFNα. Studies by Jin et al. [79, 91] also suggested deficiencies in TLR9 recruitment/signaling and production of proinflammatory cytokines in pDCs from SLE patients; however, they also showed that SLE pDC had an increased ability to stimulate T cells. Importantly, while pDCs from healthy donors induced suppressive T regulatory cell features (Foxp3 expression) in T cell cultures upon addition of apoptotic PMNs, SLE pDCs failed to do so.

These studies indicate that SLE is associated with phenotypic and functional changes in DCs and that these changes can affect different aspects of the DCs’ functional program in distinct and divergent ways.

6. SLE-Associated Dysfunction in In Vitro Generated DCs

Due to the paucity of DCs in leukopenic SLE patients, many studies have used in vitro generated monocyte-derived DCs (moDCs) to gain insight in DC generation, phenotype, and function in the context of SLE.

Initial studies suggest that monocyte-derived DCs had a reduced proinflammatory and T cell stimulatory activity [92] while later studies suggested accelerated differentiation and maturation concomitant with increased activity to maturation stimuli [93]. MoDCs from SLE patients expressed higher levels of HLA-DR and activating FcyRs, but decreased expression of inhibitory FcyR and expression levels correlated with disease severity [92, 94]. In addition, moDCs spontaneously overexpressed activating costimulatory molecules including CD40, CD80, and CD86 and showed increased production of stimulatory cytokines (IL-6, IL-8, and BAFF/BlyS), eventually resulting in an increased capacity to activate T cells in an MLR [93, 95]. Similarly, Nie et al. [96] demonstrated substantial phenotypic and functional aberrations in DCs generated from Flt3-ligand and GM-CSF/IL-4 stimulated bone marrow aspirates. Both immature and mature DCs from SLE donors expressed higher levels of CCR7, CD40, and CD86 and induced stronger T cell proliferation.

7. Nature versus Nurture

Drawing causative relationships between DCs frequencies, maturation status, functionality, and disease is complex as it is not clear whether aberrations in DC frequency and functionality are the driver or a result of the disease. It is likely that genetic alterations in DCs predispose to the development of accelerated maturation and abnormal behavior. Evidence for this intrinsic defect is supported by the observations that moDCs from SLE patients, generated from either PBMC or bone marrow, display accelerated maturation and increased proinflammatory status compared to moDC from healthy donors. On the other hand, serum of SLE patients has been shown to contain pro- and anti-inflammatory stimuli like type I IFN, type I IFN-inducing factors, and IL-10 that alter DC differentiation, maturation, and functionality, even in DCs from healthy donors [97–99]. This raises the question whether the aberrant behavior of DCs in SLE patients is a result from an intrinsic defect, a result of their development in an inflammatory environment, or a combination of these two [97]. To further confound the interpretation of human clinical data, various classic SLE treatments, including antimalarials, corticosteroids, and immunosuppressive drugs significantly affect DC number, maturity, and functionality [100].

8. Mouse Models to Dissect Role of DCs in SLE Pathogenesis

The availability of mouse models provides an exciting opportunity to gain cellular and molecular insight in the role of different DC populations in the development and progression of SLE. There are a variety of spontaneous models, including the F1 hybrid between the New Zealand Black (NZB) and New Zealand White (NZW) strains (NZB/W F1) and its derivatives, the MRL/1pr and BXSB/Yaa strains, as well as inducible models such as the pristane-induced model and chronic graft-versus-host-disease models (cGVHD) [101–104]. In recent years the number of models has been expanded with genetically modified mice, targeted in genes that can promote, resist, and modify lupus susceptibility [105, 106]. All of these models display their own variation of lupus-like disease reminiscent of symptoms observed in patients, including autoantibody production, lymphoid activation and hyperplasia, lupus nephritis, and skin manifestations. Although all of these models have been instrumental in the identification of several main concepts in this diseases, none of the models can completely recapitulate the complexity and variety of human disease. However, careful pairing of models with patient groups with the similar clinical manifestations can ensure the translational relevance of these preclinical models.

Mouse models have several advantages: (i) the relative homology between human and mouse DCs, (ii) the opportunity to genetically or pharmacologically eliminate specific DC populations during specific stages of disease, (iii) access to all target tissues for the assessment of tissue associated or infiltrating DCs, (iv) the opportunity to assess the effects of common treatments on the parameters, and (v) a plethora of biological and pharmacological tools to dissect the relative
contribution of specific molecules and mediators to the development and progression of disease.

9. Similarities between Mouse and Human DCs

Recent genomic, proteomic, and functional analyses of mouse and human DCs have identified high homology between the most abundant DC populations [107]. Like in human DCs, mouse DCs lineages encompass conventional DCs, pDCs, CD14+ DCs, tissue DCs, and monocyte-derived/inflammatory DCs [38, 108].

Conventional mouse DCs encompass three main subpopulations which are found in circulation as well as in secondary lymphoid organs [109]: (1) CD11c<sup>high</sup>MHCII<sup>+</sup>CD8α<sup>−</sup>33D1<sup>+</sup>Sirpα<sup>−</sup>CD11b<sup>+</sup> (CD11b DCs), which express most TLRs except Trlr3, display a preference for activation of CD4 T cells, and have high homology with the human BDCA<sup>+</sup> DCs; (2) CD11c<sup>high</sup>MHCII<sup>−</sup>CD8α<sup>−</sup>CD205<sup>−</sup>Sirpα<sup>−</sup>CD11b<sup>+</sup> (CD8α DCs), which express Xcl1, CD141, and Clec9A and express mRNAs coding for most TLRs except Trlr5 and Trlr7, and are characterized by high Trlr3 expression; and (3) CD11c<sup>high</sup>MHCII<sup>−</sup>CD8α<sup>−</sup>CD4<sup>+</sup>CD11b (generally termed “double” or “triple” negative) DCs that, like CD8α DC, express Xcl1, CD141, Clec9A, and Trlr3 [110–113]. These latter two populations have a high capacity to phagocytose dying cells and cross-present cell-associated or particulate antigens to CD8 T cells. Based on their genomic and functional analysis these two populations are considered to be homologues to the human BDCA3<sup>+</sup> DCs.

Like human pDCs, mouse pDCs produce vast amounts of type I IFN in response to viruses via TLR7/9 mediated pathways. Compared to their human counterparts, mouse pDCs show relatively poor capacity for phagocytosis and antigen presentation. However, both populations have been implied in the maintenance of peripheral tolerance [45, 114–116].

Various types of inflammatory and monocyte-derived DCs have been identified in mice as well. Tissue infiltrating CD14<sup>+</sup> DC-like cells have been found under inflammatory conditions [117, 118]. Inflammatory DCs have been shown to arise after a wide variety of immunological insults, including pathogenic infection, experimental sterile inflammation, and models of inflammatory diseases such as RA, colitis experimental autoimmune encephalomyelitis, and allergic asthma (reviewed in [119]).

10. The Role of DCs in Mouse SLE Models

Recent studies indicate an important role for DCs in the development and progression of SLE-like disease in mouse models. Similar to human disease, DCs from lupus-prone mice display a range of alterations in their numbers and their functionality [120–123]. Splenic DCs from NZB/W F1 showed enhanced maturation and a stronger ability to attract B cells and present antigens to T cells than DCs from control mice. pDCs from SLE-prone mice showed increased type I IFN producing capacity upon TLR9 stimulation and increased cell survival compared to pDCs from C57BL/6 mice. Enhanced mDC and pDC activity has also been reported in male BXSB/Mp mice that express an extra copy of Trlr7 on the Y chromosome.

Importantly, depletion studies have now shown causal relationships between DC subsets and disease manifestations. Constitutive depletion of pDCs in lupus-prone mice either through genetic ablation of IRF8, a transcription factor required for pDC and CD8αDC development, or by diphtheria toxin treatment of mice expressing the diphtheria toxin receptor on pDCs resulted in markedly reduced type I IFN production, a reduced IFN signature, reduced autoantibody production, and reduction in the severity of kidney pathology glomerulonephritis [124–126]. Importantly, transient pDC depletion during the early stages of disease was sufficient to significantly alter the course of the disease, suggesting a more prominent role for pDCs in the induction of the disease than in disease pathogenesis at later stages of disease [125]. Diphtheria toxin treatment of CD11c-DTA MLR.Fas<sup>−</sup> mice resulted in reduced T cell differentiation, plasmablast numbers, and autoantibody levels. Interestingly, these mice developed interstitial kidney infiltrates but failed to progress to glomerular or interstitial nephritis, suggesting that DCs play a role in the development of tissue damage [127]. In line with this observation, this group also showed that CD11c depletion, but not LC depletion, resulted in significantly reduced dermatitis, demonstrating that DCs other than LCs control dermatitis in this model [127].

Besides the opportunity to assess the relative and temporal contribution of different DC populations to the development of specific disease manifestations, mouse models also allow for the identification of specific processes in DCs which affect disease development. Targeted deletion of regulatory molecules associated with SLE susceptibility in humans, including Shp1, A20, Blimp-1, Lyn, or Eat-2, specifically in CD11c<sup>+</sup> cells resulted in increased DC activity and development of inflammatory and autoimmune phenotypes characterized by the production of autoantibodies and several manifestations of SLE, including severe glomerulonephritis [128–132].

Together these observations indicate that mouse models provide a useful platform for the identification, dissection, and targeting of DC intrinsic and extrinsic processes that facilitate the development, progression, and possibly a cure for SLE.

11. DC Targeted Therapies for SLE

Based on the general role of DC in the regulation of peripheral tolerance to self-antigens, the dysregulation of DCs observed in SLE, and the emerging evidence of the contribution of DCs in the initiation and perpetuation of SLE pathogenesis, it is not surprising that DC-targeting therapeutic strategies have become a topic of interest. Particularly, strategies that would promote self-antigen presentation in a tolerogenic context could be promising for the generation of an abortive or suppressive environment for the autoreactive T and B cells and restoration of peripheral tolerance [133, 134].
In recent years several *ex vivo* models have been established for the generation of human DCs with stable tolerogenic functions (reviewed in [135]). Generally, these resulting tolerogenic monocyte-derived DCs express low levels of positive costimulatory molecules and high levels of immune suppressive mediators (PDL-1, IL-10, etc.). Upon pulsing with specific antigens these DCs are anticipated to promote antigen-specific tolerance via the induction of T cell anergy, T cell apoptosis, skewing of T cell phenotypes to more Th2 or regulatory phenotypes, and the expansion of regulatory T cells.

