ABNORMALITIES IN THE ADHESION AND AGGREGATION PROFILES OF CIRCULATING MONOCYTES IN PSORIASIS

By

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For my family:
Dad, Mom, Jeff, and Tyler

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ABBREVIATIONS

AMP: antimicrobial peptide
BMI: body mass index
BSA: body surface area
CACS: and carotid artery calcium scoring
CAD: coronary artery disease
CCR: chemokine receptor
CD: cluster of differentiation
CLA: cutaneous lymphocyte antigen
CMML: chronic myelomonocytic leukemia
CVD: cardiovascular disease
CXCR: C-X-C motif receptor
DAMP: damage associated molecular pattern molecule
DC: dendritic cell
DQLI: dermatology life quality index
hBD: human beta-defensin
HMVEC-D: human dermal microvascular endothelial cells
hsCRP: high-sensitivity C-Reactive Protein
EC: endothelial cell
ECM: extracellular matrix
ERK: extracellular signal-regulated kinases
FAK: focal adhesion kinase
FDG: fluorodeoxyglucose
GWAS: genome-wide association study
IFN: interferon
IKK: IkappaB kinase
IL: interleukin
IMIAD: immune-mediated inflammatory autoimmune disease
IMQ: imiquimod
iNOS: inducible nitric oxide synthase
ICAM: intercellular adhesion molecule
IPA: ingenuity pathway analysis
ITG: integrin
JNK: c-Jun N-terminal kinase
KC: keratinocyte
LC: Langerhans cell
LN: lymph node
LPS: lipopolysaccharide
MAPK: mitogen-activated protein kinase
MCP: monocyte chemoattractant protein
MDSC: myeloid derived suppressor cell
MHC: major histocompatibility complex
MI: myocardial infarction
MMP: matrix metalloproteinase
MP: microparticle
MPA: monocyte/platelet aggregate
NET: neutrophil extracellular trap
oxLDL: oxidized LDL
PASI: psoriasis area and severity index
PBMC: peripheral blood mononuclear cell
PCR: polymerase chain reaction
pDC: plasmacytoid DC
PPPP: palmoplantar pustular psoriasis
PET/MRI: positron emission tomography-magnetic resonance imaging
PSORS1: psoriasis susceptibility gene 1
QoL: quality of life
RA: rheumatoid arthritis
RORγ: RAR-related orphan receptor gamma
SD: standard deviation
slanDCs: 6-sulfo LacNAc dendritic cells
STAT: signal transducer and activator of transcription
STEMI: ST segment elevation myocardial infarction
Tie-2: tyrosine kinase with immunoglobulin and EGF homology domains
TNF: tumor necrosis factor
Teff: effector T cell
TLR: Toll-like receptor
Treg: regulatory T cell
VEGF: vascular endothelial growth factor
WT: wild type
Abnormalities in the Adhesion and Aggregation Profiles of Circulating Monocytes in Psoriasis

Abstract

By

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Human psoriasis patients have numerous comorbidities including an increased risk of developing cardiovascular disease (CVD), thus making psoriasis truly more than skin deep. Numerous cellular immune mediators are implicated to initiate, direct, and participate in the chronic inflammation characteristic of psoriasis. A relatively neglected participant in psoriasis pathology is the monocyte. Three main populations of circulating monocytes have been identified in humans based on differential expression of the cell surface markers CD14 and CD16, defining them as “classical” (CD14++CD16−), “intermediate” (CD14++CD16+), and “non-classical” (CD14+CD16++). Intermediate (CD14++CD16+) monocytes are elevated in psoriasis, correlate with psoriasis severity and, interestingly, are predictive of increased CVD risk and outcomes including death, suggesting that monocytes may be an important cellular mediator. A unique feature observed for classical monocytes was a robust relative (p=.006) and absolute (p=.003) increase in circulating CD14+ monocyte-monocyte aggregates (doublets) in both unfractionated whole blood and PBMCs of psoriasis patients. Doublets (from sorted CD14+ cells) have mRNA expression distinct from either classical singlet monocytes or classical singlet monocytes following plastic adherence. Aggregated monocytes overexpress integrins, extracellular matrix proteins, and a unique disintegrin. Cumulative data suggest that psoriasis monocytes
are primed for adhesion and form increased circulating aggregates that express a unique mRNA profile. Co-culture experiments using HMVEC-D dermal endothelial cells and psoriasis monocytes demonstrated that a higher proportion of monocytes adhered to IL-17A/TNF-α-stimulated HMVEC-D cells, indicating that mediators released from these endothelial cells may enhance monocyte adhesion. To explore the mechanistic contributions of pro-inflammatory monocytes in mediating cardiovascular events, we used a murine model of psoriasiform skin inflammation known to be atherogenic and exhibit increased (shortened) thrombosis levels (KC-Tie2 mice). We backcrossed these mice to IL-6-/- mice and monitored changes in circulating monocytes and thrombosis clotting times. Thrombosis times were increased to control mouse levels upon depletion of IL-6 which also decreased circulating CD11b^+Ly-6C^{high} monocytes. These data reveal a potential role for skin-derived IL-6 in promoting increases in circulating monocytes and thrombosis-related psoriasiform inflammation.
CHAPTER 1

INTRODUCTION
Psoriasis

Psoriasis is a chronic inflammatory skin disease affecting 2-3% of the US population and in 2013 was estimated to affect 7.4 million individuals (1, 2). The total economic burden of psoriasis due to medical costs, reduction in quality of life, and loss of productivity totaled $35.2 billion (2). Although psoriasis is described as an autoimmune disease, the cause remains unknown and a strict mitigating signal or a single antigenic target has yet to be identified. A combination of human and animal studies has led to the understanding that in patients with a susceptible genetic background, some stimulus, perhaps an infection, leads to a coordinated series of signaling events involving the release and signaling of cytokines that occurs between keratinocytes (KCs), endothelial cells (ECs), T cells, monocytes and macrophages, and dendritic cells (DCs). Once initiated, these modified signaling patterns result in a vicious pro-inflammatory proliferative cycle that perpetuates a sustained cutaneous inflammatory response and resultant epidermal hyperplasia. Intervention by blocking key cellular and soluble targets in this cycle results in clinical improvement; however, durable remission and/or permanent clearance have not been achievable.

Psoriasis has been categorized as an immune-mediated disease based primarily on clinical observations demonstrating disease clearance following immunosuppressive therapies (3). Further evidence has demonstrated a strong T cell component to disease maintenance via interventions specifically targeting Th1, Th17, and Th22 cells, and their derived cytokines (4-7). Release of T cell-derived cytokines including interferon (IFN)-γ, interleukins (IL)-17A, -17F, and -22 following cutaneous
DC activation promotes KC hyperproliferation, further production of cytokines, and subsequent increased immune signaling.

Clinical pathogenesis and assessment

The most common variant of psoriasis, psoriasis vulgaris or plaque psoriasis, affects approximately 85 to 90% of all patients (8). Plaque psoriasis is characterized clinically by hallmark skin changes including erythema and well-demarcated raised, scaly patches. The typical skin architecture of a psoriasis vulgaris patient results from epidermal hyperplasia, an increase in the rate of KC division. This hyperproliferative state also results in parakeratosis as a result of retention of nuclei in the stratum corneum (9), and epidermal rete ridges formed by elongated, thin, downward epidermal projections into the dermis (10), as shown in Figure 1.1. The microvasculature of psoriatic skin is characterized by leaky blood vessels, increased expression of vascular endothelial growth factor (VEGF) on lining endothelial cells (11), and infiltration of leukocytes into the psoriatic plaques. The severity of the disease can be exacerbated by stress, infections, and medications such as beta blockers (12-14).
Figure 1.1: Histological features of psoriasis

(A) Healthy control skin demonstrating a thin epidermis and dermis without immune cell infiltration. (B) Lesional psoriatic skin demonstrating increases in acanthosis of the cell layers of the epidermis and infiltration of immune cells in the dermis. Rete ridge elongation (yellow arrows) and increase in the number and size of dermal blood vessels (black arrows) is also shown.
The second most common variant is guttate psoriasis, sometimes classified as “childhood psoriasis” as it presents at an earlier age of onset (15). It manifests as small, pink droplets (from the Latin, “gutta”) on the skin and is usually associated with a preceding streptococcal throat infection (16). Family history and recent stressful life events are also risk factors for developing guttate psoriasis (15).

Pustular psoriasis, characterized by tender, erythematous pustules, is very rare (17) and can also present on the palms as palmoplantar pustular psoriasis (PPPP) (18).

Inverse psoriasis can present in skin folds and other flexural body sites where two skin surfaces meet and is characterized by a lack of the characteristic scaly plaques due to warm, moist surfaces (19). Finally, some forms of psoriasis can develop exclusively on the nails and scalp (20).

Physicians use several different clinical assessments to determine or classify the severity of a patient’s psoriasis. In research studies, the most widely used measure of plaque psoriasis is the Psoriasis Area and Severity Index (PASI). PASI calculations take into consideration three components of the plaque: erythema (redness), induration (thickness), and desquamation (scaling), and assess the body surface area (BSA) involvement of the plaques over four regions: head, trunk, upper, and lower extremities, assigning them each a score of severity of involvement. Using a formula that takes into account the proportion of each body region, the severity of the psoriatic disease is assigned a single PASI score that ranges from 0 to 72. In clinical trials, the PASI 75 (defined as a 75% reduction from baseline PASI score) and PASI 50 (a 50%
reduction from baseline PASI score) are used as an endpoint to determine efficacy of a given particular drug (21).

Another clinical assessment tool, the Physician Global Assessment (PGA), is also used to measure psoriasis severity and tends to be a more simple assessment when compared to PASI, more closely mirroring assessments used by physicians in clinical practice (22). The PGA evaluates all plaques on the body based on erythema, scale, and induration without specifically identifying where the plaques are located or taking into account their BSA. This is accomplished using a scale that rates each plaque and varies from “clear” to “mild,” “moderate,” and “very severe” where each are represented numerically. However, different variations of PGA exist in terms of the number of values on the scale (anywhere from 4 points to 10 points) (23). Whether or not the patient’s disease history is considered provides yet another variation in the scale. Sometimes a static form of the PGA is used which assigns a score based only on a single time point, while other times a dynamic form is used that accounts for global improvements from baseline (24).

While PASI is the most highly validated tool, it has limitations that include low reproducibility of the BSA component of the scale and insensitivity and decreased accuracy when the patient has mild to moderate disease or small changes in disease presentation (23). PASI is also not valid to measure severity of other clinical manifestations of psoriasis such as guttate, and it does not take into account a patient’s history at baseline or other comorbidities present. When PASI and PGA scores are compared, they are found to be highly correlated, yet determining the PGA score requires less clinical experience and results in less intra-rater variation (25).
Taking this into account, all three measures – PASI, PGA, and BSA – can be used together to get a true idea of the involvement and severity of a patient’s psoriasis.

Recent studies on psoriasis patients have begun to quantify quality of life (QoL) indices (sometimes known as the Dermatology Life Quality Index (DLQI)) based upon patient-reported outcomes (26). These indices measure the patient perception of how their QoL is affected by psoriasis and how this QoL is modulated based upon therapy (27). Patient QoL has been found to moderately and positively correlate with physician-defined assessments such as PASI and the static PGA (28, 29). Patient centered outcomes are currently a preferred tool for measurement of disease improvement as they take into account patient satisfaction and/or dissatisfaction with treatment regimens.

**Psoriasis and associated comorbidities**

Epidemiological studies have shown that individuals with psoriasis have an impaired quality of life along with increased risk of psoriatic arthritis, diabetes, inflammatory bowel disease, and obesity (30-35). Metabolic syndrome, defined as a cluster of several cardiovascular risk factors, is associated with psoriasis and the association increases with increased psoriasis disease severity (36, 37).

Recently, epidemiologic evidence suggests that psoriasis is associated with an increased risk of experiencing a range of cardiovascular disease (CVD) manifestations and adverse cardiovascular events. A growing body of research has demonstrated that patients with psoriasis have increased risk factors associated with CVD (31, 38-41). Importantly, it has been shown that patients with severe psoriasis
have a significantly increased risk of developing and dying of CVD independent of these risk factors (40, 42-46) (42, 44). Patients with mild to severe psoriasis have an increased risk of hypertension (47, 48), venous thromboembolism (49), myocardial infarction (46), and stroke (50, 51) while patients who have had psoriasis for more than 8 years have a higher prevalence of coronary artery disease (CAD) than patients without psoriasis (52). However, it should be noted that this is still controversial as separate studies found that there is no significant risk of cardiovascular mortality associated with psoriasis, although this could be due to possible selection bias of patient inclusion in the particular cohort and/or other particular confounding variables (53-56).

**Genetics of psoriasis**

There is a large amount of genetic variation in psoriasis patients and as such, a single susceptibility gene has not been identified. Historically, the major locus identified in psoriasis, **PSORS1** (psoriasis susceptibility 1), was thought to account for a large proportion (35-50%) of the genetic risk of developing disease (57, 58). HLA-Cw6, which encodes a major histocompatibility complex I (MHCI) allele, was identified on the **PSORS1** locus on chromosome 6p and found to be significantly associated with susceptibility to early-onset psoriasis (59, 60). HLA-Cw6-positive psoriasis patients have been classified as having different clinical features, such as an earlier onset of disease, more plaque, and more severe disease, when compared to HLA-Cw6-negative patients (61).

Within the past 15 years, approximately 40 susceptibility loci have been identified in psoriasis patients (57, 58, 62, 63). A meta-analysis of 3 genome-wide
association studies (GWAS) identified 15 psoriasis susceptibility loci that share roles in regulating T cell function and innate host defense, including macrophage activation and NF-κB signaling (64). More recently, new loci continue to be discovered and have been shown to link psoriasis with IL-17 signaling in human keratinocytes (65, 66) and with a high-risk MHC class I haplotype (67). A study of 23 SNPs located within 19 genes/loci identified as associated with psoriasis uncovered 15 new susceptibility loci, but also confirmed four previously known loci (68). These discoveries lend strength to the theory that a common genetic variation between patients contributes to the risk of developing psoriasis, but the heritability of psoriasis has yet to be explained. Finally, evidence of a genetic component of the disease is supported by the fact that incidence of the disease is greater among relatives of affected persons than in the general population (69, 70).