Tolerogenic DC therapy is still in its infancy and little data is available on its *in vivo* potential. The first studies showed that transfer of antigen-loaded tolerogenic DCs could induce antigen-specific regulatory CD8 T cells and inhibit effector functions in antigen-specific CD8 T cells [136, 137]. A clinical trial in patients with type 1 diabetes using DCs treated with antisense oligonucleotides to silence costimulatory molecules was less successful, and although the treatment was well tolerated, only very limited tolerance outcomes were reported [138]. A subsequent trial in TID patients indicated that transfer of IL-10 and TGFβ1 generated tolerogenic DCs pulsed with pancreatic islet cells induced antigen-specific T cell hyporesponsiveness and was associated with better glycemic control [139]. Similarly, transfer of a single dose of tolerogenic DCs, derived by *ex vivo* treatment with NF-κB inhibitors, into patients with active RA resulted in a modest improvement in disease activity 3 and 6 months after injection [140]. Currently there are several trials addressing the therapeutic potential of tolerogenic DCs in multiple sclerosis, rheumatoid arthritis, type 1 diabetes, and allergic asthma [141].

To date no tolerogenic DC transfer studies have been published in preclinical models or SLE patients. However, *in vitro* data indicate that tolerogenic DCs can be generated from SLE patients [83, 142, 143] and that apoptotic cells can be used as source to load the DCs with autoantigens [143]. The insight obtained from currently ongoing tolerogenic DC treatment strategies in other chronic inflammatory diseases will help to identify critical parameters such as dose, route, and duration of treatment leading to the most efficacious outcome [144, 145]. However, a better understanding of the role of DCs in disease pathogenesis is critically needed in order to select the type of tolerogenic DC that can successfully counteract the dysfunctional adaptive immune responses that maintain the disease.

### Competing Interests

The authors declare that they have no competing interests.

### Acknowledgments

This work was supported in part by the Lupus Research Institute (to Edith M. Janssen), NCI Grant CA138617 (to Edith M. Janssen), Charlotte Schmidlapp Award (to Edith M. Janssen), the Albert J. Ryan Fellowship (to Jared Klarquist), the National Basic Research Program of China (973 program; 2014CB541902, to Nan Shen), the National Natural Science Foundation of China (no. 81230072; no. 81025016; no. 81401331, to Nan Shen), the Program of the Shanghai Commission of Science and Technology (no. 12JC1406000 to Nan Shen), and the Special Fund for Public Benefit Research from the Ministry of Health (no. 201202008, to Nan Shen).

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Mediators of Inflammation


Mediators of Inflammation


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The bm12 Inducible Model of Systemic Lupus Erythematosus (SLE) in C57BL/6 Mice

Jared Klarquist1, Edith M. Janssen1

1Division of Immunobiology, Cincinnati Children's Hospital Medical Center and University of Cincinnati College of Medicine

Correspondence to: Edith M. Janssen at Edith.Janssen@cchmc.org

URL: http://www.jove.com/video/53319
DOI: doi:10.3791/53319

Keywords: Medicine, Issue 105, T follicular helper cell (Tfh), germinal center (GC) B cell, plasma cell, ascites, flow cytometry, animal model, anti-nuclear antibody (ANA), autoimmunity, nephritis, trogocytosis, chronic graft-versus-host disease (cGVHD), type I interferon (IFN)

Date Published: 11/1/2015

Abstract

Systemic lupus erythematosus (SLE) is an autoimmune disease with diverse clinical and immunological manifestations. Several spontaneous and inducible animal models mirror common components of human disease, including the bm12 transfer model. Upon transfer of bm12 splenocytes or purified CD4 T cells, C57BL/6 mice rapidly develop large frequencies of T follicular helper cells (Tfh), germinal center (GC) B cells, and plasma cells followed by high levels of circulating anti-nuclear antibodies. Since this model utilizes mice on a pure C57BL/6 background, researchers can quickly and easily study disease progression in transgenic or knockout mouse strains in a relatively short period of time. Here we describe protocols for the induction of the model and the quantitation of Tfh, GC B cells, and plasma cells by multi-color flow cytometry. Importantly, these protocols can also be used to characterize disease in most mouse models of SLE and identify Tfh, GC B cells, and plasma cells in other disease models.

Introduction

Systemic lupus erythematosus (SLE) is a complex autoimmune disease characterized prototypically by anti-nuclear antibody (ANA) production and glomerulonephritis. Numerous other sequelae, including dermal, cardio-pulmonary, and hepatic lesions are associated with disease in some individuals. Prevalence estimates in the US vary widely, from 150,000-1,500,000, with particularly high incidence in women and minorities3. Although the etiology of SLE has been difficult to discern, it is thought to arise from the interplay of various genetic and environmental factors, which culminate in systemic autoimmunity.

Numerous animal models have been employed to study factors leading to disease onset and progression. Classic mouse models of SLE include genetically predisposed mouse strains including the NZB x NZW F1 model and its NZM derivatives, the MLR/lpr strain, and the BXB3/Yaa strain, and inducible systems, such as the pristane and chronic graft-versus-host disease (cGVHD) models5. Early reports of autoantibody production in GVHD models used various mouse strains or hamster strains for parent into F1 transfers5-8; more common methods used to study lupus-like disease currently include the DBA/2 parent→(C57BL/6 x DBA/2) F1, and the bm12 transfer model described here. Each model has its own caveats, but they generally share a common set of features that correlate with clinical features of human disease. The most often reported parameters in mouse models include splenomegaly, lymphadenopathy, nephritis, ANA production, and at the cellular level, the expansion of T follicular helper cells (Tfh), germinal center (GC) B cells, and plasma cells.

The inducible bm12 model is achieved by the adoptive transfer of lymphocytes from I-A<sup>bm12</sup>B6(C)-H<sub>2</sub>-Ab1<sup>bm12</sup>/K<sub>h</sub>EgJ (bm12) mice, a strain identical to C57BL/6 except for 3 amino acid substitutions on MHC class II, into I-A<sup>bm12</sup>C57BL/6 (B6) mice. Alloactivation of donor CD4 T cells by recipient APCs leads to cGVHD with symptoms closely resembling SLE. Specifically, these include expansion of donor-derived Tfh, expansion of recipient-derived GC B cells and plasma cells, and production of ANAs including anti-dsDNA, anti-ssDNA, anti-chromatin, and anti-RBC antibodies9. Over time, recipient mice develop glomerulonephritis associated with IgG deposits in the glomerular, interstitial, and vascular regions of the kidneys10. We have recently shown that, similar to human disease, there is also a critical role for type I IFN in this model11-12. Notably, the defining criteria for human SLE include the development of nephritis compatible with SLE in the presence of anti-dsDNA antibodies12, both of which are prominent features of this mouse model.

There are several advantages of the bm12 model over the spontaneous models. Classic models that develop SLE-like signs spontaneously rely upon either hybrid mouse strains, inbred mouse strains not on the B6 background, or large genetic loci on the B6 background, which make crossing to knockout or otherwise genetically modified mice difficult and time consuming. With the bm12 inducible model, genetically modified mice can serve as either the donor or recipient, allowing more rapid identification of the cellular compartment in which particular genes may be important for disease. Furthermore, disease development in the bm12 model is much faster, requiring only 2 weeks until the appearance of ANAs, compared to several months for most spontaneous models. Moreover, in contrast to the spontaneous models that develop disease
at different time points, the disease onset and progression in the bm12→B6 model is highly synchronized. This allows for the generation of appropriately sized cohorts that can be used for interventional or therapeutic strategies at any stage of disease development.

What follows is a detailed protocol for initiating SLE-like autoimmunity by the adoptive transfer of bm12 lymphocytes into C57BL/6 mice, or genetic variants on the B6 background. Additionally, we describe a flow cytometric staining protocol for enumerating Tfh, GC B cells, and plasma cells—cell types associated with human disease. Importantly, these protocols can also be used to characterize disease in most mouse models of SLE and identify Tfh, GC B cells and plasma cells in other disease models.

### Protocol

Animal work was performed under specific pathogen-free conditions in accordance with guidelines set by the Association for Assessment and Accreditation of Laboratory Animal Care International and our Institutional Animal Care and Use Committee (IACUC).

NOTE: Incorporate mice expressing a congenic marker such as CD45.1 on either donor or recipient animals if possible, because this allows for the monitoring of donor graft efficiency and specific expansion of the donor CD4 T cell population. If considering the use of otherwise genetically modified mice as donor or recipients, ensure the strain is properly backcrossed to the B6 background, or transferred cells may be rejected—this will be addressed in greater detail in the representative results and discussion sections.

NOTE: The following procedures detail volumes for harvesting 4 donor bm12 mice, which should yield enough cells to inject 12-16 mice. Six to twelve week-old bm12 mice yield roughly 100-140 million lymphocytes with approximately 25% CD4 T cells. If starting with different numbers of mice, or different mouse strains, scale volumes up or down accordingly. C57BL/6 mice generally give similar yields with closer to only 20% CD4 T cells.

NOTE: Perform all steps at RT and use RT media to avoid heat and cold shock, which can impair long-term lymphocyte viability. Perform tissue-harvesting and tissue-processing steps in a tissue culture hood using aseptic technique. All media in this protocol is IMDM with 10% heat-inactivated FBS, unless indicated otherwise, and will be simply referred to as “complete media.”

1. **Novel Method for Genotyping bm12 Mice**

   NOTE: A brief restriction digest-based protocol for genotyping is provided here, as a simple and inexpensive alternative to sequencing, which is currently the only published genotyping method for these mice.

   1. Isolate genomic DNA from mouse tails. Please refer to a previous JoVE article for detailed protocols on mouse tail clipping and the generation of cDNA from tail digests.
   2. Perform a reverse-transcriptase polymerized chain reaction (PCR) to amplify a common 474 bp DNA fragment from MHC-II I-A<sup>b</sup> and I-A<sup>bm12</sup> using primers and thermocycling conditions listed in Table 1.
   3. Perform restriction digest on ~7 µl of the PCR product using the enzyme PsuI, or one of its isoschizomers (BstX2I, BstYI, MflI, or XhoII), which cuts wild type I-A<sup>b</sup>, but not I-A<sup>bm12</sup> mutant. Follow digest protocol provided by the manufacturer, which will specify a mixture of water, buffer and enzyme, and an incubation of 5 min. to several hours at 37 °C.
   4. Load digest product and run on 1% polyacrylamide gel with ethidium bromide for 30-45 min. at 150V—though optimal voltage and time settings will vary depending on the PCR gel apparatus used. Visualize DNA bands with a UV illuminator. Representative results are shown in Figure 1.

   **Figure 1. Representative bm12 genotyping results.**

   To identify mice homozygous for I-A<sup>bm12/bm12</sup>, tail DNA was screened for bm12 by PCR/restriction digest genotyping (Step 1). Homozygous wild type (I-A<sup>b/b</sup>) DNA yields two bands at 227 and 247 bp (visualized as one thick band at ~250 bp); homozygous bm12 (I-A<sup>bm12/bm12</sup>) DNA yields one band at 474 bp; and heterozygous (I-A<sup>bm12/b</sup>) DNA yields two bands at ~250 and 474 bp. Please click here to view a larger version of this figure.

2. **Harvesting Donor Cells**

   1. Sacrifice donor mice using Institutional Animal Care and Use Committee (IACUC)-approved primary and secondary euthanasia methods. For example, euthanize mice by asphyxiation with CO<sub>2</sub> followed by cervical dislocation.
   2. Using aseptic technique, harvest spleens and lymph nodes (superficial cervical, mandibular, brachial, axillary, mesenteric, and inguinal) into 15 ml tubes containing 10 ml complete media as in [11].

   NOTE: Refer to previous reports for detailed lymph node dissection protocols. This model is also successful using only mouse splenocytes for transfer, but the addition of lymph nodes significantly reduces the required number of donor mice.
3. Donor Cell Counting

NOTE: To preserve the highest viability, red blood cell (RBC) lysis of the entire sample is not recommended.

1. Mix single cell suspension from step 2.4 well, remove 1 ml and transfer it to a separate 50 ml conical. Set aside remaining, untouched cells (49 ml) at RT while counting.

2. Add 3 ml of an ammonium chloride-based red blood cell lysis buffer to the 1 ml donor cell sample. Mix gently for 1 min. by rocking, then fill to 50 ml with complete media, and centrifuge for 5 min. at 400 x g and decant supernatant.

3. Resuspend RBC-lysed cells in 10 ml complete media and count (e.g., with trypan blue using a hemocytometer). Multiply result by 49 — this is the total number of cells remaining in the non-lysed sample that was set aside. Discard RBC-lysed cells.

4. Donor Cell Labeling and/or CD4 T Cell Purification

1. If desired, purify CD4 T cells at this stage, though this is not necessary. Additionally, if desired, label cells with CFSE, as in \(^{19}\), or other cell tracking dyes, an example of which is shown in Figure 4 of the representative results section.

NOTE: For CD4 T cell purification, negative magnetic selection is recommended, as it can achieve high purity and leaves cells untouched with high viability as described in \(^{20}\). It is important that endotoxin-free buffers are used; therefore, instead of BSA, make separation buffer with 2% FBS and the recommended concentration of EDTA.

5. Injection of Donor bm12 Cells

1. After counting donor lymphocytes in step 3 (and purified or labeled as in step 4, as desired), centrifuge cells for 5 min at 400 x g. Decant supernatant and resuspend cells in PBS at 120 million lymphocytes per ml (or 30 million purified CD4 T cells per ml). Transfer cells to a sterile 5 ml round-bottom tube, or other sterile tube that easily accommodates a 1 ml syringe fitted with a 27.5 G x 13 mm needle.

2. Prior to injection, set aside a small sample of donor cells from step 5.1 at 4 °C for flow staining to determine the percentage of CD4 T cells within donor samples. Stain these samples as described in Step 8 using the minimal antibody panel (Table 2). NOTE: If cells from different mouse strains are used as separate donors, this is an important consideration, and if CD4 T cell percentages vary substantially, purification may be required.

3. Mix cells gently, but thoroughly. This can be done by pipetting cells up and down using a 1 ml syringe without an attached needle. After mixing, draw cells into the 1 ml syringe. Attach needle after removing any air bubbles. Keeping the needle off while priming the syringe helps maintain cell viability.

4. Inject 250 µl per mouse (which is equal to 30 million lymphocytes, or 7.5 million purified CD4 T cells per mouse) intraperitoneally, as described in \(^{21}\).

NOTE: In experiments shown here and in our prior work \(^{11}\), each mouse is injected with 30 million total lymphocytes from bm12 donors, rather than the 100 million total splenocytes traditionally used. In unpublished data from our lab, no difference was observed in serum anti-dsDNA at day 14 following injections of 30 or 100 million lymphocytes per mouse. While this significantly reduces the number of mice needed for experiments, the development of nephritis with this number of cells has not been assessed.

6. Determine Grafting Efficiency

NOTE 1: This section will describe how to determine the degree of donor cell grafting in the recipient at day 3 in order to identify any mice which may have received suboptimal injections (e.g., the graft in one mouse is <10% of that seen in all other mice from the same group). These data can also help determine whether cells from a genetically modified mouse strain are rejected at later time points (for details, see representative results section, Figure 6).

NOTE 2: This section is only possible if donors and recipients are from mice on different congenic backgrounds, e.g., when using CD45.1 bm12 donors and CD45.2 C57BL/6 recipients, or when donor cells are labeled with a cell tracking dye. Importantly, at 3 days post-injection, CD4 T cells have undergone minimal expansion (see representative results section, Figure 4), so differences observed in the degree of grafting are due to variability in injections, not expansion.

1. Anesthetize mice with 4% isoflurane or other IACUC-approved method. Test rear foot reflexes to ensure mouse is properly anesthetized before proceeding to the blood draw.

2. Harvest 100-200 µl blood using an IACUC-approved method, such as retro-orbital puncture as in \(^{22}\). Collect blood into individual 0.5 ml microcentrifuge tubes containing an anti-coagulant, such as the plasma collection tubes referenced in the materials table, which contain lyophilized dipotassium EDTA. After blood collection, apply gentle pressure to the mouse eye using a sterile towel or gauze to ensure the bleeding stops, then place the mouse back in its home cage where it will remain until the final tissue harvest (Step 7).

NOTE: Do not leave mice unattended until mice have regained sufficient consciousness to maintain ventral recumbency, and do not return mice to the company of others until fully recovered.

3. Bring blood volume to 500 µl with 21 °C PBS and transfer to conical-bottom microcentrifuge tube, then slowly overlay 200 µl of 21 °C high density cell separation solution with a 200 µl pipet, being careful to minimize mixing between the two phases (see Materials Table for recommended solutions). Centrifuge cells at 700 x g for 20 min at 20-25 °C with centrifuge brake set to low.
7. Final Tissue Harvest

NOTE: The experiments described in the results section were harvested 14 days after injection of donor cells (or in some cases less time), as they focus on the initial development of Tfh cells and plasma cells; however, since this model is a chronic GVHD model of SLE, disease can be monitored at much later time points. The optimal timeframe will depend upon the research question posed in each individual experiment.

1. At a predetermined time point after injection of bm12 donor cells (step 5.4), sacrifice mice using an IACUC-approved method of euthanasia. Asphyxiation with CO₂ followed by exsanguination is recommended. Cervical dislocation can function as a secondary method of euthanasia, but this may reduce the blood draw yield.

2. Wet the abdomen of the mouse lightly with a spray bottle containing 70% ethanol. Make a small, superficial incision with surgical scissors approximately 1 cm above the genitalia. Draw back the skin of the abdomen toward the sternum, being careful to keep the peritoneal fascia intact.

NOTE: Diseased mice usually present with 0.5-3 ml ascites at day 14, which, although it has not yet been well characterized, can be measured and analyzed as an additional disease parameter.

3. Fit a 5 ml syringe with an 18 G needle. Insert the needle into the lower right quadrant of the abdomen with the needle directed up toward the animal's head and at 15° angle to the plane of the fascia. Position the needle tip near the cecum to help prevent the needle from getting clogged with intestine while aspirating ascites.

4. Carefully rotate the mouse on its side, then slowly draw ascites into the syringe. Once ascites has been recovered, remove the syringe and record the aspirate volume, based upon the volumetric markings on the side of the syringe.

5. Discharge ascites into a 5 ml round-bottom tube and store on ice for later processing.

6. Harvest blood via draw from the inferior vena cava (IVC) essentially as in22. Using dull forceps, gently move intestines to the left side of the mouse, uncovering the IVC. Insert a 27.5 G needle fitted to a 1 ml syringe into the IVC and slowly draw 400-500 µl of blood.

7. To minimize hemolysis, slowly inject blood into a 0.5 ml microcentrifuge serum or plasma collection tube. For later serum analysis of ANAs by ELISA, keep blood on ice.

8. Dissect and obtain additional relevant tissues (spleen and, if desired, lymph nodes and kidneys, particularly if collecting at later time points and glomerulonephritis will be scored). Remove spleen by gently placing the intestines back toward the right side of the animal, and pulling on the pancreas, which is the spleen’s primary connective tissue.

9. Remove any pancreas remaining, then weigh spleens on a high precision balance immediately after dissection, as gross splenomegaly is a commonly reported parameter in mouse models of SLE. Place lymphoid tissues into individual 1.5 ml tubes filled with 1ml complete media on ice. Fix kidneys in 10% neutral buffered formalin or snap freeze kidneys for later histology as in23.