**Psoriasis as an immune-mediated inflammatory disease: Cellular immune cells mediating psoriasis pathology**

**T-helper subtypes: Th1, Th2, Th17, and Th22**

The Th1/Th2 paradigm proposed by Mosmann and Coffman originally established two canonical subsets of CD4+ helper T cells (71). Th1 cells are classically defined by activation of the transcription factors signal transducer and activator of transcription 1 (STAT1) and T-bet, secretion of IFN-γ, and participation in directed immune responses to pathogens as well as coordination of cell mediated immune response. Th2 cells, conversely, are controlled by the transcription factor STAT6 and Gata-3, produce IL-4, IL-5, and IL-13, and mediate humoral immunity. A third subset, Th17, has been identified and is associated with initiation of autoimmune and
inflammatory conditions (72). The Th17 class of T cells exhibits a unique cytokine and transcription factor profile which is neither Th1- nor Th2-specific (73, 74). Th17 cells are controlled by the transcription factors STAT3 and retinoic acid receptor-related orphan receptor gamma (RORγt); in humans these cells primarily produce IL-17A and IL-17F and are associated with initiation of autoimmune and inflammatory conditions such as rheumatoid arthritis, inflammatory bowel disease, and psoriasis (5, 72, 75). A fourth distinct T helper subset, Th22, has also been recently identified in humans. Th22 cells produce IL-22 but not IL-17, IFN-γ, or IL-4 (7, 76, 77). It should be noted that while these cells are the major producers of IL-22 they are not the singular source, as IL-22 is also secreted at lower levels by activated Th1 and Th17 cells, NK cells, DCs, and CD8 cells (4, 78-80).

Based in large part on the response of psoriasis patients to immune-mediated therapeutics, psoriasis is now commonly defined as a Th1/Th17/Th22-biased inflammatory disease (81-86). Lesional tissue and peripheral blood of psoriasis patients contain Th1 cells that produce high levels of IFN-γ while producing low levels of IL-4 and IL-10 (87-89), Th17 cells producing IL-17 and IL-22, Th22 cells producing IL-22, and cytotoxic epithelial CD8 T cells. A large scale gene expression analysis confirmed the contribution of the Th1 population, demonstrating that IFN-related genes were differentially expressed in lesional psoriatic skin compared to non-lesional and control skin (90). Although the Th1 cell population has been well established to play a role in psoriasis, the identification of Th17 cells and a role for IL-17 has been identified as distinct from the Th1 subset in the psoriatic dermis and further exacerbates disease pathogenesis (82).
The Th17/IL-23 axis is crucial for maintaining the chronic inflammation described in psoriasis (91). IL-23 is a key cytokine involved in autoimmunity that promotes the expansion and maintenance of Th17 cells, providing a link between innate and adaptive immunity in psoriasis (92, 93). Th17 cells express the receptor for IL-23 (5, 94, 95) and overexpression of IL-23 in psoriatic lesions promotes Th17 cell cytokine production, potentiating a cycle of inflammation between IL-23-producing DCs and IL-17-producing Th17 cells present in lesional psoriatic dermis (96, 97). KCs from psoriasis patients and healthy controls express mRNA for both subunits of IL-23 (IL-23p19 and IL-12/IL-23p40) (97). Additionally, IL-23p19 expression in psoriatic skin is increased in DCs and macrophages when compared to control skin using immunohistochemistry (97, 98).

Interest in Th17 cells as mediators of psoriasis pathogenesis began to grow following clinical observations of psoriasis patients with increased numbers of cutaneous Th17 cells in lesional compared to non-lesional skin (82) and circulating Th17 cells (6, 99) as analyzed by flow cytometry (100). Immunohistochemistry has confirmed the increases in protein levels of IL-17F (101) and IL-17A in lesional psoriasis tissue. Serum levels of IL-17 protein have been found to correlate with disease severity and are increased in psoriasis (99, 102). Increases in circulating Th1 and Th22 cells are also observed in psoriasis patients, but to a lesser extent than the Th17 population (6). Importantly, novel anti-IL-17 therapeutics currently under development and clinical trials have been shown to be very effective at rapidly clearing disease in psoriasis patients. As discussed in more detail in Chapter 4, beginning on page 112, two monoclonal antibodies: ixekizumab (a neutralizing antibody directed
against IL-17A) and brodalumab (directed against the IL-17 receptor A), have led to impressive reductions in PASI of patients.

Naïve T cells are found mostly in the blood and lymph nodes, but effector memory T cells persist in the skin in order to provide a rapid response to infection (103). Resident T cells in normal skin express the skin-homing receptors cutaneous lymphocyte antigen (CLA), CCR4, and CCR6 (104). Non-lesional skin from psoriasis patients develops psoriatic lesions in the absence of infiltration of blood lymphocytes (105), highlighting the importance of skin-resident memory T cells in perpetuation of the disease.

**Regulatory T cells**

Although several populations of T regulatory (Treg) cells are described, the most well characterized population in psoriasis patients is the CD4+CD25+ subset. Tregs isolated from normal skin express a skin homing profile as most cells express CCR4, 80% express CLA, and 73% express CCR6 (106). Treg development is controlled by induction of the transcription factor FoxP3 (107, 108). Normally, Tregs play a role in the suppression of autoimmune responses by inhibiting self-reactive T cells (109-111) but in psoriasis a CD4+CD25^{high}CTLA4^{high}FoxP3^{high} subpopulation demonstrates dysfunctional regulation and decreased suppression of T effector cells (Teff) (112). This failure to suppress Teff cells is due in part to a psoriatic tissue microenvironment rich in IL-6, which enhances the Teff resistance to Treg suppression (113, 114), leading to phosphorylation of STAT3 and expansion of the pathogenic T cell population (115).
CD8+ T cells

Although CD4+ T cells make up the majority of T cells in the lesional skin, CD8+ T cells are also found in involved plaque tissue (116, 117). CD8+ cells isolated from psoriatic lesions produce both IL-22 and IL-17 (118). An in vitro analysis of isolated T cells that spontaneously emigrated from cultured dermal skin tissue demonstrated that CD8+ T cells were capable of producing IL-17A (80). This study demonstrated that CD8+ T cells from psoriasis skin contained a significantly higher percentage of IL-17A producing cells when compared to normal skin (3.39 versus 0.35%, respectively), suggesting a possible role for CD8 T cells in contributing to the overall Th17 environment of psoriasis.

Dendritic cells

Dendritic cells in psoriasis are heterogeneous and serve primarily to present antigen and activate naïve T cells. Three main subsets of DCs are classified as Langerhans cells (LCs), resident dermal DCs, and plasmacytoid DCs (pDCs). Langerhans cells (CD14negCD1a+CD207+) are immature DCs, present in the epidermis, and come into close contact with keratinocytes (119). In psoriatic plaques, CD14+ dermal DCs are increased and have the capacity to prime CD4+ T cells while CD14negCD1a+ DCs reside in the dermis and activate CD8 T cells more effectively than the CD14+ DCs (85, 120). Plasmacytoid DCs, which mediate antiviral immunity, are also upregulated in psoriatic plaque and produce IFN-α and IFN-β (121). Another subset of DCs resident in the dermis of the inflamed skin of psoriasis patients has been referred to as “inflammatory DCs.” These are characterized as CD14negCD1c+CD11c+ myeloid-derived DCs which express HLA-DR, CD40, and
CD86, with lesser amounts of DC-LAMP and CD83, and can produce TNF, inducible nitric oxide synthase (iNOS), and IL-23 (122), as reviewed in (123). Finally, an inflammatory population of human blood DCs, 6-Sulfo LacNAc DCs (slanDCs), with a surface phenotype characterized as CD14\textsuperscript{neg}CD1c\textsuperscript{-}CD11c\textsuperscript{+}CD16\textsuperscript{+} and produce high levels of IL-12 and TNF-\(\alpha\) (124). These cells were then identified in the psoriatic plaque and found to release TNF, IL-23, and iNOS, giving them a strong capacity to induce Th1 and Th17 cell responses (125).

**Neutrophils**

Neutrophils in psoriasis are known to localize to the epidermis and stratum corneum (in Munro’s microabscesses) (123). More recently, they have been shown to form neutrophil extracellular traps (NETs), as a form of antimicrobial activity, through a process called NETosis (126). These NETs are composed of fine, sticky chromatin threads that may contain myeloperoxidase or antimicrobial peptides (AMPs) such as cathelicidin and interestingly have been shown to also contain IL-17 when identified in psoriatic lesions (127). The presence of IL-17-producing neutrophils was confirmed in a study of patients undergoing treatment with an anti-IL-17A antibody (secukinumab) (128). These patients demonstrated clearance of cutaneous neutrophils observed at a similar time as when keratinocyte abnormalities were resolved, suggesting that there may be a neutrophil-keratinocyte crosstalk important in the IL-17A/IL-23 pathway in psoriasis.
Monocytes and macrophages

Although the adaptive immune response is likely the major phenotypic driver of psoriasis pathogenesis, the innate immune response is also critical in recruiting and activating T cells at the site of pathology. Although monocytes are not believed to have a major anti-inflammatory role, they are key effectors of the innate immune response.

Three main populations of circulating monocytes have been identified in humans based on differential expression of the cell surface markers CD14 and CD16. The three monocyte subsets are currently defined as “classical” (CD14++CD16−), “intermediate” (CD14++CD16+), and “non-classical” (CD14+CD16++) (129, 130), see Figure 1.2.

CD14 acts as a co-receptor for lipopolysaccharide (LPS) along with the complex of Toll-like receptor (TLR) 4 and MD-2, where MD-2 associates with TLR4 to confer responsiveness to LPS (131). CD14 is expressed in two forms, a membrane-anchored form (mCD14; via a phosphatidylinositol tail) and a soluble form (sCD14) (132, 133). Signaling through this receptor can lead to downstream gene activation of intracellular signaling pathways such as mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinases (ERK) 1 and 2, IkappaB kinase (IKK), p38, and c-Jun N-terminal kinase (JNK), as reviewed in (134). Subsequent downstream activation of transcription factors results in monocyte-release of many pro-inflammatory cytokines.

Two isoforms of CD16 exist – the low-affinity FcγRIIIa and high-affinity FcγRIIIb; FcγRIIIb is a GPI-linked protein, coupled to the outer leaflet of the plasma
membrane, and predominantly expressed on neutrophils (135), while FcγRIIIa exists as a transmembrane receptor expressed on monocytes and natural killer cells (136, 137). Aside from its function as an Fc receptor, little is known about: 1) the expression of CD16 in psoriatic monocytes; 2) the additional functions that CD16 confers on monocytes, and; 3) the regulation of CD16 in the psoriatic monocytes. Neither CD14 nor CD16 appear to play a functional role in monocyte subset differentiation, but it could be possible that these surface markers are simply upregulated or downregulated in response to ligands that signal through receptors such as TLR4 or integrins, therefore, further research is needed in this area.

In healthy individuals, the classical CD14++CD16- subset represents the main population of circulating monocytes while the intermediate and non-classical populations account for only approximately 5-10% of the total (130, 138). Following T cell infiltration into the plaque, interaction between monocytes and specific CD4+ T helper cells results in differentiation of monocytes to specific DC subsets that have distinct phenotypic functions (139). Each monocyte subset has been suggested to execute a different function and each could be hypothesized to give rise to a different subset of DCs.

Classical CD14++CD16- cells express genes related to phagocytosis and in functional analysis have the highest phagocytic capacity of all 3 subsets (130). The intermediate CD14++CD16+ subset was found to have the highest expression of the surface markers Tie2, CXCR4, CD163, CD115, receptors to ICAM-1, VEGF, and the highest surface levels of apolipoprotein B and ferritin when compared to the classical CD14++CD16- and non-classical CD14+CD16++ populations (140). The intermediate
CD14^{++}CD16^{+} population has a unique genomic expression pattern, differentiating it from the other two subsets and linking it to antigen processing, inflammation, monocyte activation, and angiogenesis (141). The non-classical CD14^{+}CD16^{++} subset patrols the vasculature, respond to viruses and nucleic acids via TLR7 and TLR8, and responds poorly to TLR1, TLR2, and TLR4 agonists (142). The recognition of three monocyte subsets has only been defined within the past decade (129). Consequently, previous studies lumped the two CD16^{+} populations (intermediate and non-classical) together, and as a result, the intermediate CD14^{+}CD16^{++} subset is not well characterized as a distinct population. Importantly, both the classical (143) and intermediate subsets (144-146) have been implicated in cardiovascular disease events (this is discussed in more depth in Chapter 2, pages 44-45 and 79-80). Intermediate monocytes have also been linked to conditions such as myocardial infarction (145), peripheral artery disease (147), rheumatoid arthritis (148), acute liver failure (149), and kidney disease (150). Thus far, neither monocyte subset has been extensively examined in psoriasis.

Macrophage differentiation was originally proposed to occur as a response to the local tissue environment, resulting in either M1 or M2 macrophages that are “classically activated” through classical Th1 signaling pathways or “alternatively activated” though alternative Th2 signaling pathways, respectively (151), but it has been demonstrated that the functional phenotype of macrophages may repolarize based on local tissue cues (152) and/or Th1 or Th2 cytokines (153). Classically activated M1 macrophages are involved in defending the host from bacterial and viral infections while alternatively activated M2 macrophages play more of a role in general
anti-inflammatory functions (as reviewed in (154)). A third set of macrophages termed "wound healing macrophages" were more recently classified and are responsible for supporting tissue repair (as reviewed in (155)). Currently, the pathways which lead to differentiation of these subtypes of macrophages in the skin are not well elucidated.

Psoriasis lesions contain macrophages localized to the dermal compartment and along rete ridges in proximity to proliferating keratinocytes (156). CD163 is a scavenger receptor that marks normal, mature macrophages (157, 158). An increased number of CD163+ macrophages that produced pro-inflammatory cytokines have been reported in psoriasis skin (159). These macrophages produced IL-23p19 and IL-12/23p40 as well as TNF and iNOS, indicating that these cells represent a pro-inflammatory "classically activated" population present in the psoriatic plaque (159). While the function of monocytes and macrophages in psoriasis is not yet well defined in humans, the inflammatory psoriatic milieu may provide signals for the differentiation of these cells in tissue. Interestingly, recent studies have identified CD14+-macrophages in human thrombotic carotid lesions, indicating a potential link between the increase in these cells in psoriasis and a cardiovascular co-morbidity (160).
Figure 1.2: Subsets of circulating human monocytes

(A) Three subsets of human monocytes differentiate from a single myeloid progenitor. Classical monocytes are defined as being high in CD14 expression but having little to no CD16 expression (CD14++CD16neg). (B) Representative flow cytometry dot plot from a healthy control; CD14 expression is on the x-axis and CD16 expression is on the y-axis. The three monocyte populations are distinct yet are part of a continuum of expression. Gates are determined by isotypes. For a more detailed gating strategy, see Figure 2.1.
**Keratinocytes**

Acceleration of basal keratinocyte hypoproliferation and a lack of engagement in a terminal differentiation pathway results in the epidermal hyperplasia and additional clinical features observed in psoriasis such as parakeratosis, retention of nuclei in the KCs, and thickening of the plaques (10, 161). Keratinocytes in psoriasis skin are major producers of cytokines, chemokines, and growth factors (162) as well as mediators of innate immune response molecules such as S100 proteins or human beta defensins (hBDs) (163-165). Cellular products (cytokines and chemokines) released from keratinocytes influence immune cell activation and interact (sometimes synergistically) with pro-inflammatory mediators secreted from activated immune cells influencing keratinocyte proliferation and contributing to a constant cycle of inflammation. One example of this positive feedback loop is overexpression of the pro-inflammatory, small calcium-binding proteins S100A7, S100A8, and S100A9 in the psoriatic epidermis (163, 164, 166) and serum of psoriasis patients (163). Psoriatic epidermal keratinocytes upregulate secretion of S100A8 and S100A9 which in turn activate adjacent keratinocytes to produce pro-inflammatory cytokines such as IL-6 and TNF-α (165).