10. Centrifuge blood within 2 hr of collection for 3 min. at 10,000 x g (4 °C). Remove serum and store at -80 °C for later analysis of ANA by ELISA. Refer to previous reports for detailed ANA ELISA protocol24,25. Store serum in multiple 10-20 µl aliquots to minimize freeze/thaw cycles, and allow a greater number of future assays.

11. Centrifuge ascites 400 x g for 5 min. Remove the supernatant with a 1 ml pipet and aliquot into several 0.5 ml tubes. Freeze supernatant at -80 °C for later analyses of anti-nuclear antibodies (ANAs) or other soluble inflammatory mediators.

12. Resuspend cellular fraction in approximately 1 ml complete media and transfer 200 µl to a 96-well U-bottom plate for flow staining (as in Step 8).

13. Mash each spleen through 70 µm cell strainers into separate tubes and rinse with complete media. Resuspend splenocytes with 1 ml cold RBC lysis buffer for 1 min. and mix gently by rocking. Bring volume to 10 ml with cold complete media and centrifuge for 5 min at 400 x g and decant supernatant.

14. Resuspend cell pellet in 5 ml complete media and count using a hemocytometer. Adjust volume such that 200 µl of complete media contains 1-3 million cells. Begin staining for flow cytometry (Step 8) using an extended panel (Table 2) to quantify donor CD4 T cell and recipient B cell differentiation and expansion.

NOTE: Depending upon what laser, photomultiplier tube (PMT), and filter set combinations are available, separating the T and B cell analysis panel into multiple panels may be necessary.

8. Flow Staining

1. Transfer 1-3 million splenocytes in 200 µl complete media into individual 5 ml round-bottom tubes, or into separate wells of a 96-well U-bottom plate.

NOTE: Using a 96-well plate is an efficient way to stain multiple samples, but take care to plate samples in every other well in order to prevent cross-contamination. One 96-well plate can accordingly hold 24 samples.

2. Centrifuge plate at 500 x g for 3 min., then flick supernatant from plate into appropriate (biohazard) container.

3. Resuspend the cell pellets with 100 µl of flow buffer (1% FBS in PBS) containing a fixable viability dye at the manufacturer’s recommended concentration and purified anti-CD16/anti-CD32 antibody cocktail at 1 µg/ml. Incubate for 10 min at 20-25 °C, then add 100 µl cold complete media to quench viability dye.

NOTE: Splenocytes from diseased mice usually contain relatively high numbers of dead or dying cells; therefore, the inclusion of a viability dye is recommended to avoid non-specific antibody labeling of dying cells, resulting in cleaner, more reliable data. Similarly, the anti-CD16/anti-CD32 cocktail is included to block non-specific fluorescently-labeled antibody binding by Fc receptors.

4. Centrifuge plate at 500 x g for 3 min., then flick supernatant from plate into biohazard container. Resuspend cells in 200 µl flow buffer.

Centrifuge plate at 500 x g for 3 min., then flick supernatant from plate into biohazard container.

5. Resuspend cells in 50 µl flow buffer containing antibody cocktail (Table 2). Incubate for 20 min at 4 °C, then add 150 µl flow buffer. Repeat wash step 8.4.
6. Resuspend cells with 100 µl 2% paraformaldehyde in PBS. Incubate for 30 min at 4 °C, then add 100 µl flow buffer. Centrifuge plate at 500 x g for 3 min., then flick supernatant from plate into biohazard container.

7. Resuspend in 200 µl flow buffer, transfer to flow tube inserts or standard flow tubes, add an additional 100-200 µl flow buffer, and store at 4 °C in the dark until acquiring on flow cytometer.

8. Acquire on a flow cytometer equipped with the appropriate lasers and PMTs for the chosen antibody panels within several days of staining. Record forward scatter width and/or height in addition to forward scatter area, side scatter area, and area of the fluorescent parameters utilized. For reliable results, acquire ≥1,000 donor cells in each WT sample, or an equivalent number of total lymphocytes in genetically modified or otherwise manipulated mice that show minimal expansion of donor cells, where collection of so many events may not be feasible. NOTE: Flow cytometry analyses are described in detail in the representative results section (Figures 3-6).

**Representative Results**

Diseased mice develop splenomegaly in as little as 14 days, exhibiting spleens 2-3 times the size of healthy mice in terms of mass and cellularity (Figure 2).

Splenocytes are sequentially gated on light scatter (FSC-A by SSC-A), elimination of doublets (FSC-W or -H by FSC-A), viable cells (low staining of viability dye), and CD4<sup>+</sup>TCR<sub>β</sub><sup>+</sup> (Figure 3A). Donor cells are distinguished from recipient cells based on CD45.1 and CD45.2 (Figure 3B, C). A portion of the recipient CD4 T cell population also differentiates into Tfh (Figure 3B, bottom right). After an initial die-off and/or migration of transferred cells, the expansion of donor-derived Tfh is logarithmic, reaching 10-20 million cells in the spleens of mice 14 days after injection (Figure 3D).

CFSE-labeling of transferred lymphocytes demonstrated that donor CD4 T cells differentiate into Tfh early after activation; essentially all divided cells observed at days 3, 7, and 14 upregulated CXCR5 and PD-1 (Figure 4). The proliferation peak profiles also suggest that a relatively low percentage of donor CD4 T cells underwent alloactivation and division. By day 14, most of the detectable donor cells are those that have divided beyond the maximum number of divisions measurable by CFSE.

The expansion of donor-derived Tfh is accompanied by a corresponding accumulation of endogenous GC B cells and plasma cells (Figure 5A). Plasma cell accumulation in the spleen is delayed compared to that of Tfh, exhibiting no increase over naïve animals on days 3 or 7 (Figure 5B). Accordingly, anti-nuclear antibodies are not readily detectable prior to day 9 (data not shown), but can be reliably quantified on day 14<sup>11</sup>.

Through the use of congenic markers, we have observed rejection of donor cells in multiple strains. While this is a common problem when mice are not sufficiently backcrossed to the C57BL/6 background, we also observed rejection when bm12 cells were transferred into B6.PL-Thy1<sup>a</sup>/CyJ (CD90.1) mice that are generally considered to be fully backcrossed. Therefore, mice are routinely screened at day ~3 by flow staining blood samples to assess the efficiency of the initial CD4 T cell graft; we know from CFSE proliferation experiments that grafted cells have expanded very little at this early time point. These results are then compared to results obtained from the day 14 harvest. In an example case of rejection, 30 million CD45.1<sup>+</sup> bm12 lymphocytes were transferred into Cardif<sup>−/−</sup> mice which had been backcrossed to C57BL/6 mice for 12 generations. At day 5, all mice displayed equivalent grafting (2-3% of circulating CD4 T cells), but by day 14, bm12 donor cells were completely eliminated from the genetically modified recipient (Figure 6).

**Figure 2. Spleen growth kinetics after injection of bm12 lymphocytes.** Spleens were weighed on a high precision balance directly after excision (left). Live cell numbers were determined by counting with a hemocytometer using trypan blue to exclude dead cells (right). Results are depicted as mean ± SEM, where n = 9, 4, 3, and 6, respectively. Please click here to view a larger version of this figure.
Figure 3. Analysis of T follicular helper cell expansion in the bm12 model of SLE. CD45.1+ bm12 lymphocytes were transferred into C57BL/6 recipients and spleens were analyzed 14 days later. (A) Representative gating strategy showing "lymphocytes" (first panel), "single cells" (second panel), "live cells" (third panel), and "CD4 T cells" (fourth panel). (B) Donor cells are distinguished from recipient CD4 T cells by CD45.1 and CD45.2 staining and analyzed for expression of PD-1 and CXCR5. (C) Donor cells adopt Tfh phenotype, as indicated by the upregulation of several proteins commonly associated with Tfh. (D) Typical results showing the expansion of donor-derived Tfh (defined as CD4+CD45.1+PD-1+CXCR5+ live cells) at days 3, 7, and 14 post transfer. Results are depicted as mean ±SEM, where n = 5, 4, 3, and 6, respectively. Please click here to view a larger version of this figure.
Figure 4. PD-1 and CXCR5 are upregulated on dividing cells. Bm12 cells were labeled with CFSE prior to injection into C57BL/6 recipients. Representative flow plots are shown for donor CD4 T cells at 3, 7, and 14 days after injection. Please click here to view a larger version of this figure.

Figure 5. Expansion of splenic plasma cells and GC B cells. (A) Representative flow plots from naïve mouse spleens, or those from mice 14 days after CD45.1\textsuperscript{+} bm12 transfer. Plasma cells are defined as CD138\textsuperscript{+}CD19\textsuperscript{low} live cells (top panels). GC B cells are a subset of CD19\textsuperscript{+} B cells, which express GL-7 and Fas (bottom panels). (B) Quantitative data showing the accumulation of splenic plasma cells over time. Results are depicted as mean ± SEM, where n = 5, 4, 3, and 6, respectively. Please click here to view a larger version of this figure.
Figure 6. Bm12 grafts are rejected by some genetically modified recipient mice. CD45.1^+ bm12 lymphocytes were transferred into either genetically modified mice (top panels) or C57BL/6 (bottom panels) recipient mice. Mice were bled at 5 days post injection and assessed for graft efficiency. Splenocytes from the same mice were analyzed 14 days later. Please click here to view a larger version of this figure.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5' - 3')</th>
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<tr>
<td>Bm12F</td>
<td>CGTGGTCGCCGCTGCC</td>
<td>°65 °C</td>
</tr>
<tr>
<td>Bm12R</td>
<td>GGCGAGGCGAGGAGG</td>
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Table 1: Primers and thermocycling conditions for bm12 genotyping. Optimal thermocycling conditions will depend upon the exact reagents and instruments used. These conditions have been optimized for a thermocycler capable of changing annealing temperature at every step, though the protocol may also work using a static annealing temperature.
Table 2: Antibody panels for splenic Tfh, GC B and plasma cell identification by flow cytometry. Presented are panels we have used successfully for assessing graft efficiency at day 3 (left) and the analysis of T and B cells at day 14 after bm12 cell injection (middle). These have been optimized for an LSRII with 5 lasers (355, 405, 488, 561, and 640 nm). Recommended filter sets for each fluorochrome are listed (right), though these may not be the best option for every machine. Depending upon what laser, PMT, and filter set combinations are available, separating the T and B cell analysis panel into multiple panels may be necessary.