**Treatment of psoriasis**

Historically, psoriasis was postulated to occur due to an inherent dysfunction of keratinocytes resulting in aberrant proliferation. However, the clinical observation that cyclosporine A (CsA), an immunosuppressant, was found to be highly effective at treating psoriasis led to the revised hypothesis that some immune mediated response must also be necessary for the observed pathogenesis (3). Based upon these initial
observations and subsequent years of study, the focus for psoriasis mediated
keratinocyte hyperproliferation has shifted toward immune-mediated mechanisms and
subsequent therapeutics have targeted immune cells and their soluble mediators
present in psoriatic plaques.

Additional support for the importance of immune cells as pathogenic mediators
in psoriasis is derived from the efficacy of so called “biologic” agents used in the
treatment of psoriasis including therapies which target T cells/T cell activation directly,
and those which target pro-inflammatory cytokine pathways present in lesional
psoriasis skin. Methotrexate, a pan-immunosuppressant, has been used since the
1960s to successfully treat psoriasis (167). T cell interventions, initially using
cyclosporine A (3) and then FK506, DAB-IL-2, anti-CD4, CTLA4-Ig, and anti-CD3 as
systemic therapy, proved that T cell activation is critical for sustaining a psoriatic
lesions (reviewed in (122, 168-172)). Proof that dermal memory effector T cells (CD2+
CD45RO+, Tmem/eff) are pathogenic comes from the specificity of biological therapies
for psoriasis such as the initial FDA-approved biological therapy for psoriasis LFA3-Ig
(alefacept), which binds CD2^{high} activated Tmem/eff cells and selectively depletes
these cells (173).

Clinical improvement is also accompanied by a reduction in T cells and Th17-
related mediators following treatment by several biological therapies including TNF
inhibitors (e.g., infliximab, etanercept, adalimumab) (102, 174) and the IL-12/IL-23p40
inhibitor ustekinumab (175) which indirectly inhibit T cell activation/differentiation and
maintenance. TNF-α inhibitors produce psoriasis remission by blocking the cytokine
itself or its receptor and preventing the activation and expansion of T cells by DCs
and/or monocytes/macrophages, leading to a decrease in overall inflammation and allowing the lesion time for repair (176, 177). Three inhibitors, infliximab, adalimumab, and etanercept, specifically block the role of the cytokine itself. Ustekinumab, targeted toward the cytokines IL-12 and IL-23, appears to result in potentially more durable remission (178, 179), perhaps reflecting a direct effect on reducing the circulating numbers of DCs and decreases in the DC- and macrophage-derived IL-23p19 and IL-12p40 levels in psoriasis lesions (159, 169, 180, 181). This in turn leads not only to the marked decrease in IFN-γ production (IL-12 effect), but also to a decrease in Th17 activation (IL-23 effect) (182) and subsequent release of IL-17 and IL-22 from these cells. Recently reported anti-IL-17 therapeutics (e.g. ixekizumab, broadalumab) (183, 184) directly target IL-17 and IL-17 receptors have been successful (as introduced previously in this Chapter on pages 25-26 and further explained in Chapter 4).

**Murine models recapitulate aspects of human psoriasis**

Although no single murine model accurately exemplifies all the clinical components of human psoriasis, current models exhibit similar phenotypes to human disease (comprehensively reviewed in (185)). These include the K14-ARGE mouse that demonstrates increased inflammatory cell infiltration of the dermis with concomitant increased epidermal cell proliferation (186) and the K5.Stat3C mouse that has increased numbers of dermal blood vessels (187). Another recent mouse model that has been used to acutely induce psoriasis is the imiquimod (IMQ) model where psoriasis-like symptoms, such as increased epidermal proliferation, neutrophil accumulation in microabcesses, and increased immune cell infiltrates, are observed after topical application of the IMQ cream (188). Whole-genome microarray analysis
comparing the global transcriptional signatures of lesional skin of murine models to human psoriasis skin revealed that murine models expressed a change in the gene signature of epidermal development and keratinization similar to what is observed in human psoriasis (189). However, while all of the above models demonstrate phenotypic characteristics of psoriasis, the only model shown so far to recapitulate cardiovascular complications seen in psoriasis patients is the KC-Tie2 mouse.

The KC-Tie2 mouse, a murine model of psoriasis developed in the laboratory of Dr. Nicole Ward (190), is driven by ectopic Tie2 (tyrosine kinase with immunoglobulin and EGF homology domains) gene expression introduced and confined to KCs. Tie-2 is a tyrosine kinase receptor essential for vascular maturation predominately expressed on endothelial cells (ECs) which regulates blood vessel stabilization and angiogenesis (as reviewed in (191)). The main ligands of Tie-2 are angiopoietins, a family of secreted glycoproteins that include 4 members (Ang1, -2, -3, and -4) which act on the vasculature and control blood vessel development (192).

This model exhibits a psoriasiform phenotype similar to human disease which is characterized by skin lesions with areas of uninvolved and involved skin, erythema, extensive acanthosis, and increased expression of cytokines and chemokines associated with psoriasis (190). On a cellular level, increases in dermal immune cell infiltrate including T cells skewed towards the Th1/Th17 profile, increases in DCs and macrophages and their derived cytokines, in particular IL-6, IL-22, IL-12/23, IL-17A, IFN-γ, and TNF-α, and upregulation of STAT3 and phospho-STAT3 have been reported (190). These mice also exhibit increases in biomarkers associated with cardiovascular risk such as serum TNF-α, IL-17A, vascular endothelial growth factor
(VEGF), IL-12, monocyte chemotactic protein-1 (MCP-1), and an increase in the inflammatory markers S100A8/A9 in both serum and skin, yet the mice do not demonstrate increased levels of total cholesterol, triglycerides, or low-density lipoprotein (190). STAT3 phosphorylation is upregulated in the epidermis when compared to control animals (190), providing evidence for another common mechanism between human disease pathology and this murine system, as STAT3 phosphorylation has previously been shown to be critical in human disease (113, 115, 187).

Interestingly, recent work using the KC-Tie2 mouse demonstrates that long-term skin-specific inflammation may in fact have the capacity to cause systemic levels of inflammation sufficient to drive atherogenesis (193). Spontaneous aortic vascular root lesions developed by one year of age in 33% of these mice. Furthermore, they were pro-thrombotic as demonstrated by arterial thrombosis clotting times being significantly shortened compared to controls (193). Of particular relevance in treating psoriasis patients, skin disease regressed following repression of the transgene with doxycycline. This also lead to elimination of the vascular inflammation and the time to thrombus formation returned to control levels, suggesting that pro-thrombotic events are a result of the skin-specific disease. This suggests that chronic sustained skin inflammation may be sufficient to elicit levels of systemic inflammation sufficient to induce atherothrombosis, indicating that aggressive treatment of inflammatory skin disease may prevent cardiovascular disease co-morbidities.
Murine monocyte populations

In mice, at least two subsets of circulating blood monocytes exist and are defined by surface expression of Ly6C (Ly6C\text{high}CD11b^+CCR2^+Gr1^+ and Ly6C\text{low}CD11b^+CCR2^-Gr1^-) (194). The pro-inflammatory Ly6C\text{high} population migrates to infected tissues in a CCR2-dependent manner (195) and participates in chronic disease while the Ly6C\text{low} population patrols resting blood vessels (196) and contributes to tissue repair (197).

Unlike human monocytes which can be classified distinctly based on CD14 and CD16 expression, only the non-classical murine population can be distinctly identified based on surface expression (Ly6C\text{low}) as both the classical and intermediate murine populations express high levels of Ly6C on their surfaces. Three subsets were proposed based on expression of the surface marker CD43, where the Ly6C\text{high} population is divided into two subsets based on CD43: Ly6C\text{high}CD43^+ (classical) and Ly6C\text{high}CD43^{++} (intermediate), with the remaining population defined as Ly6C\text{low}CD43^{++} (non-classical) (129), but this classification is not currently widely applied. This lack of a definitive correlation between human and mouse classical and intermediate subsets is acknowledged in the literature (129).

The CD11b^+Ly6C\text{high} subset has been likened to the intermediate CD14^{++}CD16^- population in humans based on the pro-inflammatory function common to both populations (129), and a prominent role for these cells has been identified in murine atherosclerosis. It must be noted, though, that it is also possible that the CD11b^+Ly6C\text{high} population resembles the human classical CD14^{++}CD16^- population.
as it is also pro-inflammatory. This area needs further research in order to definitively characterize the murine Ly6C\text{high} population(s) and function(s).

**Objectives and Significance**

Psoriasis is an immune-mediated inflammatory disease accompanied by comorbidities including hypertension, obesity, metabolic syndrome, and cardiovascular disease (CVD) (40, 42-44, 53, 54). The psoriatic tissue milieu contains activated inflammatory cells and pro-inflammatory cytokines including monocytes, macrophages, T cells, and DCs along with Th1, Th17, and Th22-derived cytokines including IFN-\(\gamma\), IL-17A, IL-17F, IL-17C, IL-22, and IL-6 (113). Recent studies have indicated that elevated levels of the pro-inflammatory, intermediate monocyte subset independently predict adverse outcomes in patients with high cardiovascular risk (144). Therefore, increases in the intermediate monocyte subset observed in psoriasis patients suggests a potential link between psoriasis disease pathology and associated CVD co-morbidities (see Figure 1.3).

A mechanism has not yet been elucidated linking psoriasis pathogenesis and onset of CVD, but a recent GWAS study reported that psoriatic individuals have common genetic variants that predispose them to increased risk of dyslipidemia, hypertension, and CAD, revealing an association between cardiovascular and metabolic disease genes with psoriasis (198). Vascular inflammation and atherosclerosis share common pathogenic mediators with lesional psoriasis, such as similar inflammatory cytokines and pro-inflammatory cell infiltration including T cells, monocytes/macrophages, and neutrophils. Additionally, a recent study of 951 patients at risk for CVD found increases in cell counts of monocytes of the intermediate
CD14^{++}/CD16^{+} population independently predicted incidence of cardiovascular events in these individuals (199). Therefore, the hypothesis that aggressive treatment of the cutaneous disease (psoriasis) may decrease the risk of developing other co-morbidities, such as CVD, is currently an area of intense investigation (200-203).

As a long-term study of a large cohort of psoriasis patients developing CVD is not feasible, therefore we performed iterative studies to validate associative data observed in humans with a mechanistic model in mice. The KC-Tie2 murine model of psoriasis has been previously shown to demonstrate increased levels of proinflammatory cytokines and cell types consistent with human psoriasis (190, 193). These mice also have been shown to be pro-thrombotic and develop aortic root vascular lesions (193). We hypothesized that the observed increase in CD11b^{+}/Ly6C^{high} cells mediate skin pathology and development of cutaneous lesions and therefore propose mechanistic studies using the murine model to establish a more causal rather than correlative link between Ly6C^{high} cells and skin inflammation.

The results from our study provide evidence that the chronic pro-inflammatory psoriatic milieu of the skin elicits an increase in circulating pro-inflammatory monocytes. Common mechanisms and/or cellular mediators may be responsible for the chronic inflammation observed in both psoriatic and cardiovascular disease. This induced skewing of monocytes via chronic skin-derived inflammation could provide a mechanism that lends further insight into maintenance of the psoriatic plaque as well as associated cardiovascular comorbidities. Intervention of skin disease by targeting proinflammatory monocytes may have the added benefit of reducing associated comorbidities.
Figure 1.3: Overall hypothesis

Known associations exist between psoriasis and cardiovascular disease, and cardiovascular diseases and increases in intermediate and classical monocytes. We hypothesize that there is potentially a common cellular mediator that may participate in and/or result from the chronic inflammation characteristic of both diseases.
CHAPTER 2

CHRONIC PSORIATIC SKIN INFLAMMATION LEADS TO INCREASED MONOCYTE ADHESION AND AGGREGATION

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Chronic Psoriatic Skin Inflammation Leads to Increased Monocyte Adhesion and Aggregation
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Introduction

Psoriasis is a chronic inflammatory disease of the skin affecting 2-3% of the US population in which expression is modified by susceptibility genes and environmental triggers (204). The pathogenesis of psoriatic tissue hyperplasia is thought to be driven by an interplay of macrophages, dendritic cells, and pathogenic and resident memory T cells, with enhanced representation of the IL-23-Th17/Th22 and IL-12-IFN-γ/TNF pathways (10, 161). In addition to an enormous negative impact on quality of life, psoriasis patients exhibit numerous co-morbidities including destructive psoriatic arthritis, stigmatization, depression and anxiety, inflammatory bowel disease, lymphoma, obesity, metabolic syndrome-associated conditions, and notably, increased risk of early death from cardiovascular disease (CVD) (42, 44, 53, 54, 205, 206). A mechanism has not yet been elucidated linking psoriasis pathogenesis and onset of CVD, but recent genome-wide association studies (GWAS) found psoriatic individuals have common genetic variants that predispose them to increased risk of dyslipidemia, hypertension, and coronary artery disease (CAD), revealing an association of cardiovascular and metabolic disease genes with psoriasis (198).

Efforts to identify circulating inflammatory transducers of CVD revealed that increases in circulating intermediate monocyte subpopulations are associated with CVD (144), acute ischemic heart failure (146), myocardial infarction (145), peripheral artery disease (147), and acute coronary syndrome associated with HIV (207). Within human peripheral blood, three distinct monocyte populations have been identified and genotyped: classical monocytes (CD14^{++}CD16^{neg}), intermediate monocytes (CD14^{++}CD16^{+}), and non-classical monocytes (CD14^{+}CD16^{++}) (129, 130, 208-212).
Psoriasis patients, who also exhibit an increased risk of death by CVD, have been reported to have elevated levels of circulating CD16$^+$ cells which contain the intermediate monocyte population (213, 214). Induction of CD16 on the intermediate monocyte population can occur as a result of platelet interaction (215), which also increases monocyte adhesion to vascular endothelium and subsequent trans-endothelial migration (216). Indeed, circulating monocyte/platelet aggregates (MPAs) are considered a robust marker of platelet activation and indicator of coronary artery disease (CAD) (217), ST segment elevation myocardial infarction (STEMI) (145), and acute myocardial infarction (218), as reviewed in (219).

In murine CVD models, a proinflammatory monocyte subset (CD11b$^+$Ly6C$^{high}$) infiltrates murine atherosclerotic plaques and promotes atherogenesis (220) and also plays a role in myocardial infarction (221). Interestingly, in the skin-specific KC-Tie2 murine model of psoriasis, elevated levels of circulating CD11b$^+$Ly6C$^{high}$ cells are observed and precede the spontaneous formation of aortic root lesions. Moreover, these mice also develop a pro-thrombotic clotting phenotype (193) consistent with the idea that skin-contained chronic inflammation may have the capacity to promote atherothrombosis.

In this report, we demonstrate that psoriasis patients have both a relative and absolute increase in circulating monocyte aggregates as well as an increase in intermediate monocytes, correlating with an increase in disease severity assessed by psoriasis area severity index (PASI), compared to healthy controls. Interestingly, control intermediate monocytes demonstrate increased adhesiveness to Human Dermal Microvascular Endothelial Cells (HMVEC-D) following endothelial cell
stimulation with proinflammatory cytokines known to be increased in psoriasis skin (TNF-α and IL-17A). Circulating monocyte-monocyte aggregates are also present in the KC-Tie2 murine psoriasiform model. We also show that monocyte aggregation in humans is associated with a distinct transcriptional profile and can occur in the presence or absence of platelets. Taken together, this data suggests a novel role for monocyte adhesion and subsequent aggregation as a potential link between the pathogenesis of psoriasis and CVD.

Materials and Methods

**Human subjects**: All studies of human subjects were approved by the Institutional Review Board of University Hospitals Case Medical Center (Cleveland, OH). Peripheral blood samples and/or punch biopsies were obtained from volunteer healthy controls and psoriasis patients following informed consent. Psoriasis patients were not on any systemic psoriasis medications and those patients using any topical therapeutics discontinued use for at least two weeks prior to entering the study. For patient demographics, see **Table 2.1**.
Table 2.1: Patient demographics

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Age (mean ± STD)</th>
<th>Gender</th>
<th>PASI (mean ± STD)</th>
<th>Duration of Disease (mean years ± STD)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Original Cohort</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>23</td>
<td>29.8 ± 7.6</td>
<td>10 male (43.5%)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>13 female (56.5%)</td>
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<td>Psoriasis</td>
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<td>43.4 ± 12.3</td>
<td>11 male (57.9%)</td>
<td>11.7 ± 12.9</td>
<td>19.8 ± 11.2</td>
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<td></td>
<td></td>
<td></td>
<td>8 female (42.1%)</td>
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<td><strong>PCR Array Cohort</strong></td>
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<td></td>
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<tr>
<td>Control</td>
<td>5</td>
<td>39.6 ± 9.5</td>
<td>3 Male (60%)</td>
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<td>N/A</td>
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<td></td>
<td></td>
<td></td>
<td>2 Female (40%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Psoriasis</td>
<td>5</td>
<td>42.2 ± 11.4</td>
<td>3 Male (60%)</td>
<td>6.3 ± 5.9</td>
<td>10.0 ± 7.2</td>
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<td></td>
<td></td>
<td></td>
<td>2 Female (40%)</td>
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<td><strong>HMVEC-D co-culture Cohort</strong></td>
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<tr>
<td>Control</td>
<td>4</td>
<td>32.8 ± 9.3</td>
<td>1 male (25%)</td>
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<td></td>
<td></td>
<td></td>
<td>3 female (75%)</td>
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</table>

*Note: One of the psoriasis patients from the PCR cohort (first on the heatmap) was on Soriatine at the time of the blood draw for a non-psoriasis condition
**Cell culture:** PBMCs were isolated from 23 controls and 19 psoriasis patients using Ficoll-Paque centrifugation, washed, and red blood cells were lysed using ACK (Invitrogen, Carlsbad, CA) and then immediately stained for surface markers. Experiments were performed using total PBMCs and electronically gated for monocyte subpopulations. For adhesion studies, PBMCs were plated on tissue culture-treated 6 well dishes for 30 minutes, 1 hour, 4 hours, or overnight in RPMI media supplemented with FBS and P/S in 5% CO₂ at 37°C.

**Flow Cytometry:** Flow cytometric data collection was performed using a BD FACS-Aria instrument and analyzed using FlowJo software (Tree Star, Ashland, OR). Monocytes were initially gated using a FSC-A vs SSC-A discrimination plot and then analyzed for specific CD14 versus CD16 staining (see Figure 2.1 for gating strategy and Table 2.2 for antibody clone and source). Doublet analysis was performed by gating FSC-W versus FSC-H on total events. For experiments that required sorted monocytes, samples were sorted using an 85μm tip at a speed of 1.0 and pressure of 45psi. Surface adhesion analysis of CD11b, CD11c, CD18, VLA-4, VCAM-1, and ICAM-1 was performed on gated classical monocyte populations.
Figure 2.1: Monocyte and doublet gating strategy

(A) All PBMC events are gated on FSC-W vs. FSC-H to separate singlets from doublets. 
(B) Monocytes are electronically selected based on their SSC-A vs. FSC-A profile. 
(C) The left side of the classical population gate is determined based on CD14 isotype and the 
upper limit is determined by CD16 isotype. The intermediate population gate is also 
determined by CD14 isotype combined with all events above the CD16 isotype. The non-
classical population is determined by the CD14 isotype for the right side of the gate and 
negative events for the left side of the gate. 
(D) Representative images obtained on the Amnis Imagestream cytometer of cells in the doublet gate post-sort demonstrating the 
doublet population used in subsequent transcriptomic analyses. Dotted line in panel A 
indicates doublet gate used in sort.
## Table 2.2: Antibodies used in Flow Cytometry and Immunofluorescence

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<th>Company</th>
<th>Clone</th>
<th>Cat #</th>
<th>Application</th>
<th>Corresponding Isotype</th>
<th>Company</th>
<th>Cat #</th>
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<td>PerCP</td>
<td>BD</td>
<td>SP34.2</td>
<td>552851</td>
<td>Flow Cytometry</td>
<td>Used in Annexin analysis, no isotype</td>
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<td>APC-Cy7 Ms IgG1k</td>
<td>BD</td>
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<td>BV510</td>
<td>BD</td>
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<td>BD</td>
<td>M5E2</td>
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<td>Alexa 488 Ms IgG2ak</td>
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<td>eBio</td>
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<td>MG38</td>
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<td>Flow Cytometry</td>
<td>BV510 Ms IgG1k</td>
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Whole Blood Method: 50μl of whole blood from 4 healthy controls and 4 psoriasis patients was stained with mouse anti-human CD14 and CD16 for 15 minutes at RT. The blood was then lysed at RT with 450μl of 1X FACS Lysing Solution (BD, Franklin Lakes, NJ) for 12 minutes and analyzed immediately by flow cytometry.

Monocyte and HMVEC-D co-culture experiments: Monocytes were negatively selected from PBMCs using the Pan-Monocyte kit (Miltenyi, Cologne, Germany). HMVEC-D cells (Lonza, Basel, Switzerland) were cultured in complete media (Lonza) at 37°C, 5% CO₂. HMVEC-D cells were stimulated for 4 hours in serum free media at 37°C, 5% CO₂ with TNF-α (1ng/ml; R&D, Minneapolis, MN) and IL-17A (100ng/ml; R&D) to mimic a psoriatic and cardiovascular cytokine profile, as published in (222). The cells then rested for 30 minutes before addition of monocytes. HMVEC-D cells were used at P2-P3 and 90-100% confluency in a 6 well dish. 4-5 million monocytes were plated per well and adhered for 1 hour before harvesting the adherent and supernatant fractions and staining for CD14 and CD16 surface markers.

Imaging Flow Cytometry: Heterogeneous doublet population data was collected using an Amnis Imagestream cytometer and analyzed using IDEAS software v6.0. PBMCs were stained for surface expression of CD14, CD16, CD42b, CD3, and CD56 (for antibodies, see Table 2.2). Events were first selected on focused events. Next, to gate singlets versus doublets, area vs. aspect ratio of the brightfield channel was plotted. Doublets were defined as having an aspect ratio less than 0.6 with an area of 100-400, while singlets had aspect ratios above 0.6 and an area of 75-200. The singlets and doublets were then separately gated into CD14 APC vs. CD16 FITC for three monocyte subset analysis. From the CD14 vs. CD16 scatter plot of doublets,
each subset was plotted again as area vs. aspect ratio of each respective channel. This allowed us to separate out homogeneous versus heterogeneous cell pairs. For example, a doublet that contained two CD14\(^+\) cells would have a larger area on the CD14 channel than a doublet that contained one CD14\(^+\) cell and one CD14\(^\text{neg}\) cell (such as a T cell).

**Gene Expression Array:** RNA was extracted from flow-sorted CD14\(^++\)CD16\(^\text{neg}\) singlet cells and CD14\(^++\)doublet cells using the QIAGEN RNeasy Minikit in combination with the QIAGEN QIAshredder kit per the manufacturer's protocol from psoriasis patients (n=5) and controls (n=5). A portion of the CD14\(^++\)CD16\(^\text{neg}\) singlets were adhered for 4 hours on tissue culture plastic before RNA extraction. cDNA was made using the RT\(^2\) First Strand Kit (SA Biosciences, Valencia, CA). cDNA was combined with RT\(^2\) SYBR Green Mastermix and dispensed into a PCR Array for Human Extracellular Matrix & Adhesion Molecules (Qiagen) with a 384 (4 x 96) E, G format. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Golden et al., 2015) and are accessible through GEO Series accession number GSE70327 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE70327). A heat map was generated using R: A Language and Environment for Statistical Computing (Vienna, Austria) with fold changes calculated from ratios using the following equation: 

\[ \text{fold change} = \text{IF}(\text{value}>1,\text{value},(1/\text{value})^{-1}) \]

Fold change values of greater than 20 were changed to 20, and fold change values of less than -20 were changed to -20 to normalize the range to the majority of the values and avoid an unclear gradient within the heat map. The psoriasis top doublet network, representing the leukocyte extravasation pathway,
was generated through the use of QIAGEN’s Ingenuity Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity) with an average of all control CD14+ doublets as the comparator.

**Immunofluorescence:** Frozen 4mm punch biopsies from involved psoriasis plaque of human volunteers were sectioned into 8μm slices and four serial sections were mounted on each slide to provide proper controls for staining. Tissue sections were fixed in acetone for 10 minutes, washed in PBS buffer (HyClone, Thermo Scientific, Waltham, MA), rehydrated using antibody diluent solution (MP, Santa Ana, CA), and blocked in 20% secondary isotype specific serum (R&D) for 30 minutes. Primary antibodies were incubated either overnight at 4°C or for 1h at room temperature dependent on the target and amplified using an isotype-specific corresponding secondary antibody for 1h at room temperature (see Table 2.2). Nuclei were stained using one drop (approximately 15μL) of Prolong Gold anti-fade reagent with DAPI (Molecular Probe, Life Technologies, Eugene, OR).

**Fluorescent image acquisition and analysis:** All images were acquired using the UltraVIEW VoX™ spinning disk confocal system (PerkinElmer, Waltham, MA) which is mounted on a Leica DMI6000B microscope (Leica Microsystems, Inc., Bannockburn, IL) at 20x magnification. Confocal images were collected using solid state diode lasers, with 640-nm, 488-nm, 561-nm and 405-nm excitation light, respectively, and with appropriate emission filters (see Table 2.2 for antibodies). All confocal images were analyzed using Volocity™ (PerkinElmer, Waltham, MA), MetaMorph™ Premier software (Molecular Devices Corporation, Sunnyvale, CA), and
SigmaPlot™ (Systat Software, Inc., San Jose, CA). The white line designates the dermal-epidermal junction of skin sections.

**Mouse studies:** The KC-Tie2 binary, tet-repressible psoriasiform mouse model, its genetic engineering, and the characterization of its skin and vascular phenotypes have been described at length previously (190, 193, 223). Mice spontaneously develop a chronic inflammatory skin phenotype following transgenic introduction of the angiopoietin receptor, Tie2, into keratinocytes (using the keratin 5 promoter). The skin inflammation phenocopies human psoriasis (190), is characterized by a robust Th1/Th17 skewed immune response, and is responsive to antibodies targeting TNF-α or antigen cell depletion (223). Mice spontaneously develop systemic inflammation, elevated circulating CD11b^+^Ly6C^high^ moncytosis, aortic root vascular inflammation, and are pro-thrombotic; these are reversed following targeted inhibition of the skin inflammation (193). Psoriasis-like inflammation was observed in all animals at the time of experiments.

Spleens from male and female, adult, age-matched KC-Tie2 transgenic mice (1 year old mice on a CD1 outbred background, n=10) and littermate controls (1 year old, n=5) were removed and homogenized in serum-free media containing 50μg/ml DNase I (Sigma, St. Louis, MO) and 2mg/ml collagenase D (Roche, Basil). Red blood cells were lysed using ACK and then cells were pelleted, resuspended, and filtered 2x through a 70μm filter in wash buffer containing 5% FBS. The cells were immediately stained for the cell surface markers Ly6C-Alexa Fluor 700 (eBiosciences, San Diego,
CA) and CD11b-eFlour450 (BD), using 7AAD to determine live cells. Cells were gated as previously published (193).

All animal protocols were approved by the Case Western Reserve University Institutional Animal Care and Use Committee and conformed to the American Association for Accreditation of Laboratory Animal Care guidelines.

**Data Analysis:** Normality of distributions was tested using the Kolmogorov-Smirnov statistic, and upon non-rejection of this assumption, t-tests for independent samples were used to compare mean values. Equality of variances was tested in order to select the appropriate resultant p-values. The Mann-Whitney test was used when the assumption of normality was not met. Correlations were estimated using Pearson correlation coefficients. Results are expressed as mean ± standard error. Data analysis was done using SPSS v21 and graphs were generated using GraphPad Prism 6.