Discussion

The bm12 inducible model is a relatively easy and efficient way to study the cellular and molecular processes of SLE. Chronic activation of adoptively transferred CD4 T cells directed against self antigens leads to the accumulation of Tfh, GC B cells, and plasma cells which can be measured by flow cytometry, as described here. Future studies using this model can quickly and easily interrogate the role of candidate genes and novel therapies in the autoimmune germinal center processes which resemble those occurring in patients with SLE, and ultimately govern the pathological accumulation of autoantibodies. Furthermore, the flow cytometric analysis described here can be used to study additional mouse models that involve the development of immunoglobulins including, but not limited to autoimmunity, infection, and allergy.

Like all animal models of human disease, this model also has its limitations. Given the speed with which disease develops and its magnitude, not all genes involved in the development of SLE are likely to be necessary for pathogenicity in this model. Additionally, care must be taken to rule out graft rejection when data suggest minimal expansion of Tfh in genetically modified recipients. Congenic markers should be used to confirm the presence of donor cells at harvest especially since recipient cells can also differentiate into Tfh (Figure 3B), which could otherwise mask the absence of donor cells. Using congeneric markers, we observed complete elimination of donor cells that evidently harbored rejection antigens by day 14 (Figure 6). We have successfully used CD45.1+ bm12 and CD45.2+ bm12 mice as donors, and CD45.1+ BoyJ (B6.SJL-PtprcaPepcβ/BoyJ) and CD45.2+ C57BL/6 mice as recipients (Figures 3, 6, 7, and unpublished data). However, wild type congenic CD90.1+ cells from the B6.PL-Thy1.2/CyJ mouse strain do not graft well, nor do CD90.2+ bm12 cells graft well in a CD90.1+ recipient (unpublished data), a phenomenon that is evidently not unique to this model.

Either C57BL/6 or bm12 mice can serve as the donor or the recipient, as initially reported by Morris et al. However, in our lab we find the transfer of bm12 cells into B6 mice produces more consistent expansion of T cell and B cell populations at day 14. Furthermore, donor and recipient mice from different groups should be gender- and age-matched. Although experiments reported in the first description of the bm12 model found no significant difference in any disease parameter between male→male and female→female transfers, male→female transfers are not advised, as male antigen (H-Y) expressed by transferred CD4 T cells may induce graft rejection in female recipients.

When choosing antibodies and fluorochromes for congeneric markers, it should be noted that donor cells become positive for the recipient congeneric marker to varying degrees, such that a CD45.2+ gate would not constitute the entire CD45.1+ graft. The phenomenon is clearly illustrated in a comparison of CD45.2 expression by CD45.1+ naïve, CD45.1+ donor, and CD45.2+ recipient CD4 T cells (Figure 7). Notably, donor cells also seem to upregulate expression of their own congeneric marker, compared to naïve controls (Figure 7). Similar results are also seen with CD45.2+ bm12 transfer into CD45.1+ BoyJ mice (data not shown). Presumably, acquisition of recipient CD45 results from trogocytosis by activated CD4 T cells following repeated interactions with recipient B cells. In fact, the bm12 model may prove a useful tool in studying the process of trogocytosis in vivo.
It has been shown that the transfer of purified bm12 CD4 T cells into C57BL/7 mice is sufficient to initiate disease; however, equivalent disease develops when whole lymphocytes are transferred, and purification is not necessarily required to study disease dependence upon T cell-intrinsic gene expression. Eisenberg and colleagues, clearly demonstrated that the antibody-producing cell in the bm12 model is almost exclusively of recipient origin. Furthermore, the requirement for recipient CD4 T cells is limited to the ‘nurturing’ of B cells during their development, which can be off-set by the addition of exogenous IL-4, although our data indicate that recipient T cells may participate somewhat in the germinal center response, as some of them do develop a Tfh phenotype (Figure 3B, bottom right).

One critical decision that must be made for every bm12 experiment is when to harvest tissues for analyses. Of course the decision depends upon exactly what factors are important to the current study, which may vary. The experiments described here take up to 14 days, as they focus on the initial development of Tfh cells and plasma cells; however, since this model is a chronic GVHD model of SLE, disease can be monitored much longer. In fact, certain clinical features of SLE, including glomerulonephritis, develop later. Proteinuria has been detected as early as 2 weeks post-injection, but reaches peak levels at 4-8 weeks post-injection. Additionally, the flow cytometric analyses described here have been optimized for experiments lasting 2 weeks. We find a considerable number of GC B cells and plasma cells at this time point; although, given additional time, a large percentage of ANA-secreting plasma cells may reside in the bone marrow. Moreover, the plasma cells identified by this flow staining panel likely also include plasmablasts—in order to distinguish between these two cell types, additional antibodies would be required. Tfh expansion presumably reaches a peak at some point after the 14-day window on which we have focused. However, to our knowledge, no studies have reported bm12 donor cell number beyond 2 weeks, or used congenic markers to track adoptively transferred cells.

Disclosures

Acknowledgements

This work was supported in part by the Lupus Research Institute, NCI grant CA138617, NIDDK grant DK090978, Charlotte Schmidlapp Award (to E.M.J.), and the Albert J. Ryan Fellowship (to J.K.). We are grateful for the support and instrumentation provided by the Research Flow Cytometry Core in the Division of Rheumatology at Cincinnati Children’s Hospital Medical Center, supported in part by NIH AR-47363, NIH DK78392 and NIH DK90971.

References

STING-Mediated DNA Sensing Promotes Antitumor and Autoimmune Responses to Dying Cells

Jared Klarquist,*1 Cassandra M. Hennies,*1 Maria A. Lehn,* Rachel A. Reboulet,* Sonia Feau,† and Edith M. Janssen*

Adaptive immune responses to Ags released by dying cells play a critical role in the development of autoimmunity, allograft rejection, and spontaneous as well as therapy-induced tumor rejection. Although cell death in these situations is considered sterile, various reports have implicated type I IFNs as drivers of the ensuing adaptive immune response to cell-associated Ags. However, the mechanisms that underpin this type I IFN production are poorly defined. In this article, we show that dendritic cells (DCs) can uptake and sense nuclear DNA-associated entities released by dying cells to induce type I IFN. Remarkably, this molecular pathway requires STING, but not TLR or NLR function, and results in the activation of IRF3 in a TBK1-dependent manner. DCs are shown to depend on STING function in vivo to efficiently prime IFN-dependent CD8+ T cell responses to tumor Ags. Furthermore, loss of STING activity in DCs impairs the generation of follicular Th cells and plasma cells, as well as anti-nuclear Abs, in an inducible model of systemic lupus erythematosus. These findings suggest that the STING pathway could be manipulated to enable the rational design of immunotherapies that enhance or diminish antitumor and autoimmune responses, respectively. The Journal of Immunology, 2014, 193: 6124–6134.

The immune system carefully balances its response to dead and dying cells to maintain homeostasis and prevent the development of autoimmunity. Although uptake and clearance of dying cells is generally considered a tolerogenic process, the existence of immunogenic cell death has been well described (1). Depending on the nature of the cell death, dying cells can emit damage-associated molecular patterns (DAMPs) (2) that act as danger signals and increase a dying cell’s immunogenicity. Many of these DAMPs seem to use the sensing and signaling pathways that are normally associated with the recognition and elimination of pathogens. Given the importance of immune responses to cell-associated Ags in autoimmunity, allograft rejection, and tumor rejection, the identification of these DAMPs, their cognate sensors, and their proinflammatory sequelae have become topics of intense research.

Ample studies have implicated type I IFNs in the development or progression of immune responses to self-Ags in autoimmune diseases such as rheumatoid arthritis, type I diabetes mellitus, Sjögren syndrome, and systemic lupus erythematosus (SLE) (3). However, type I IFNs were only recently identified as a crucial mediator in the priming of CD8+ T cells to cell-associated Ags in cancer and cancer treatments. Mice that lack type I IFN sensing, either by genetic IFN receptor (IFNAR) deletion or treatment with blocking Ab, develop more chemically induced tumors and show poorer rejection of transplanted immunogenic tumors than wild-type (WT) mice, highlighting the requirement for type I IFN in spontaneous tumor rejection (4, 5). Additional studies showed that the spontaneous induction of tumor-specific CD8+ T cells in tumor-bearing mice was predominantly mediated by type I IFN sensing in dendritic cells (DCs) (6, 7). A similar role for type I IFN was seen in therapy-induced tumor elimination. Burnette et al. (8) and Kang et al. (9) showed increased intratumoral production of type I IFN upon ablative radiotherapy or chemotherapy. The ablative effect of the therapy was associated with enhanced (cross)priming capacity of tumor-infiltrating DCs and could be abolished by eliminating IFNAR from the hematopoietic compartment. Our previous work, using tumor cell therapy in vaccination and therapeutic settings, showed a comparable dependency of type I IFN in the induction of protective antitumor CD8+ T cell responses (10–12).

Although the general immunostimulatory effects of type I IFN on DCs are well studied, little is known on the cellular source of type I IFN, the type I IFN–inducing ligand, and the receptor–signaling pathways involved in its induction upon the sensing and clearance of dying cells. Our previous work indicated that DCs can produce type I IFN upon phagocytosis of dying cells (10–12). Importantly, DCs from MyD88−/−/TRIF−/− mice showed normal type I IFN production upon phagocytosis of dying cells, and type I IFN–dependent CD8+ T cell priming to tumor cell vaccines was comparable in WT and MyD88−/−/TRIF−/− mice, indicating that the type I IFN induction requires an unidentified TLR-independent sensing pathway (11).