**Results**

*Psoriasis patients have a higher percentage of circulating CD14^{++}CD16^{+} intermediate monocytes that correlates with disease severity*

Peripheral blood mononuclear cell (PBMC) preparations were obtained from either psoriasis patients or healthy controls and analyzed for the percentage of circulating monocyte subsets (classical (CD14^{++}CD16^{neg}), intermediate (CD14^{++}CD16^{+}), or non-classical (CD14^{+}CD16^{++})). Higher percentages of circulating intermediate monocytes were observed among psoriasis patients compared to control (16.5% ± 2.7 vs. 11.9 ± 1.4, n=19, n=23, respectively; p=0.056) (**Figure 2.2A**).
Classical and non-classical subsets showed no significant differences between psoriasis patients and healthy controls (65.5% ± 3.2 vs. 70.2% ± 1.7, CD14++CD16<sup>neg</sup>, and 4.1% ± 0.59 vs. 3.8% ± 0.42, CD14<sup>+</sup>CD16<sup>++</sup>; Figure 2.2B). Representative individual scatter plots are shown in Figure 2.2C. Interestingly, the percentage of circulating classical and intermediate cells correlate with psoriasis disease severity (measured by the Psoriasis Area Severity Index (PASI)) as shown in Figure 2.2D. While the classical subset negatively correlates with PASI (r= -0.541, p=0.017), the intermediate subset positively correlates with PASI (r=0.638, p=0.003), and non-classical monocytes do not demonstrate a significant correlation (r= -0.082, p=0.738, data not shown). When absolute numbers of cells are calculated, psoriasis patients have increased numbers of total monocytes and doublets compared to healthy control individuals (Table 2.3).
Table 2.3: Absolute cell count\textsuperscript{a}

<table>
<thead>
<tr>
<th></th>
<th>Doublets\textsuperscript{b}/ PBMC</th>
<th>Monocytes\textsuperscript{c}/ Singlet PBMC</th>
<th>Classical\textsuperscript{d} Monocytes</th>
<th>Intermediate\textsuperscript{e} Monocytes</th>
<th>Non-classical\textsuperscript{f} Monocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.83 ± 0.12</td>
<td>18.8 ± 1.93</td>
<td>12.9 ± 1.38</td>
<td>2.46 ± 0.40</td>
<td>0.69 ± 0.10</td>
</tr>
<tr>
<td>Psoriasis</td>
<td>2.53 ± 0.82</td>
<td>32.3 ± 15.9</td>
<td>21.6 ± 11.2</td>
<td>4.89 ± 2.06</td>
<td>1.11 ± 0.45</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Determined by percentage of monocytes/total PBMCs.

\textsuperscript{b} Doublets defined based on a FSC-W vs. FSC-H analysis of all events.

\textsuperscript{c} Monocytes defined on a FSC-A vs. SSC-A analysis from the singlet events.

\textsuperscript{d} Defined as CD14\textsuperscript{**}CD16\textsuperscript{neg} from the monocyte and singlet gates.

\textsuperscript{e} Defined as CD14\textsuperscript{**}CD16\textsuperscript{*} from the monocyte and singlet gates.

\textsuperscript{f} Defined as CD14\textsuperscript{*}CD16\textsuperscript{**} from the monocyte and singlet gates.
Figure 2.2: CD14**CD16* (intermediate) cells are increased in psoriasis patients compared to healthy controls.

(A) The percentage of intermediate monocytes (CD14**CD16*) is increased in psoriasis patients (n=19, open squares) when compared to healthy controls (n=23, solid squares; p=.056) while (B) the percentage of classical (circles) and non-classical monocytes (triangles) do not differ between psoriasis patients and healthy controls. (C) Representative flow plots showing gates and population distribution. (D) Classical monocytes (red, open circles) negatively correlate with disease severity measured by PASI (r=-0.541, p=0.017), while intermediate monocytes (blue, open square) positively correlate with disease severity (r=0.638, p=0.003).
Monocyte doublets are increased in psoriasis patients compared to controls

We noted that a prominent population of cells was expressed within the intermediate CD14\(^{++}\)CD16\(^{+}\) gate in the scatter plot of psoriatic PBMCs (Figure 2.3A). Back-gating this population revealed that these cells were larger on FSC-A vs. SSC-A scatter plots and mapped to a region expected to contain doublets (Figure 2.3B). Indeed, cell width vs. height analysis on forward scatter of total events confirmed the accumulation of doublets (Figure 2.3C, top row). Doublet discrimination analysis demonstrated that psoriasis patients have a > 2.5 fold increase in total PBMC doublets compared to controls (Figure 2.3D; 2.56\% ± 0.54 vs. 1.06\% ± 0.17, n=19, n=23, respectively; p=0.006). The top quartile of psoriasis patients (5/19) with increased doublets have a positive correlation that approaches significance with disease duration (r=.804, p=.101), suggesting that increased doublet percentage may be an additional indicator of disease severity. To ensure that doublet formation was relevant to in vivo psoriasis circulation, we also measured monocyte subset and doublet formation using whole blood, a technique commonly used to quantify the percentage of circulating monocyte subsets (224). As shown in Figure 2.3C, bottom row, the increase in doublet formation can also be captured in whole blood assays.
Figure 2.3: Psoriasis patients have increased total doublets within PBMCs

(A) The prominent intermediate monocyte population in psoriasis patients was selected and in (B), cells were overlaid electronically into a monocyte scatter gate as shown in the FSC-A vs. SSC-A plot. (C) The majority of the cells were doublets, as shown in representative plots of FSC-W vs. FSC-H, demonstrating singlet and doublet populations in PBMCs (n=42) and whole blood (n=8). (D) Analysis of all patients demonstrated that the total doublet percentage is significantly increased in psoriasis patients when compared to controls (open diamonds versus solid diamonds, n=19 and n=23; respectively; p=.006).
In order to confirm which cells form aggregates, we used an Amnis Imagestream flow cytometer to acquire an image of each cellular event. This analysis is capable of visualizing cellular events by collecting a photographic image that corresponds to each flow cytometric event acquired. PBMCs were stained using mouse anti-human CD14 APC, CD16 FITC, and DAPI to discriminate among classical, intermediate, and non-classical monocytes as shown in Figure 2.4A. Imagestream analysis recapitulated our standard flow cytometric analysis confirming that monocyte doublets were 2x more likely in psoriasis PBMCs compared to healthy control samples (Figure 2.4B). Interestingly, analysis of the doublets revealed that monocytes were capable of forming pairs with different subsets of lymphocytes. This included different monocyte subsets such as an intermediate: classical pair (a CD14++CD16+ cell binding to a CD14++CD16neg cell) (Figure 2.4C, representative images in 2.4D) as well as monocytes binding to other lymphocytes including T cells (Figure 2.4C, inset), although monocyte:NK cell binding was not observed. Although some monocyte doublet pairs contain platelets, doublets (monocyte:monocyte) can also be formed in the absence of platelets. In the intermediate gate, the majority of doublets are represented by classical:non-classical (CD14++CD16neg:CD14+CD16++) pairs. Intermediate (CD14++CD16+) monocytes binding to other lymphocytes are also evident, although they represent a minority of the cells comprising the intermediate doublet population. Interestingly, both classical and intermediate monocytes exhibit the capacity to bind either CD14+ or CD16+ monocytes, in addition to other lymphocytes, although the classical monocytes appear to have an enhanced capacity over the intermediate cells to form these pairs (Figure 2.4C).
Figure 2.4: Amnis Imagestream visualization of cell surface expression of CD14 (APC) and CD16 (FITC) and monocyte-monocyte doublets

(A) Representative images of each type of monocyte (classical, intermediate, and non-classical) as imaged by the Amnis imagestream cytometer. CD14 (red) is expressed on the surface of the classical cells, CD16 (green) is expressed on the surface of the non-classical cells, while both CD14 and CD16 (orange) co-localize to the surface of the intermediate monocyte. (B) Psoriasis patients (n=3) have increased monocyte doublets when compared to controls (n=3) (black bar versus white bar, respectively; as expressed by a ratio of (% monocyte doublets/% monocyte singlets)). (C) Monocytes form aggregate pairs in the form of monocyte:monocyte or monocyte:lymphocyte. The classical CD14+ cells participate in the most doublet formation as a homogenous pair (CD14+ binding to another CD14+) or as a heterogeneous pair (CD14+ binding to a lymphocyte). The CD14+ cells within the intermediate doublet gate also participate in homo- and heterogeneous pairs, but not to the same extent. Additionally, double positive CD14+CD16+ monocytes participate in binding to CD3+ lymphocytes (as shown in D). Non-classical CD16+ cells rarely participate in homogenous doublet pairs. (D) Representative images of the doublet pairs described in C. Platelets (labeled with CD42b (pink)) participate in doublet formation but are not necessary for monocyte:monocyte aggregation.
Intermediate CD14++CD16+ cells are detectable in psoriasis tissue

Although intermediate CD14++CD16+ monocytes have been proposed to be dendritic cell (DC) precursors, the evolution/differentiation of these cells remains controversial (225). As shown in Figure 2.5, intermediate monocytes (yellow arrows) can indeed be detected in psoriasis involved papillary dermal perivascularature, as confirmed by co-localization with CD31 (Figure 2.6A), indicating that they do gain entry into lesional tissue. Some monocytes appear to be undergoing differentiation to DCs as evidenced by co-expression with DEC-205 (Figure 2.6C), or to macrophages based on CD68 co-expression (Figure 2.6B). Given the known criticality of T cells in psoriasis, we also identified CD3+ cells present in the plaque adjacent to, but not overlapping with, CD14+ cells (Figure 2.6D).
Figure 2.5: CD14++CD16+ cells are present and detectable in psoriatic plaques

Frozen involved human psoriatic plaque stained for CD14 (red) and CD16 (green) with nuclei stained by DAPI (blue). Yellow arrows represent CD14++CD16+ (intermediate) monocytes. Representative image from a psoriasis patient with a PASI score of 33.4 (n=7).
Figure 2.6: CD14, CD16, and CD3 staining with CD31, CD68, or DEC-205 co-localization

(A) Frozen involved human psoriatic plaque stained for CD14 (red), CD16 (green), and CD31 (blue) to identify vasculature (n=3). (B) Frozen involved human psoriatic plaque stained for CD14 (red), CD16 (green), and the macrophage marker, CD68 (blue) (n=2). (C) Frozen involved human psoriatic plaque stained for CD14 (red), CD16 (green), and the DC marker, DEC-205 (blue) (n=2). (D) CD14++ cells do not co-localize with CD3+ cells in involved psoriasis tissue. Frozen involved human psoriatic plaque stained for CD14 (red) and CD3 (green) with nuclei stained by DAPI (white) (n=3). In all sections, yellow arrows indicate double positive CD14/CD16 staining while white arrows indicate triple positive cells.
Monocyte modulation of CD16 following adherence

Based on our results demonstrating increased intermediate monocytes in psoriasis circulating blood and tissue and the participation of CD14+ cells in monocyte:monocyte and monocyte:lymphocyte doublets, we anticipated that these monocytes would demonstrate increased adhesiveness. Indeed, exposure of monocytes to tissue culture plastic significantly increases the expression of CD16, confirming previously published observations (213). The classical monocyte population is significantly diminished, and the majority of monocytes take on an intermediate monocyte phenotype beginning as early as 4 hours and becoming nearly exclusively CD14++CD16+ following overnight adherence to plastic (Figure 2.7A). Interestingly, monocytes derived from either psoriasis or healthy control peripheral blood exhibited the capacity to up-regulate CD16 expression upon binding. To examine CD16 induction on a more physiologically relevant substrate, we co-cultured control monocytes on pre-stimulated HMVEC-D endothelial cells (TNF-α and IL-17A) and compared them to monocytes cultured on unstimulated HMVEC-D cells. After one hour, the supernatants of unstimulated HMVEC-D (Figure 2.7B, middle panels) or stimulated HMVEC-D cells (Figure 2.7B, right panels) were isolated and stained for CD14 and CD16 expression on monocytes. Although a brief exposure to endothelial cells did not induce CD16 to the same extent as culturing on plastic, intermediate monocytes exhibited an average 2-fold increase in adhesion to stimulated HMVEC-D cells, based on monocyte number in the supernatant population, compared to unstimulated HMVEC-D cells (23.9% ± 4.5% vs. 12.4% ± 1.2%, respectively, n=4,
p=.029; representative images in Figure 2.7B, middle and right panels, supernatant intermediate population).
Figure 2.7: Upregulation of CD16 expression and enhanced adhesion

(A) Representative images: PBMCs at baseline (left panel) were adhered for 4 hours (middle panel) or overnight (right panel) to plastic and then stained for CD14 and CD16 (n=3). After 4 hours, expression of CD16 begins to increase; after overnight culture almost the entire classical population has upregulated CD16 and transitioned into the intermediate gate. (B) Representative images of monocyte gating of negatively selected control monocytes at baseline (left panels) and after 1 hour culture with unstimulated endothelial HMVEC-D cells (middle panels) and HMVEC-D cells stimulated with TNF-α (1ng/ml) and IL-17A (100ng/ml) (right panels); (n=4).
Psoriatic classical monocytes have increased β2 integrin expression at baseline

After determining that monocytes are capable of forming homogeneous pairs, and adhesion to plastic significantly increased CD16 expression, we asked whether or not psoriasis classical monocytes (Figure 2.8, solid red lines; n=3) had a different adhesion marker profile compared to classical cells from healthy controls (Figure 2.8, solid blue lines; n=3) at baseline. Psoriasis patients had moderately increased surface expression of the β2 integrins CD11b (Figure 2.8A) and CD11c (Figure 2.8B) with a slight elevation of CD18 (Figure 2.8C and Table 2.4), while psoriasis and control patients had similar levels of ICAM-1 (Figure 2.8D), VCAM-1 (Figure 2.8E), and VLA-4 (Figure 2.8F) expression. Isotype control curves are shown in grey.

Although control and psoriasis classical monocyte subsets upregulate CD11b and CD11c at baseline, control monocytes induce greater upregulation of these markers after a 30 minute adhesion (Figure 2.8, dashed blue lines) when compared to psoriasis monocytes (Figure 2.8, dashed red lines). This suggests that the psoriasis cells may already be “primed” to adhere, resulting in less upregulation of CD11b and CD11c than controls. CD18 is moderately upregulated in both control and psoriasis cells after adhesion, whereas levels of ICAM-1, VCAM-1, and VLA-4 do not change, suggesting that these markers are not likely to mediate the observed adhesion phenotype.
Figure 2.8: Increased β2 integrin expression on psoriasis classical monocytes

At baseline, psoriasis patients (solid red lines; n=3) demonstrate increased surface expression of (A) CD11b and (B) CD11c with a slight upregulation of (C) CD18 on the classical population when compared to controls (solid blue lines; n=3). After a 30 minute plastic adhesion, control (dashed blue lines) and psoriasis (dashed red lines) classical monocytes upregulate (A) CD11b, (B) CD11c, and (C) CD18, while there is no change in (D) ICAM-1, (E) VCAM-1, or (F) VLA-4. Isotypes are shown on all panels in grey.
Table 2.4: MFI changes

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<th>Adhesion Molecule</th>
<th>Baseline&lt;sup&gt;a&lt;/sup&gt;: Psoriasis/Control</th>
<th>Control&lt;sup&gt;b&lt;/sup&gt;: 30min/Baseline</th>
<th>Psoriasis&lt;sup&gt;c&lt;/sup&gt;: 30min/Baseline</th>
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<tr>
<td>ICAM-1</td>
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</tr>
<tr>
<td>CD11c</td>
<td>1.5</td>
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<td>CD18</td>
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</tr>
<tr>
<td>VLA-4</td>
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<td>1.1</td>
<td>1.0</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>1.2</td>
<td>1.1</td>
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</tr>
</tbody>
</table>

<sup>a</sup> Change in mean fluorescence intensity (MFI) of psoriasis classical monocytes compared to control classical monocytes. These cells were not adhered to plastic.