Using various murine cancer and tumor cell vaccination models and in vitro approaches, we show that the type I IFN production
upon sensing of dying cells is not only TLR independent, but also RLR independent, and requires STING-IRF3-mediated sensing of apoptotic cell–derived nuclear DNA structures by DCs. The ensuing type I IFN production enhances DC functionality in an autocrine manner, resulting in the increased clonal expansion, polyfunctionality, and memory formation of tumor-specific CD8+ T cells. Importantly, the role of the STING/IRF3/IFNAR nexus was not limited to CD8+ T cell priming or tumor models; elimination of STING or IFNAR significantly impacted the development of CD4+ follicular T cells (Th9), plasma cells, and anti-nuclear Abs in an inducible model of SLE. Collectively, our results demonstrate that STING/IRF3 sensing of nuclear DNA-structured by DCs broadly drives the priming of adaptive immune responses to dying cells.

Materials and Methods

Mice, cells lines, and peptides

Mice were maintained under specific pathogen-free conditions in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care International. C57BL/6J, B6.PL-Thy1a/b-CyJ (B6/CyD90.1), B6.SJL-Piper+/− (B6/CyD45.1), CD11c-DTR, and B6(C5-H2-AhiBm12/KhEgl) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and H2-Kb−/− mice from Harlan (Indianapolis, IN). IFNAR−/−, MyD88−/−/TRIF−/−, IFNAR−/−/TRIF−/−/IRF3−/−, and Act-mOVA/H2-K−/− mice were bred in our facility. IRF3−/− and IRF7−/− mice were a gift from Dr. K. Fitzgerald (University of Massachusetts Medical School, Worcester, MA). STING−/− mice a gift from Dr. R. Vance (University of Berkeley, Berkeley, CA), and IPS-1+/− mice a gift from Dr. J. Tschopp (University of Berkeley, Berkeley, CA).

Mixed bone marrow (BM) chimeric mice were generated using donors on different congenic backgrounds in 1:1 ratios. All BM chimeric mice were rest for 12 wk before experiments were started.

T cells, B cells, Mph, and DCs were sorted by flow cytometry using markers for TCRβ, CD4, CD8, CD11c, CD11b, CD19, CD45R, and MHC class II as previously described (12). Purity of sorted cells was generally ≥88% and viability was ≥97% as determined by 7-AAD staining.

Erythrocytes, normoblasts, reticulocytes, and RBCs were generated grown without cytokines. Cells were sorted based on size gating and nucle-catechol staining with guidelines by the Association for Assessment and Accreditation of Laboratory Animal Care International. C57BL/6J, B6.PL-Thy1a/b-CyJ (B6/CyD90.1), B6.SJL-Piper+/− (B6/CyD45.1), CD11c-DTR, and B6(C5-H2-AhiBm12/KhEgl) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and H2-Kb−/− mice from Harlan (Indianapolis, IN). IFNAR−/−, MyD88−/−/TRIF−/−, IFNAR−/−/TRIF−/−/IRF3−/−, and Act-mOVA/H2-K−/− mice were bred in our facility. IRF3−/− and IRF7−/− mice were a gift from Dr. K. Fitzgerald (University of Massachusetts Medical School, Worcester, MA). STING−/− mice a gift from Dr. R. Vance (University of Berkeley, Berkeley, CA), and IPS-1+/− mice a gift from Dr. J. Tschopp (University of Lausanne, Lausanne, Switzerland).

In vitro and ex vivo type I IFN analyses were performed by quantitative real-time PCR using SYBR Green and primers for β-actin (forward, 5′-TTGCTGACAGGAGTCAGGA-3′; reverse, 5′-GACTTGCCGTCAGGACGAGG-3′) and pan IFN-α (forward, 5′-TCTGATGACGGATGGTTG-3′; reverse, 5′-AGGGCTTCCAGGTCTCCTG-3′). Samples were treated with DNase to eliminate genomic DNA contaminations. Gene expression was analyzed using the relative standard curve method and was normalized to Gapdh and β-actin expression.

In vivo type I IFN analyses were performed by cytokine production and polyfunctionality, and memory formation of tumor-specific CD8+ T cells. Importantly, the role of the STING/IRF3/IFNAR nexus was not limited to CD8+ T cell priming or tumor models; elimination of STING or IFNAR significantly impacted the development of CD4+ follicular T cells (Th9), plasma cells, and anti-nuclear Abs in an inducible model of SLE. Collectively, our results demonstrate that STING/IRF3 sensing of nuclear DNA-structured by DCs broadly drives the priming of adaptive immune responses to dying cells.

In vitro models

Immunizations. Mice were injected s.c. or i.p. with irradiated cells (OVA-Kb+−/−/−/−) splenocytes, 108–109; B6-OVA, B16/OVA, and B16/F10, and 1–5 × 106). Alternatively, mice received i.v. 2 × 107 purified DCs that had been pulsed with OVA peptide or exposed to irradiated cells in vitro (10–12, 21). For depletion studies, CD11c-DTR mice and mixed BM chimeric mice were treated 24 h before and 24 h after immunization with 4 mg/diphtheria tox in i.p.

Tumor models. For the cytoablation model, mice were s.c. injected with 4 × 105 B6-OVA or B16/F10 in PBS. Ten days later, tumors (7–10 mm) were cryoablated using Verruca-Freeze armed with a 6-mm probe (Brymill Cryogenic Systems) (22) for 3 × 25-s cycles. To test long-term tumor protection, we challenged the mice with 3 × 108 B6-OVA or B16/F10 40 d after the ablation of the primary tumor and monitored for tumor growth. In the EL-4–mOVA challenge model, mice were immunized with 107 irradiated OVA-Kb−/−/−/−/− splenocytes as described earlier and 40 d later challenged with 105 EL-4–mOVA cells s.c.

SLE model. In the SLE model (23), mice of induced genetic backgrounds received 3 × 106 B6Cy-H2-AhiBm12/KhEgl (bm12) splenocytes i.p. Spleen B and T cell composition B was analyzed by flow flow within 2 wk. Serum levels of anti-dsDNA IgG, IgG1, and IgG2a were determined by ELISA as previously described (24).

T cell analysis

Unless stated differently, analyses were performed 7 d after immunization. CD8+ T cells were enumerated in spleens and draining lymph nodes using OVA257–264 Kb tetramers (Beckman Coulter) or E1B92–200 D decamers (ImmuKnow) together with staining for CD80, CD44, and 7-AAD. In parallel, cytokine production and polyfunctionality was determined directly ex vivo by intracellular cytokine staining after a 5-h stimulation with cytokine peptide in the presence of brefeldin A as described previously (11, 16). Samples were collected on an LSR II flow cytometer with Diva software (BD Pharmingen), and data were analyzed with FlowJo software (Tree Star). In B16/F10 studies, responses to TRP2180–188 were determined by ELISPOT directly ex vivo.
Capacity for secondary expansion in vitro was determined by stimulating the splenocytes or purified CD8+ T cells on irradiated MEC.B7.SigOVA (OVA-specific) or I1.2 cells (E1B specific) for 6 d and dividing the absolute number of Ag-specific CD8+ T cells at the beginning of the culture by the absolute number of Ag-specific CD8+ T cells at the end of the culture as described previously (16, 25). In vivo secondary expansion was determined by reinjecting the mice with a 10-fold higher number of irradiated cells than used during immunization. Four days after the secondary challenge, the frequency of Ag-specific CD8+ T cells was compared with nonchallenged immunized mice (16).

Statistical analyses

Data were analyzed using Prism software (GraphPad Software). Unless stated otherwise, the data are expressed as means ± SEM. Survival responses were analyzed by Kaplan–Meier using a log-rank test. All other data were evaluated using a two-way ANOVA followed by a Dunnett’s test. A p value <0.05 was considered statistically significant.

Results

Protective antitumor immunity requires type I IFN sensing by DCs

We and others recently showed that type I IFN sensing is critical for the induction of protective antitumor responses in various tumor models in both vaccination and therapeutic settings (6–8, 22). Cryoablation of B16-OVA tumors in WT mice resulted in effective priming of OVA257–264-specific CD8+ T cells that provided protective immunity upon subsequent B16-OVA challenge. In contrast, IFNAR−/− mice failed to induce adequate OVA257–264-specific CD8+ T responses and succumbed upon tumor rechallenge (Fig. 1A, 1B). Similarly, immunization with gamma-irradiated, OVA-expressing Kβ−/− splenocytes (OVA-Kβ−/−) induced a significantly more robust OVA257–264-specific CD8+ T cell response

![Graph showing percent survival and fold expansion](image-url)
in WT mice than in IFNAR−/− mice (Fig. 1C, 1D). The OVA257–264–specific CD8+ T cells from WT mice, but not IFNAR−/− mice, underwent expansion upon secondary encounter with Ag in vitro and in vivo, and protected mice from EL-4-mOVA challenge in vivo (Fig. 1E, Supplemental Fig. 1A). Importantly, this response was not restricted to OVA or the selected pathways of cell death. Similar results were found when the parental line B16/F10 was used and the response to self-Ag TRP-2 was probed (Supplemental Fig. 1B, 1C). In addition, direct immunization with gamma-irradiated, UV-irradiated, Fas-cross-linked, or etoposide-treated cells showed significantly decreased CD8+ T cell responses in IFNAR−/− mice, illustrating a central role for type I IFN in CD8+ T cell priming to cell-associated Ags in various scenarios of cell death (Fig. 2).

Because nearly all cells express IFNAR, we first used BM chimeras to identify which cells required type I IFN sensing in the CD8+ T cell response to dying cells. BM chimeric mice (WT→IFNAR−/−, WT→WT, IFNAR−/−→WT, IFNAR−/−→IFNAR−/−) demonstrated that type I IFN needed to be sensed by the hematopoietic compartment (Fig. 3A, 3B). Additional studies with WT/IFNAR−/→WT mixed BM chimeric mice indicated that the diminished CD8+ T cell response could not be attributed to the lack of IFNAR on the CD8+ T cells because IFNAR−/− CD8+ T cells showed only a marginal decrease in clonal expansion (Fig. 3C). Given the important role of DCs in cross-presentation of cell-associated Ags, we next examined the role of type I IFN sensing on DCs. Mixed BM chimeras were generated in which IFNAR−/− recipients received a combination of WT-CD11c-DTR and IFNAR−/−/CD45.1 BM or IFNAR−/−CD11c-DTR and WT/CD45.1 BM. In this model, the administration of diphtheria toxin yielded animals that contained either WT or IFNAR−/− DCs with an otherwise comparable composition of hematopoietic cells. Depletion of WT DCs significantly decreased the number and frequency of Ag-specific CD8+ T cells. In contrast, depletion of IFNAR−/− DCs had no effect on the number or frequency of Ag-specific CD8+ T cells (Fig. 3D). Similar results were observed when purified WT or IFNAR−/− DCs were pulsed with irradiated cells and transferred into WT and IFNAR−/− recipients, illustrating the critical role of type I IFN sensing by DCs in CD8+ T cell priming to cell-associated Ags (Fig. 3E).

**DCs predominate the type I IFN production elicited by dying cells**

Besides sensing type I IFNs in the context of CD8+ T cell priming to dying cells, we reasoned that DCs may also be the main producers of type I IFN, thereby establishing a positive IFN-feedback loop. In vitro studies using purified cell populations indicated that type I IFN was produced by DCs upon incubation with dying cells, whereas only nominal IFN production was observed in cultures depleted of DCs (Fig. 4A–C) (11, 12). The in vitro data were supported by our observation that IFN-α mRNA was readily detectable in the draining lymph nodes of control-treated, but not diphtheria toxin (DT)-treated, CD11c-DTR mice upon s.c. immunization with irradiated cells (Fig. 4D).

**STING/IRF3 pathway drives type I IFN in response to cell-associated Ag**

Induction of type I IFNs is generally associated with innate sensing of pathogenic danger signals. To identify which innate sensing pathways facilitated the type I IFN production, we tested primary DCs from WT, MyD88−/−/Trif−/−, IRF3−/−, IRF7−/−, IPS1−/− (MAVS/Cardif), and STING−/−-deficient mice for their type I IFN production upon culture with dying cells. Type I IFN production was similar in WT, MyD88−/−/Trif−/−, IPS1−/−, and IRF7−/− mice.

**FIGURE 2.** Impaired CD8+ T cell priming in IFNAR−/− mice is independent of the method of cell death or cell type. Frequency of splenic Ag-specific CD8+ T cells in WT and IFNAR−/− mice 7 d after s.c. immunization with actmOVA-Kb splenocytes (which were gamma irradiated [1500 rad], UV-irradiated [120 mJ/cm²], or treated with FAS cross-linking Ab [Jo-1, 20 μg/ml; 4 h, 37˚C]), 5E1-TAKO cells (3000 rad, 240 mJ/cm²), or treated with etoposide [10 μM]), or B16-OVA or B16/F10 cells (3000 rad or 240 mJ/cm²). Ag-specific T cell frequencies were determined by intracellular cytokine staining upon incubation with OVA257–264, E1B192–200, TRP-2180–188, or control peptide GP33–41 (white bar, control peptide; black bar, specific peptide). Data in all experiments are expressed as mean ± SEM with n = 5. *p < 0.05.
DCs, indicating that the IFN induction was TLR, RIG-I, and Mda5 independent. In contrast, significant reductions in type I IFN production were seen with DCs deficient in STING and the transcription factor IRF3 even though their subset compositions, maturation status, and phagocytic capacity were similar to WT DCs (Fig. 5A, 5B, and data not shown). Immunoprecipitation and Western blotting studies indicated rapid IRF3 phosphorylation in WT DCs and a significant reduction and delay in IRF3 phosphorylation in STING-/- DCs upon exposure to dying cells (Fig. 5C, 5D).

**FIGURE 3.** Selective requirement for type I IFN sensing in the DCs. (A) Bone-marrow chimeric mice (WT→IFNAR<sup>−/−</sup>, WT→WT, IFNAR<sup>−/−</sup>→WT, IFNAR<sup>−/−</sup>→IFNAR<sup>−/−</sup>) were immunized i.p. with irradiated 5E1-TAKO cells and the frequency of E1B<sub>192–200</sub>-specific CD8<sup>+</sup> T cells in the spleens was determined 7 d later (white bar, control peptide; black bar, E1B<sub>192–200</sub> peptide). (B) The effect on memory formation using these chimeras was determined by measuring Ag-specific T cell expansion ex vivo. Secondary expansion of E1B<sub>192–200</sub>-specific CD8<sup>+</sup> T cells was calculated by dividing the absolute number of E1B<sub>192–200</sub>-specific CD8<sup>+</sup> T cells at the end of a 6 d culture by the absolute number at the start of the culture. (C) WT/CD90.1 mice were irradiated and reconstituted with WT, IFNAR<sup>−/−</sup> or a 1:1 ratio of WT/CD45.1:IFNAR<sup>−/−</sup> (CD45.2) bone-marrow. Mice were i.p. immunized with irradiated 5E1-TAKO cells and the frequency of splenic E1B<sub>192–200</sub>-specific CD8<sup>+</sup> T cells in each graft was determined 7 d later. (D) WT<sup>CD11c-DTR</sup>:IFNAR<sup>−/−</sup> and WT:IFNAR<sup>−/−</sup>/CD11c<sup>DTR</sup> mixed bone-marrow chimeras were treated with DT or vehicle and immunized with irradiated 5E1-TAKO cells. The frequency of E1B<sub>192–200</sub>-specific CD8<sup>+</sup> T cells in the WT compartment was determined 7 d later. (E) Freshly isolated WT and IFNAR<sup>−/−</sup> DCs were incubated with irradiated 5E1-TAKO cells, sorted and i.v. injected into WT and IFNAR<sup>−/−</sup> mice. The frequency of splenic E1B<sub>192–200</sub>-specific CD8<sup>+</sup> T cells was determined 7 d later. Data of representative experiments (out of three to five) are shown (mean ± SEM, n = 5/group, *p < 0.05).

**FIGURE 4.** Type I IFN production by DCs in vitro and in vivo. Total and subset-depleted splenocytes (A) or purified cell populations (B) from spleens of naive WT mice were cultured with irradiated OVA-K<sub>b</sub>-/- splenocytes, and type I IFN in the supernatant was determined 20 h later (black bars, irradiated cells; white bars, no cells). Data are expressed as mean ± SEM with n = 4. (C) Splenocytes from CD11c-DTR mice (treated with PBS or DT 24 h prior) were isolated and cultured with irradiated OVA-K<sub>b</sub>-/- splenocytes. Type I IFN in the supernatant was determined 20 h later. (D) CD11c-DTR mice were treated with vehicle or DT and 24 h later s.c. injected with irradiated cells. Type I IFN mRNA in draining lymph nodes was assessed 6–8 h later. Representative data of one experiment (out of three to four) are shown (mean ± SEM, n = 5, *p < 0.05).
p-IRF3 signal was associated with the frequency and size of phagocytosed particles, we incubated WT and STING−/− DCs with VT-labeled, irradiated splenocytes. Uptake of VT materials was determined 6 h later by flow cytometry. Data are expressed as percentage of DCs that contain VT. Representative data of one experiment (out of three to four) are shown (mean ± SEM, n = 4). (C and D) p-IRF3 kinetics in WT and STING−/− DCs upon incubation with irradiated IRF3−/− splenocytes. (E) ImageStream images of p-IRF3 nuclear translocation in WT DCs 8 h after incubation with VT-labeled irradiated IRF3−/− cells. Original magnification ×60. (F) ImageStream analysis of nuclear p-IRF3 intensity and intensity of cytosolic phagocytosed material in WT and STING−/− DCs. Tight masking on the cytosol was done to discriminate between bound and internalized cellular material. At least three experiments were performed with 800–1000 analyzed cells/condition. *p < 0.05

Nuclear DNA-derived structures induce type I IFN production by DCs

We next set out to determine the ligand upstream of STING that was responsible for inducing type I IFN. STING facilitates immune responses to various nucleotide structures, including cytosolic dsDNA, and in some cases dsRNA (26–28). Because dsRNA sensing uses the RIG-I/IPS-1 pathway and IPS1−/− DCs have normal type I IFN production, it is likely that the IFN-inducing species released by dying cells is a DNA-based and not an RNA-based entity. Indeed, addition of DNases, but not RNases, to WT DC/irradiated cell cocultures significantly reduced type I IFN production without affecting uptake of cellular material or responses to non-nucleic TLR ligands (Fig. 6A, Supplemental Fig. 2C, 2D). To more rigorously address the role of DNA complexes in the type I IFN induction, we exploited the process of erythropoiesis where RBC precursors sequentially lose their nuclei, mitochondria, and ribosomes. Timed RBC cultures were sorted, irradiated and treated with thrombospondin-1 (irr/TSP) to induce comparable phosphatidylserine expression on the membrane and facilitate an “apoptotic phenotype” in the non-nucleated cells (Fig. 6B). ImageStream analysis showed comparable uptake of the irr/TSP cell subsets by DCs (Fig. 6C). Nucleated irr/TSP
erythroblasts readily induced type I IFN in DCs, whereas enucleated reticulocytes and RBCs failed to do so (Fig. 6D). These data indicate that the IFN-inducing species is nuclear DNA derived.