<sup>b</sup> Change in MFI of control classical monocytes after a 30 min adhesion relative to baseline expression.

<sup>c</sup> Change in MFI of psoriasis classical monocytes after a 30 min adhesion relative to baseline expression.
PCR analysis demonstrates a different mRNA expression profile for singlet, doublet, and adhered monocytes

In an attempt to interrogate the adhesive interaction observed in doublets, we compared the mRNA expression profiles of singlet classical monocytes (CD14++CD16neg) to adherent monocytes (singlet classical monocytes adhered to tissue culture plastic for 4 hours), and monocytes forming doublets in peripheral blood using an RNA array specific for adhesion molecules (SA Biosciences Extracellular Matrix and Adhesion Array) in both healthy controls (n=5) and psoriasis patients (n=5).

Interestingly, we found doublet cells (sorted from CD14+ cells) have an mRNA expression pattern that is distinct from classical monocytes that have been adhered to tissue culture plastic (Figure 2.9A, heatmap). As shown, doublet monocyte pairs (both psoriasis and healthy controls) express an upregulated cluster of genes distinct from post-plastic adherent monocytes including integrins (ITGA3, ITGA6, ITGB3), disintegrins (ADAMTS1, ADAMTS8, ADAMTS13), a matrix metalloproteinase (MMP11), collagens (COL5A1, COL6A1, COL6A2), and other cellular adhesion molecules (NCAM1, CDH1, LAMA2). Similarly, post-plastic adherent monocytes have a unique upregulated gene set compared to baseline singlet monocytes and monocyte doublets that included numerous matrix metalloproteinases (MMP1, MMP2, MMP9, MMP10, MMP14), fewer integrins (ITGAV), a laminin (LAMB3), and an adhesive glycoprotein (THBS1). Interestingly, adhered singlet monocytes from healthy control individuals also appear to downregulate several cellular adhesion genes including an intercellular adhesion molecule (ICAM-1), a sarcoglycan (SGCE), an extracellular matrix protein (KAL1), and a laminin (LAMA3), when compared to psoriasis adhered
singlet monocytes. Each individual patient’s classical singlet monocytes were used as the comparator. A comparison of the average psoriasis classical singlet monocytes to the average expression of control classical singlet monocytes also revealed a distinct gene expression profile (Figure 2.9B), although the changes were not as robust as those observed between doublet and adherent cell populations.
Figure 2.9: Gene networks in doublets and adhered classical monocytes

(A) Heat map of different gene expression patterns of CD14⁺-doublets (Dblt) and tissue culture adhered classical singlet monocytes (Adh) in control (C; n=5) and psoriasis (P; n=5) individuals. Each individual patient’s singlet classical monocytes were used as the comparator. (B) Heat map of gene expression patterns of psoriasis singlet classical monocytes. An average of the classical singlet monocytes was used as the comparator.
Analysis of the psoriasis versus control doublets indicates upregulation of several integrins, L-selectin (SELL), extracellular matrix proteins (VCAN, FN1, HAS1), and TGFB1 (Figure 2.10A). IPA analysis of the psoriasis doublet population identified the leukocyte extravasation pathway (shown graphically in Figure 2.10B) as the top canonical pathway. Network analysis from this pathway identified several directly upregulated alpha-integrins (ITGAV, ITGAL, ITGAM, ITGA4, ITGA5), beta-integrins (ITGB1, ITGB2, ITGB3, ITGB5) and cellular adhesion molecules (ICAM1, VCAM1) as well as several imputed genes of interest in psoriasis versus control doublets, including IL-1, the NFkB complex, the IL-12 complex, and genes known to play a role in cell surface adhesion such as fibrinogen, fibrin, collagen type II, and focal adhesion kinase (FAK).
Figure 2.10: Gene network of psoriasis doublets

(A) Psoriasis doublet gene network generated using the average of all psoriasis doublets (n=5); an average of all control doublets (n=5) is the comparator. (B) Leukocyte extravasation signaling pathway network of psoriasis doublets generated by IPA.
Monocyte doublets are increased in KC-Tie2 mice

In order to address what mediates the doublet formation, we used the KC-Tie2 skin-specific psoriasiform mouse model. KC-Tie2 mice have been previously shown to develop elevated systemic monocytosis comprised of circulating, proinflammatory CD11b+Ly6Chigh monocytes (193); this precedes the spontaneous development of aortic root vascular inflammation. This CD11b+Ly6C\textsuperscript{high} monocyte population has been previously correlated with the classical and intermediate human monocyte populations (129), thus we were curious whether KC-Tie2 mice would also demonstrate increases in circulating monocyte-monocyte doublets. Similar to our observations in psoriasis patient blood, KC-Tie2 mice also demonstrated an approximate 4 fold increase in monocyte:monocyte doublet formation compared to wild type (WT) controls (0.79% ± 0.17 vs. 0.20% ± 0.08, n=10, n=5, respectively; p=.008; Figure 2.11B, representative images in Figure 2.11A), indicating that chronic skin-specific inflammation may influence circulating monocyte aggregation.
Figure 2.11: Monocyte doublets are increased in KC-Tie2 mice

(A) Representative figures of doublet gating strategy. Percentages represent doublets within the CD11b^+Ly6C^high gate. (B) Splenic doublets are significantly increased in KC-Tie2 mice (n=10) when compared to littermate WT controls (n=5) (p=.008).
Discussion

Psoriasis is an immune-mediated inflammatory autoimmune disease (IMIAD) that has been demonstrated at the epidemiologic level to place patients at a higher risk for cardiovascular complications (44). The linkage of a number of IMIAD’s to cardiovascular disease (CVD) points to a common pathology, but there is a major gap in understanding how cellular inflammation at distant sites predisposes vascular tissue to CVD. The advanced state of validation of specific psoriasis pathogenesis pathways via biologic therapies provides a unique opportunity to link pathomechanisms of IMIADs with CVD. Intervention with current biological therapeutics for psoriasis has demonstrated that psoriasis may be dependent upon LFA-1 expressing leukocytes (myeloid cells and T cells), TNF-producing cells (monocytes, T cells, others), IL-23 (monocytes and DCs), and IL-17 (Th17 cells) (179, 183, 184, 226-229). In humans, a newly defined intermediate monocyte subset (CD14++CD16+) is predictive of CVD, myocardial infarction, and death (144-146). Several publications have implicated CD14++CD16+ intermediate cells as critical mediators of inflammation (140, 230, 231). Importantly, there is compelling epidemiologic evidence connecting increases in intermediate monocytes as predictive of fatal cardiovascular complications (144) as well as compelling epidemiologic evidence connecting psoriasis to cardiovascular risk (53, 54). Several relevant review articles have described the critical relationship among monocytes and cardiovascular disease (220, 232) and the concept of chronic inflammation driving cardiovascular outcomes has been validated in numerous murine models, including one psoriasiform mouse (193, 233, 234). The potential cellular mechanism(s) connecting psoriasis with elevated CVD risk has not been definitively
addressed. As elevated levels of the intermediate subset have been shown to contribute to CVD (150), we hypothesized that increases in this CD14++CD16+ subset seen in human psoriasis patients may also mediate a link between psoriasis disease pathology and its associated CVD co-morbidities. Increased MPAs in psoriasis patients suggest that circulating monocytes in these individuals may have increased adhesive properties and may play a potential role in the common pathology between psoriasis progression and CVD.

In this study, we asked if psoriasis patients possessed elevated levels of intermediate monocytes and if these monocytes demonstrated elevated adherence properties compared to healthy controls. Increased levels of intermediate (CD14++CD16+) cells in psoriasis circulation confirms a recent study among psoriasis patients (214) as well as a previously reported increase in total CD16++ cells in patients with psoriatic arthritis (213). We demonstrate here that intermediate monocytes correlate with disease severity, are present in involved psoriatic plaque tissue, and preferentially bind to stimulated endothelial HMVEC-D cells. Upon back-gating of the circulating intermediate monocyte population, we noted that many of these cells appear consistent with larger cell populations judged by side scatter. Additionally, when areas where doublets appear were included in the gating strategy, the intermediate population was more prominent in these larger forward scatter areas. Since intermediate monocytes are believed to contribute to, or correlate with, numerous co-morbid conditions for psoriasis, we hypothesized that doublets made up of two intermediate monocytes would be predominant in psoriasis pathology and plaque formation.
Using Amnis Imagestream technology, we demonstrated that monocytes can form monocyte:monocyte and monocyte:lymphocyte pairs in the presence or absence of platelets. Specifically, the predominant monocyte:monocyte doublet pairs consist of one CD14++CD16\textsuperscript{neg} classical monocyte binding to either another CD14++CD16\textsuperscript{neg} classical monocyte, a CD14++CD16\textsuperscript{+} intermediate monocyte, or a lymphocyte. To better understand which adhesion molecules may mediate the monocyte doublet formation, we screened control and psoriasis PBMCs using a panel of typical surface adhesion markers. Monocytes from psoriasis patients express moderately increased levels of β2 integrins at baseline levels and do not upregulate expression of these adhesion markers to the same extent as healthy control monocytes upon adherence to plastic, suggesting that psoriatic monocytes may be previously “primed” by the circulating psoriasis milieu and are not able to be further stimulated. This potential priming and baseline elevation of CD11b and CD11c may lead to the observed increase in circulating monocyte:monocyte doublets of psoriasis patients. Additionally, co-culture of control monocytes on TNF-α and IL-17A-stimulated HMVEC-D endothelial cells results in an average 2-fold increase in adhesion of intermediate monocytes to stimulated HMVEC-D cells when compared to monocytes cultured on unstimulated HMVEC-D cells, indicating that myeloid cell interaction with activated psoriatic endothelium may contribute to observed enhanced adhesiveness.

To further define the gene expression pattern of classical singlet monocytes, doublets containing CD14\textsuperscript{+} cells, and monocytes post-adherence, we used an mRNA expression array specific for extracellular matrix and adhesion gene expression and compared monocytes from doublet pairs or monocytes post-adherence on tissue
culture plastic to singlet classical monocytes. After adherence to tissue culture plastic, singlet classical monocytes isolated from healthy individuals upregulate genes involved in cellular adhesion including integrins (ITGA3, ITGA7, ITGA8), a cadherin (CDH1), and VCAM-1 to a greater extent than psoriasis monocytes, suggesting some disease-specific adhesion gene response profiles. Singlet plastic-adherent classical monocytes from both control and psoriasis patients upregulate several matrix metalloproteinases (MMP-1, -2, -10, and -14), thrombospondin, and secreted phosphoprotein 1 (SPP1), while downregulating P-selectin (SELP), indicating that plastic adhesion is distinct from cell-cell adhesion. The observed upregulation of MMPs in plastic adhesion when compared to the doublet cell populations suggests that the cell-cell interaction of monocyte-doublet pairs initiates signaling pathways that are distinct from a monocyte plastic-adherence phenotype. MMPs may play an important role in this adhesion as they are known to participate in atherosclerotic plaque stability (235) and are predominantly upregulated upon cellular contact with extracellular matrices. A major difference in gene expression patterns among psoriasis patients compared to healthy individuals occurs primarily following adherence of classical monocytes to tissue culture plastic.

A readily apparent difference between monocytes in doublet pairs compared to monocytes post-plastic adherence highlights upregulation in doublets of integrins, sarcoglycan, collagen type VI, alpha 1 and 2, and disintegrins. Interestingly, upregulation of CD56 (NCAM1) is also observed in the doublet pairs compared to plastic-adherent monocytes. CD56 expression may indicate upregulation of the CD56⁺ monocyte population (236), previously demonstrated in other autoimmune
disorders (237). Specific genes upregulated in doublet pairs, such as ITGA-3 and ADAMTS1 (a disintegrin), suggest that integrins may participate in the cell-cell adhesion we observed using Amnis Imaging technology. Although the function of ADAMTS1 in monocytes is unclear, one report showed that ADAMTS1 expression can be induced during monocyte to macrophage differentiation (238). Interestingly, a polymorphism in the ADAMTS1 allele has been linked to an increased risk of fatal coronary disease, indicating that it may play a role in mediating CVD (239). IPA pathway network analysis of the leukocyte extravasation pathways in psoriasis doublets identified several imputed genes of interest, including genes known to play a role in cell surface adhesion such as fibrin, fibrinogen, a collagen, and focal adhesion kinase, while also including molecules known to participate in important signal transduction pathways impinging upon STAT3 regulation such as the NFκB signaling complex. At baseline, comparison of singlet classical monocytes from psoriasis and healthy control patients demonstrates a distinct gene expression pathway in psoriasis monocytes that may account for their increased propensity to form doublets.

To determine what was mediating the doublet formation, we took advantage of the KC-Tie2 mouse, a psoriasiform model that exhibits chronic skin-specific inflammation and is known to have increases in circulating monocytes and thrombosis formation, as well as to develop aortic root vascular lesions. We show here that these mice also have increased circulating monocyte-monocyte doublets, suggesting that skin inflammation may drive cell differentiation and aggregation, although the precise mechanisms mediating this outcome require further study. Cumulatively, the data suggest that cells forming doublets in circulation upregulate distinct adhesion
molecules that potentiate cellular adhesion and may increase the risk of cardiovascular co-morbidities observed in psoriasis patients.
CHAPTER 3

CHRONIC, NOT ACUTE, SKIN-SPECIFIC INFLAMMATION PROMOTES THROMBOSIS IN PSORIASIS MURINE MODELS

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Introduction

Psoriasis vulgaris is a chronic inflammatory skin disease that affects ~2% of Americans and is characterized by red, scaly, well-demarcated plaques containing activated immune cells (20, 123). The disease is associated with numerous co-morbidities including psoriatic arthritis (240), diabetes (241-243), kidney disease (244, 245), and metabolic syndrome (246). Psoriasis patients also experience increased depression and societal stigmatization (20, 247, 248). Importantly, individuals with psoriasis have an increased risk of developing and dying of cardiovascular disease (CVD) (40, 42, 44, 54, 249). Likewise, patients with severe psoriasis have an increased risk of experiencing an adverse cardiovascular event, such as stroke or myocardial infarction (MI) (50, 243, 250), and this occurs independently of other CVD risk factors including age, gender, smoking, diabetes, hypertension, and hyperlipidemia (42, 44). Causality between psoriasis and CVD is challenging to explore; however, many commonalities exist at the cellular and molecular levels between the two diseases (251, 252) and treating psoriasis patients with systemic anti-inflammatory drugs may improve associated CVD outcomes (253).