**DC-intrinsic STING regulates CD8+ T cell responses to dying cells**

Given the importance of STING-mediated DNA sensing in the type I IFN production, we next assessed the relative contribution of STING in the priming of CD8+ T cells to dying cell-associated Ags. WT, MyD88-/-, TRIF-/-, IPS-/-, and IRF7-/- mice showed comparable CD8+ T cell priming as determined by tetramer staining, intracellular cytokine staining, and capacity for secondary expansion upon immunization with irradiated 5E1-TAKO cells (Fig. 7A, 7B). In contrast, IFNAR-/-, IRF3-/-, and STING-/- mice showed significantly reduced CD8+ T cell priming (Fig. 7C, 7D). Moreover, the Ag-specific IFNAR-/-, IRF3-/-, and STING-/- CD8+ T cells displayed less cytokine polyfunctionality and impaired capacity for secondary expansion (Fig. 7E). The defect in CD8+ T priming was fully DC regulated as similar results on CD8+ T cell clonal burst, secondary expansion, and cytokine polyfunctionality were seen when purified IFNAR-/-, IRF3-/-, and STING-/- DCs were exposed to irradiated cells in vitro and transferred into WT recipients (Fig. 7F–H). The priming defect of the IFNAR-/-, IRF3-/-, and STING-/- DCs could not be attributed to a decrease in overall DC functionality as peptide-pulsed IFNAR-/-, IRF3-/-, and STING-/- DCs induced comparable CD8+ T cell responses as WT DCs upon transfer in WT recipients (Fig. 7I). Together, these data demonstrate the requirement for STING, IRF3, and IFNAR in DCs in the cross-priming of CD8+ T cells to cell-associated Ags.

**STING regulates CD4+ T cell and B cell responses in the bm12 SLE model**

To assess whether STING has a broader role in the priming of adaptive immune responses to dying cells, we assessed the induction of CD4+ T cell and B cell responses in the bm12-cGVHD model where H-2b B6 hosts develop lupus-like disease upon transfer of B6.C-H2bm12 CD4+ T cells (29). Both IFNAR-/- and STING-/- mice developed considerably less activated CD4+ T cells and Th than WT recipients upon transfer of bm12 CD4+ T cells (Fig. 8A, 8B). Moreover, both IFNAR-/- and STING-/- mice developed considerably less activated B cells, plasma cells, and pathogenic anti-dsDNA IgG2a Abs than WT recipients (Fig. 8A, 8C, 8D). Given that the transferred bm12-CD4+ T cells were IFNAR and STING sufficient, these data further support the role for Ag-presenting, cell-intrinsic STING and IFNAR in the induction of adaptive immune responses to dying cell-derived Ags.

**Discussion**

Type I IFNs have been implicated as the upstream events precipitating autoimmune disease and a prerequisite for effective antitumor radiotherapy. In this study, we identify DC sensing of cell-derived nuclear DNA entities via the STING/IRF3 pathway as a key component in the early type I IFN response to dying cells.

Dying cells can emit a plethora of structurally distinct DAMPs, and it is likely that the molecular pathways involved in the sensing of these DAMPs are equally diverse. Although many DAMPs can contribute to the final adaptive immune response to cell-associated Ags, our data identified type I IFN as the dominant proinflammatory factor and STING/IRF3 signaling as the principle pathway in the initiation of the immune responses to cell-associated Ags.
Ags in our tumor models, as well as the autoimmune responses in our SLE model. Although all nucleated cells can sense type I IFN, and type I IFN has been shown to directly act on T cells, our data indicate that the early STING/IRF3-mediated type I IFN predominantly acts on DCs. Transfer of IFNAR\textsuperscript{2/2} DCs (pulsed with irradiated cells) into WT recipients resulted in similar deficiencies in CD8\textsuperscript{+} T cell expansion, functionality, and memory formation as the direct immunization of IFNAR\textsuperscript{2/2} mice. Moreover, immunization of WT/IFNAR\textsuperscript{2/2} mixed BM chimeric mice did not show any significant difference in CD8\textsuperscript{+} T cell clonal expansion or polyfunctionality between the WT and IFNAR\textsuperscript{2/2} grafts. Importantly, transfer of STING\textsuperscript{2/2} or IRF3\textsuperscript{2/2} DCs into WT recipients resulted in similar defects in CD8\textsuperscript{+} T cell responses as the transfer of IFNAR\textsuperscript{2/2} DCs. Likewise, STING\textsuperscript{2/2} recipients showed identical reduction in Tfh and plasma cell formation as IFNAR\textsuperscript{2/2} recipients in our SLE model. Together with the observation that STING\textsuperscript{2/2} and IRF3\textsuperscript{2/2} DCs have significantly reduced type I IFN induction upon phagocytosis of dying cells, these data implicate that the STING/IRF3 pathway is the critical component in the type I IFN–dependent T cell priming to cell-associated Ags.

It has been suggested that different types of death may induce different DAMPs. We observed comparable type I IFN induction and STING/IRF3 engagement in DCs upon phagocytosis of cells treated with gamma irradiation, UV irradiation, Fas–cross-linking Ab, or etoposide, suggesting that these different types of cell death generated a similar nuclear DNA-derived DAMP (Supplemental Fig. 2B and data not shown). It is also likely that similar nuclear DNA-associated DAMPs become available in vivo as type I IFN induction, and type I IFN–dependent CD8\textsuperscript{+} T cell priming was readily observed in WT and MyD88/Trif-deficient mice, but sig-

![Image of STING regulation of CD8\textsuperscript{+} T cell responses to dying cells in vivo.](https://www.jimmunol.org/DownloadedFromHttp://www.jimmunol.org/)
Importantly, the crucial role for type I IFN in the priming of protective adaptive antitumor responses is not restricted to situations where massive tumor cell death occurs, as is the case for radiotherapy, chemotherapy, and cryoablative tumor therapies. Recent publications indicate that spontaneous and limited tumor cell death in tumor-bearing mice also resulted in type I IFN–dependent protective immune responses. Fuertes et al. (7) and Diamond et al. (6) showed spontaneous antitumor CD8+ T cell induction and tumor rejection in tumor-bearing mice that was critically dependent on type I IFN sensing by cross-priming DCs. In this light, it is interesting to note that STING−/− mice, like IFNAR−/− mice, develop significantly more lung metastases than WT mice upon i.v. injection of low numbers of untreated B16 melanoma cells, illustrating a role for the STING/IFNAR nexus in the antitumor response when cell death is limited (data not shown).

STING can facilitate innate responses to cytosolic bacterial cyclic dinucleotides (cyclic-di-GMP) (30, 31), dsDNA, and in some cases cytosolic dsRNA (26–28). Our data strongly suggest that the main type I IFN–inducing ligand is a nuclear DNA species. STING-mediated dsRNA sensing requires RNA with 5′-tri-phosphate groups in combination with the IPS-1/RIG-I pathway. The absence of IPS-1 recruitment to STING in WT DCs upon phagocytosis of cellular materials, as well as the normal type I IFN production and CD8+ T cell responses in IPS1−/− mice, effectively argue against a role for dsRNA sensing in the STING/IFN phenotype. Our hypothesis that the type I IFN–inducing ligand is a DNA structure is strongly supported by our in vitro data that show significantly reduced type I IFN induction upon addition of DNases to the dying cell/DC coculture. Moreover, the use of enucleated cells dramatically reduced type I IFN production and cross-priming by WT DCs in vitro. Importantly, the latter experiment also suggested that mitochondrial or ribosomal nucleotide structures had no notable role in the type I IFN production as reticulocytes, enucleated but still containing mitochondria and ribosomes, failed to induce type I IFN.

Recent studies indicate direct binding of cyclic-di-GMP and cyclic-GMP-AMP (cGAMP) to STING but have not provided evidence for direct dsDNA–STING interactions (32, 33) suggesting the involvement of upstream DNA sensors. Over the last few years, several candidate sensors have been identified, including cGAMP synthase (cGAS), which has been shown to signal through STING via the production of cGAMP (34–37). DExD/H-box protein family member DDX41 and IFI16 (p204) have also been implicated as possible DNA sensors acting through STING, but their precise molecular interactions have not been fully elucidated (38, 39). Currently, these candidate DNA sensors...
are studied in in vitro systems where the DNA is directly delivered into the cytosol via transfection, transduction, or infection pathways. However, for the phagocytosed DNA-derived structures to be sensed by the STING pathway, either its key components should be recruited to the phagosome or the DNA-derived species should escape into the cytosol. Although STING can translocate from the endoplasmic reticulum to the Golgi and autophagosome-like compartments, we and others did not observe STING in phagosomes or phagolysosomes (data not shown) (40–44). However, phagosomal DNA sensing via STING could still be possible by phagosomal recruitment of p204 that has reported migratory capacity or cGAS that produces the highly mobile secondary messenger cGAMP (34, 36, 39). In contrast, phagosomal escape is a well-reported process in cross-presentation where protein structures escape into the cytosol to be processed for presentation in MHC class I (20, 45). The exact mechanism by which proteins escape into the cytosol is not known, but it is strongly associated with alkalinization of the phagosome and prevention of phagosomal acidification (20, 45, 46). Consistent with the latter possibility, STING-mediated type I IFN production was strongly associated with inhibition of phagosomal acidification (45); in vitro treatments of DCs with agents that accelerated phagosomal acidification decreased type I IFN production, whereas alkalization or the delay of endosomal acidification significantly enhanced type I IFN production (Supplemental Fig. 3). Moreover, we found that DC populations that have the greatest capacity for cross-presentation and slowest phagosomal acidification rate also produced the most type I IFN upon phagocytosis of dying cells (11, 12, 47, and data not shown).

Although the exact mechanism by which the nuclear DNA-derived structure activates the STING pathway needs further elucidation, our data strongly demonstrate its potent role in antitumor immunity and autoimmunity. Our observations are in line with the findings that mice that lack DNase II, responsible for degradation of phagocytosed DNA, die of TLR-independent proinflammatory findings that mice that lack DNase II, responsible for degradation of 

**References**


**Acknowledgments**

We are grateful to Dr. H. Singh for comments and discussion on the manuscript, Dr. T. Kalfa for providing reagents and advice for the in vitro erythropoiesis experiments, A. White and M. Delaey for support with the Annuis ImageStream, and the Research Flow Cytometry Core at Cincinnati Children’s Hospital Medical Center.

**Disclosures**

The authors have no financial conflicts of interest.