Recently, we generated and reported a murine model of psoriasis driven by keratinocyte-specific overexpression of Interleukin (IL)-17C, the K5-IL-17C mouse (254). IL-17C is an IL-17 family member believed to signal through the receptors IL-17RE/RA (255). IL-17A and IL-17F are the most highly characterized IL-17 cytokines in the context of psoriasis pathogenesis (256); however, IL-17C is the most abundant IL-17 isoform expressed in lesional human psoriasis skin (254, 257), and is rapidly responsive to efficacious biologics, implicating a potential pathogenic role in disease
(254, 256). To explore this possibility, K5-IL-17C mice were genetically engineered to model increased epidermal IL-17C expression (254), and develop a spontaneous skin phenotype similar to human psoriasis that includes the development of disease after birth, well-demarcated skin lesions with clear gross demarcation between uninvolved and involved skin, an IL-12/23-Th1/Th17 immune cell phenotype, and improvement of skin pathology upon treatment with TNF-α antagonists (254).

This model system offers a unique opportunity to confirm a pro-thrombotic phenotype resulting from chronic skin-specific inflammation and demonstrate the importance of exposure time to inflammation, or chronicity to this cardiovascular outcome. Our prior work demonstrated keratinocyte-containment of the transgene using genetic-reporter approaches (193); thus, we hypothesize that K5-IL-17C mice will develop enhanced thrombosis as a direct result of chronic skin-specific inflammation in the presence of systemic monocytosis, similar to that observed in the KC-Tie2 mouse model (193) and in psoriasis patients. Of translational importance, recent work done by our group (Golden et al., J Immunol 140:2307; doi:10.4049/jimmunol.1402307) and others (Dr. Nehal Mehta, NHLBI, personal communication) have identified circulating pro-inflammatory monocytes in psoriasis patients that resemble the increased CD11b^+Ly6C^high cells observed in the KC-Tie2 model, and which we hypothesize will also be elevated in K5-IL-17C mice, providing a potential link between chronic skin inflammation and the CVD co-morbidities.

The contributions of specific genes to skin inflammation have recently been investigated pre-clinically using acute (5 days) topical application of Aldara (5% imiquimod; a TLR7/8 agonist) (188, 258). Controversy over whether this model is
appropriate for studying psoriasis pathogenesis has ensued in part due to the timing
of this elicitation and the fact that it does not model chronicity as observed in human
psoriasis, but does model some early events in psoriasiform plaque formation, such
as increased acanthosis (259, 260). Both the K5-IL-17C (chronic) model and the
Aldara (acute) model result in increased infiltrating dermal T cells, dendritic cells, and
macrophages into lesional tissue (188, 254). Moreover, similar patterns of elevated
pro-inflammatory gene transcripts are observed in lesional skin of both models,
including IL-12/23, TNF-α, IL-17A, and IL-17C (188, 254, 261). These unique
experimental models provide the opportunity to compare acute vs. chronic skin-
specific inflammation and their effects on systemic monocytosis and thrombosis
outcomes.
Materials and Methods

Mice: K5-IL-17C mice on a C57Bl/6 background were bred and genotyped as previously described (254) in the Case Western Reserve University (CWRU) animal vivarium. Littermates carrying a single non-expressing transgene (either K5tTA or Tet^osIL-17C) or no transgenes (C57Bl/6, wildtype (WT)) were used as littermate controls. For Aldara experiments, C57Bl/6 mice were purchased (Jackson Laboratories, Bar Harbor, ME) and allowed to acclimatize to the CWRU animal vivarium for at least 14 days before beginning topical application of either Aldara or control cream. All mice used in the experiments were of similar age (10-16 weeks of age), and both male and female mice were used for all experimental outcomes. For Aldara experiments, mice were shaved one day prior to application of Aldara, and topical Aldara or control cream was spread on the dorsal surface of the mouse (5% Aldara, 3M Pharmaceuticals; 62.5mg) daily for a period of 5 days. As needed, mice were provided with IP saline to supplement fluid loss associated with Aldara treatment, a well-known side-effect (262). On day 6, mice underwent the thrombosis protocol outlined below. All animal protocols were approved by the CWRU Institutional Animal Care and Use Committee and conformed to the American Association for Accreditation of Laboratory Animal Care guidelines.

Rose Bengal occlusive thrombosis: The Rose Bengal thrombosis vascular occlusion assay was completed as previously described (193). Briefly, male and female K5-IL17C mice (n=14; male (5), female (9)) or littermate controls (n=21; male (10), female (11) or C57Bl/6 WT mice treated with either Aldara (n=25; male (11), female (14)) or control cream (n=21; male (10), female (11)) were deeply anesthetized
and had their right common carotid artery exposed and monitored by a Doppler flow probe. Animals received a tail-vein injection of Rose Bengal (50mg/kg) followed by laser illumination of the carotid artery (540nm) to initiate thrombosis as described previously (263-265). Blood flow was monitored until occlusion occurred, defined as cessation of blood flow for 10 minutes.

**Tissue harvesting and flow cytometry:** Following thrombosis, inflamed skin was harvested and processed as described previously for histology and immunohistochemistry (190, 254). Skin draining axial and inguinal lymph nodes were isolated from a subset of K5-IL-17C transgenic mice and littermate controls (n=6 and n=15; respectively) and Aldara- or control cream-treated C57Bl/6 WT mice (n=12 and n=11; respectively) and were then pooled. Spleens were isolated from a subset of K5-IL-17C transgenic mice and littermate controls and Aldara- or control cream-treated C57Bl/6 WT mice (n=4-9). These tissues were homogenized in serum-free media containing 50µg/ml DNase I (Sigma, St. Louis, MO) and 2mg/ml collagenase D (Roche, Basil). Isolated cells were then pelleted, re-suspended, and filtered 2x through a 70µm filter in wash buffer containing 5% FBS. The cells were immediately stained for the cell surface markers Ly6C-Alexa Fluor 700 (eBiosciences, San Diego, CA), Ly6G APC (Abcam, Cambridge, MA), and CD11b eFlour450 (BD, Franklin Lakes, NJ). Flow cytometry data collection was performed using a BD FACS-Aria instrument and analyzed using FlowJo software (Tree Star, Ashland, OR). For cell gating, monocytes were first selected on an FSC-A vs. SSC-A plot. The monocytes were then analyzed for Live/Dead cell populations using the 7-AAD cell exclusion dye. From the live monocyte gate, singlet cells were selected and the doublet events were excluded. Live
singlet cells were then selected for CD11b⁺Ly6G⁻ cells. From this population, Ly6C was plotted versus SSC-A, and cells that are high on Ly6C and low on SSC-A were considered CD11b⁺Ly6C<sup>high</sup>, excluding CD11b⁺Ly6C<sup>low</sup> cells and eosinophils. See Figure 3.1 for gating strategy.
Figure 3.1: Gating strategy to identify the CD11b^+Ly6G^{neg}Ly6C^{high} monocyte population

(A) FSC-A vs. SSC-A plot was used on all events to identify monocytes. (B) The monocytes were next analyzed for live/dead cell populations based on 7-AAD cell exclusion. (C) From the live monocyte gate, cells were gated for singlet and doublet events, and the doublet events were excluded. (D) The singlet cells were selected for CD11b^+Ly6G^{neg} cells (based on isotypes for CD11b and Ly6G). (E) From CD11b^+Ly6G^{neg} gate, Ly6C expression was plotted versus SSC-A, and cells that expressed high levels of Ly6C (based upon isotype) and low on SSC-A (i.e., non-granular) were considered CD11b^+Ly6C^{high} monocytes. Cells that expressed low levels of Ly6C (based upon isotype) and low on SSC-A (i.e., non-granular) and eosinophils (high on SSC-A) were excluded.
Statistics: Data analysis and graphs were generated using GraphPad Prism 6 and Microsoft Excel. Results are expressed as mean (± standard error of the mean). The assumption of normality was tested using the D’Agostino and Pearson omnibus normality test. When normality was met, a parametric, unpaired, two-tailed t-test with Welch’s correction was used to compare mean values. When the assumption of normality was not met, the non-parametric Mann-Whitney U test was used with a t-test for independent samples to compare mean values. Significance was defined as p<0.05.

Results and Discussion

Aldara was applied to C57Bl/6 WT mice in an area that mimicked (approximated) the K5-IL-17C mice for body surface involvement (Figure 3.2A) and involved skin from both models developed similar increases in acanthosis (epidermal thickness), an often-used surrogate measure of inflammation for murine skin (185, 266), compared to control cream-treated and littermate controls (Figure 3.2B, bottom row compared to top row). We and others have previously reported the increased presence of skin-infiltrating T cells, myeloid cells, and concomitant increases in pro-inflammatory cell-derived cytokines, including elevated TNF-α, IL-12, IL-23, IL-17A and IL-17C in post-Aldara treated animals and in the chronic K5-IL-17C psoriasiform mouse model (188, 254, 261). Thus, Aldara, or chronic stimulation of keratinocytes by increased levels of IL-17C leads to cutaneous infiltration of pro-inflammatory cells.

To determine whether the chronicity of skin-specific inflammation affects thrombotic clotting times, we performed the Rose Bengal photochemical carotid artery
injury model on the acute inflammatory Aldara-induced model (and control cream-treated mice) as well as the chronic K5-IL-17C mice (and their littermate controls). C57Bl/6 WT mice treated topically with Aldara for 5 days had clotting times similar to C57Bl/6 WT mice treated with control cream (Figure 3.2C, triangles vs. circles; 32.2 ± 3.0 min vs. 31.4 ± 2.5 min, p=0.841; n=25, n=21, respectively). In contrast, K5-IL-17C mice had significantly reduced clotting times (enhanced thrombosis) compared to littermate controls (Figure 3.2C, diamonds vs. squares; 15.7 ± 2.1 min vs. 26.5 ± 3.4 min., p=0.0071; n=14, n=21, respectively). No differences were observed between male and female mice within any treatment or genetic grouping, thus male and female results were grouped together. These data suggest that chronic, but not acute, skin inflammation promotes thrombosis, and validates our prior observations describing shortened thrombosis times in the KC-Tie2-psoriasiform model in a second skin-specific chronic inflammatory mouse model (193) and extend these findings to suggest an important role for exposure time to skin inflammation in the promotion of this outcome.
Figure 3.2: Chronic, but not acute, skin inflammation promotes shorter arterial clotting times in the Rose Bengal thrombosis assay

(A) Representative photographs of a C57Bl/6 WT mouse treated for 5 days with topical 5% Aldara (left panel) and a K5-IL-17C mouse (right panel). (B) Representative images of H&E stained mouse back skin from a C57Bl/6 WT mouse treated for 5 days with topical Aldara and a K5-IL-17C mouse demonstrate increases in acanthosis compared to controls. (C) C57Bl/6 WT + Aldara clotting times (n=25) are not different than C57Bl/6 WT + control cream (n=21); however K5-IL-17C mice (n=14) have significantly decreased clotting times compared to controls (n=21), p=0.0071. Scale bar in B = 100μm.
This observation is comparable to clinical reports demonstrating that CVD risk is highest for young psoriasis patients with moderate-to-severe disease, presuming that they are exposed to chronic unrelenting inflammation for a more sustained period of time (46), and that psoriasis patients with severe disease have increased risk of thromboembolism (267). Interestingly, recent epidemiological data suggests that chronicity, or the length of time exposed to persistent inflammation, may play a role in longitudinal increased risk of a myocardial infarction (MI) event, with the more severe psoriasis patients at the greatest risk (243). Importantly, a tandem study from our group (this issue) demonstrates that psoriasis patients have 2.7-fold higher odds of developing atherosclerosis (after adjusting for age, gender, race, BMI, and smoking status), and this difference becomes especially pronounced in young psoriasis patients (age 30-39) where 49% of the patients had atherosclerosis, defined as present if a patient had one or more of the following: CAC score ≥1 (or a coronary stent), right or left CIMT >75th percentile for age, or the presence of visible carotid plaque, compared to only 15% of age matched controls. Psoriasis patients also had elevated levels of HsCRP (high-sensitivity C-reactive protein), a frequently used measure of systemic inflammation and a surrogate predictor of CVD events (268-272). Taken together, these results provide additional evidence that the length of time an individual is exposed to systemic inflammation, such as that derived from inflamed psoriasis skin, appears to increase the risk of developing CVD.

Increased risk of cardiovascular complications has been reported for patients with other chronic organ-specific inflammatory diseases, including inflammatory bowel disease and rheumatoid arthritis, where risk of thromboembolism or levels of unstable
carotid artery plaque are also significantly elevated (respectively) compared to healthy controls; during a flare in either disease, this risk further increases (273-275), and then decreases during disease remission (274, 275). Whether similar increases occur in psoriasis patients during acute flare remains to be examined.

Importantly, retrospective meta-analyses suggest some decrease in the incidence of myocardial infarctions in psoriasis patients treated with either TNF inhibitors or anti-IL-12/23 antibody, suggesting that aggressively targeting chronic inflammation and its cellular drivers may reduce life-threatening co-morbidities associated with psoriasis (276-278). Our prior findings using the KC-Tie2 mouse model support this, such that reversal of the skin disease, following gene repression, returned thrombosis times to control mouse levels; and reversed aortic root inflammation (193). More recently, additional clinical support for our pre-clinical results has been reported demonstrating that psoriasis patients treated with systemic biologic anti-inflammatory agents showed a lower association with CVD events (cardiovascular death, myocardial infarction, stroke) compared to patients treated with other anti-psoriatic therapies (279).

To determine if the observed increase in frequency of skin-infiltrating, pro-inflammatory CD11b⁺Ly6C^high monocytes is responsible for the promotion of thrombosis, we isolated skin-draining axial and inguinal lymph node (LN) cells from a subset of acute Aldara-treated C57Bl/6 WT mice, chronic K5-IL-17C animals and their relative controls. Using flow cytometry, we measured surface expression of CD11b, Ly6G, and Ly6C on LN cells (representative images in Figure 3.3A). Interestingly, CD11b⁺Ly6C^high pro-inflammatory monocytes were significantly increased in both the
acute (Aldara-treated) and chronic (K5-IL-17C) skin inflammation models compared to their respective controls in both the LN (Figure 3.3B; 66.6 ± 4.4 vs. 17.4 ± 2.6, p=0.0286, n=43, n=4; and 74.2 ± 1.2 vs. 21.6 ± 2.8, p=0.0286, n=3, n=6; respectively) and spleen (Figure 3.3C; 81.7 ± 1.2 vs. 40.9 ± 4.8, p=0.0079, n=5, n=5; and 73.9 ± 8.7 vs. 21.2 ± 1.3, p=0.0028, n=4, n=9; respectively). These data suggest that CD11b+Ly6C<sup>high</sup> monocytes accumulate rapidly in draining LN and spleen following acute skin inflammation. Previously, we demonstrated that circulating pro-inflammatory CD11b+Ly6C<sup>high</sup> monocytes are increased in the KC-Tie2 mice (193), and other investigators have implicated these cells as mediators of atherogenesis (234, 280). Despite the increase in spleen and LN-CD11b+Ly6C<sup>high</sup> cells, thrombosis times failed to change significantly between Aldara-treated and control mice (Figure 3.2C), suggesting that acute monocytosis alone does not promote thrombosis. Although increased levels of circulating monocytes have been previously categorized as a risk factor for coronary heart disease (281), our data suggests that increases in cutaneous and circulating CD11b+Ly6C<sup>high</sup> monocytes alone do not lead to shortened thrombosis times.
Figure 3.3: CD11b⁺Ly6C⁺ monocytosis is increased in acute and chronic inflammatory skin disease mouse models

(A) Representative flow cytometry dot plots of CD11b and Ly6C surface staining in skin-draining axillary and inguinal lymph nodes (LN). (B) C57Bl/6 WT mice treated for 5 days with topical Aldara (n=4 pooled samples) and K5-IL-17C mice (n=3 pooled samples) have increases in skin-draining LN-CD11b⁺Ly6C⁺ cells compared to their respective controls (n=4 and n=6 pooled samples; p=0.0286 and p=0.0286, respectively). Each point represents lymph nodes pooled from 2-3 animals. (C) Increased splenic-CD11b⁺Ly6C⁺ are also observed in Aldara-treated WT mice (n=5) and K5-IL-17C mice (n=4) when compared to their respective controls (n=5, n=9; p=0.0079 and p=0.0028, respectively). Each point represents a single animal.
In other chronic illnesses, such as HIV, increased levels of circulating lymphocytes and leukocytes have also been observed and proposed to be responsible for the increased risk of cardiovascular events (282). More important than numerical increases, perhaps, is the functional activation of these monocytes and lymphocytes, as suggested by Funderberg et al. (283, 284), who demonstrate that monocytes can become activated by exposure to oxidized LDL (oxLDL), leading to increased cardiovascular risk. Interestingly, psoriasis patients are dyslipidemic and have increased circulating and plaque oxLDL (39, 276, 285, 286). Moreover, stimulation of macrophages with psoriasis patient-isolated LDL increases production of IL-6 and TNF-α, and also results in increased monocyte adhesion to human umbilical vein endothelial cells (287). Thus, increases in oxLDL found in psoriasis patients and oxLDL-mediated effects on lymphocytes may provide further support for how skin-inflammation promotes distant vessel inflammation and the promotion of atherothrombosis.

Indeed, in several chronic inflammatory diseases associated with increased cardiovascular risk, chronic inflammation can extend to inflamed vessels (arteries) that are likely to signal for additional pro-inflammatory leukocytes and lymphocytes (288), leading to increased infiltration of pro-inflammatory cells. This contextual activation of both monocytic and endothelial cells may be dependent on the length of exposure to chronic inflammation; such that increased exposure, like that induced in the chronic K5-IL-17C model, but not acute exposure, similar to that elicited by Aldara application, are necessary to compromise endothelial-monocyte (dys)function. Supporting this concept are reports that recent-onset plaque psoriasis patients fail to present with
endothelial dysfunction (289) whereas established-plaque psoriasis patients do (290, 291), consistent with the idea that duration of disease may lead to the observed dysfunction in chronic patients. In addition to endothelial dysfunction, psoriasis patients also have increases in circulating endothelial cells and microparticles (MPs), which may promote coronary artery disease, acute coronary syndromes and atherothrombosis (291-295). Importantly, these decrease following treatment with anti-TNF-α therapy (292) and could provide mechanistic insight into why TNF-α inhibition reduces risk of MI in psoriasis patients (276, 278).

The contribution of elevated monocytes and/or the activation status of these recruited monocytes, or potentially the micro-milieu the monocytes encounter, may all contribute to the thrombotic potential of the mice. Targeted experiments designed to eliminate monocyte egress from the bone marrow, thus depleting these cells from circulation in psoriasiform mice (i.e., backcross CCR2-/- and/or CCR5-/- mice with either KC-Tie2 or KC-IL-17C) should address the necessity and importance of monocytosis for thrombosis alterations. In addition, alternative cellular mediators of inflammation may play additional, as yet unidentified roles, in the thrombotic process. Duration, or chronicity of the skin inflammation, appears to be more correlative of shortened thrombosis times rather than accumulation of pro-inflammatory cellular mediators. However, the duration of the cutaneous cellular response and resultant prolonged exposure to circulating immune cells and derived cytokines may also be a critical factor rather than the appearance of transient pro-inflammatory cells.
Conclusions

Acute, as well as chronic, skin-specific inflammation promotes the circulation and infiltration of CD11b⁺Ly6C<sup>high</sup> monocytes into the skin, but only chronic skin-specific inflammation is correlated with an increase in occlusive distant vessel thrombosis. Our findings provide evidence in an independent second genetic skin-contained mouse model that chronic cutaneous inflammation itself has the capacity to promote thrombosis; however our findings also suggest that despite similar levels of skin involvement (body surface area) in acute and chronic models, the length of exposure to skin-elicited inflammation, and potentially systemic monocytosis, appears most critical to poor thrombosis outcomes. Further work delineating the cellular and molecular mechanisms by which psoriasis promotes systemic inflammation and poor CVD outcomes, at the pre-clinical and clinical levels, are needed to better understand the link between psoriasis and CVD.
CHAPTER 4

IL-17 IN PSORIASIS: IMPLICATIONS FOR THERAPY AND CARDIOVASCULAR CO-MORBIDITIES

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IL-17 in psoriasis: Implications for therapy and cardiovascular co-morbidities.

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Introduction

Psoriasis is a chronic inflammatory skin disease; its cause remains unknown as a strict mitigating signal or a single antigenic target has yet to be identified. A combination of human and animal studies has led to the understanding that in patients with a susceptible genetic background, some stimulus, perhaps an infection, leads to a coordinated series of signaling events involving release and signaling of cytokines that occurs between keratinocytes, endothelial cells, T cells, monocytes and macrophages, and dendritic cells (DCs) that once initiated results in a vicious pro-inflammatory proliferative cycle that perpetuates a sustained cutaneous inflammatory response. Intervention at several key points in this cycle results in clinical resolution; however durable remission and/or permanent clearance have not been achievable.

Psoriasis as an immune-centric disease stems from clinical observations associated with the disease clearance following immunosuppressive therapies (3). Further evidence has demonstrated a strong T cell component to disease maintenance via Th1, Th17, and Th22 cells and their derived cytokines (4-7). Release of cytokines from these T cells including IFN-γ, IL-17A, IL-17F, and IL-22 following cutaneous DC activation induces keratinocyte hyperproliferation and further proinflammatory immune responses. Although psoriasis primarily affects the skin and joints (psoriatic arthritis), several co-morbidities, including inflammatory bowel disease (IBD), lymphoma, obesity, and metabolic syndrome are associated with psoriasis (206, 296). Most recently, growing epidemiologic evidence has shown that psoriasis patients have a significantly increased risk of developing and dying of cardiovascular disease (CVD) (40, 42-46). Because vascular inflammation and atherosclerosis share
common mechanisms of pathogenesis with lesional psoriasis including similar inflammatory cytokine profiles and proinflammatory cell types including T cells, monocytes/macrophages, and neutrophils (Table 4.1), the hypothesis that aggressive treatment of the primary disease (psoriasis) may decrease the risk of developing the other co-morbidities (CVD) is now currently being investigated.

**Psoriasis pathogenesis and treatment**

Psoriasis is a chronic inflammatory skin disease affecting 2-3% of the US population (1) and the most common variant of psoriasis, psoriasis vulgaris, affects approximately 85 to 90% of all patients (8). Psoriasis is clinically characterized by erythematous, raised, well-demarcated scaly patches of skin. Skin lesions form as a result of epidermal hyperplasia, premature keratinocyte maturation, parakeratosis due to retention of nuclei in the stratum corneum (9), and the formation of epidermal rete ridges: elongated, thin, downward projections of keratinocytes into the dermis (10). In addition to the epidermal changes, changes to the skin microvasculature occur, including increased expression of vascular endothelial growth factor (VEGF) leading to the formation of leaky vessels (11), and increases in skin infiltrating leukocytes. The disease can be exacerbated by stress, infections, and medications such as beta blockers (12-14).

Treatments for psoriasis include topicals such as corticosteroids, as well as agents such as anthralin, synthetic Vitamin D3, and Vitamin A; phototherapy including broad and narrowband-UVB, laser UVB, and psoralen UVA (PUVA); systemics such as cyclosporine, methotrexate, and retinoid receptor inhibitors (Acitretin); and biological therapeutics including TNF-α inhibitors and cytokine inhibitors such as anti-
IL23p40 and IL-17 inhibitors. Historically, psoriasis was believed to originate due to keratinocyte dysfunction until the discovery that cyclosporine A (CsA), a calcineurin inhibitor that blocks cytokine production and interleukin release resulting in reduced function of effector T-cells and ultimately immunosuppression, was found to be highly effective (3). Since this time, the focus has shifted toward immune-mediated mechanisms to explain the keratinocyte hyperproliferation and subsequent therapeutics have targeted immune cells present in psoriatic tissue.

Additional support for the importance of T cells as pathogenic in psoriasis is derived from the efficacy of biologic agents used in the treatment of psoriasis, including therapies which target T cells/T cell activation directly and those which target pro-inflammatory cytokine pathways present in lesional psoriasis skin. T cell-specific interventions, such as FK506 (tacrolimus, another calcineurin inhibitor that blocks T cell signal transduction and IL-2 transcription), DAB-IL-2 (denileukin diftitox, a fusion protein of interleukin-2 (IL-2) and the enzymatically active and translocating domains of diphtheria toxin which induces apoptosis in IL-2 receptor bearing cells that take up the drug), anti-CD4 (Hu-Max, targeting helper T cells, regulatory T cells, DC subtypes and monocytes and therefore highly immunosuppressive), CTLA4-Ig (abatacept, a fusion protein of the Fc region of immunoglobulin IgG1 with the extracellular domain of CTLA-4 which prevents antigen-presenting cells (APCs) from delivering the costimulatory signal to T cells), and anti-CD3 (ala-ala, a humanized non-FcR binding derivative of the anti-human CD3 monoclonal antibody OKT3 (huOKT3y1), which has been proposed to lead to a preferential depletion of pathogenic effector T cells via apoptosis and concomitant stabilized expression of Helios positive regulatory T cells)
as systemic therapy, proved that T cell activation is critical for sustaining psoriatic lesions (reviewed in (122, 168-172)). Effector memory T cells persist in the skin in order to provide a rapid response to infection (103) and resident T cells in normal skin express the skin-homing receptors cutaneous lymphocyte antigen (CLA), CCR4, and CCR6 (104). Non-lesional skin from psoriasis patients develops psoriatic lesions in the absence of infiltration of blood lymphocytes (105), highlighting the importance of skin-resident memory T cells in perpetuation of the disease. While memory effector T cells play a role in psoriasis, it should be noted that the first FDA-approved biological therapy for psoriasis LFA3-Ig (alefacept), which binds CD2\textsuperscript{high} activated Tmem/eff cells and selectively depletes these cells (173), has been voluntarily discontinued in the U.S. for the treatment of psoriasis due to, among many factors, minimal response rates.

Clinical improvement is also accompanied by a reduction in T cells and Th17-related mediators following treatment by several biological therapies including TNF inhibitors (e.g., infliximab, etanercept, adalimumab) (102, 174), the IL-12/IL-23p40 inhibitor ustekinumab (175), and the new IL-23p19 antibody (MK-3222) (298) which indirectly inhibit T cell activation/differentiation and maintenance, as well as recently reported anti-IL-17 therapeutics (e.g. ixekizumab, brodalumab) (183, 184) which directly target IL-17 and IL-17 receptors.

Taken together, the efficacy of agents described above demonstrates that depleting T cells or their cytokines directly, or targeting their signaling pathways, can be an effective strategy in the treatment of psoriasis. TNF-\(\alpha\) inhibitors produce psoriasis remission by blocking the cytokine itself or its receptor and preventing the
activation and expansion of T cells, leading to a decrease in overall inflammation and allowing the lesion time for repair (176, 177). Ustekinumab’s effects appear to result in potentially better durable remission (178, 299), perhaps reflecting a direct effect on reducing the circulating numbers of DCs, and decreases in IL-23p19 and IL-12p40 levels in psoriasis lesions (169, 180, 181) which in turn leads not only to the marked decrease in IFN-γ production (IL-12 effect), but also to a decrease in Th17 activation (IL-23 effect) (182). Evaluation of MK-3222 efficacy is still in early clinical trials; however preliminary reports of initial results appear promising (298). Two new humanized antibodies that target IL-17 through either neutralization of IL-17 or blocking the IL-17 receptor, ixekizumab and broadalumab respectively, are effective in the treatment of moderate-to-severe-psoriasis and will be discussed in more detail below (183, 184).

**Psoriasis immunology and the role of IL-17**

**T-helper subtypes important in psoriasis pathogenesis**

Psoriasis is now defined as a Th1/Th17/Th22-biased inflammatory disease (81-86). Lesions and peripheral blood of psoriasis patients contain Th1 cells that produce high levels of IFN-γ and low levels of IL-4 and IL-10 (87-89), Th17 cells that produce IL-17 and IL-22, Th22 cells which produce IL-22, and cytotoxic epithelial CD8 T cells. A large scale gene expression analysis confirmed the contribution of the Th1 population as it demonstrated IFN-related genes were differentially expressed in involved psoriatic skin than in noninvolved and control skin (90). While historically the Th1 cell population has been well established to play a role in psoriasis, Th17 cells have been recently identified as distinct from the Th1 subset in the psoriatic dermis.
Increases in circulating Th1 and Th22 cells are also observed in psoriasis patients, but to a lesser extent than the Th17 population (6).

Interest in the Th17 population in relationship to psoriasis advanced when patients were shown to have increased numbers of circulating Th17 cells (6, 99) as well as cutaneous Th17 cells in lesional compared to non-lesional skin (82). The Th17 class of T cells exhibits a unique cytokine and transcription factor profile which is neither Th1- nor Th2-specific (73, 74). Th17 cells are controlled by the transcription factors STAT3 and RORγt; in humans they primarily produce IL-17A and IL-17F, and are associated with initiation of autoimmune and inflammatory conditions (5, 72, 75). Immunohistochemistry has confirmed the increases in IL-17F (101) and IL-17A protein in lesional psoriasis tissue. Serum levels of IL-17 protein have been found to correlate with psoriasis severity (99, 102).

Th22 cells exhibit epidermal homing and express the chemokine receptor CCR6 and the skin homing receptors CCR4 and CCR10, and consequently have been implicated in psoriasis and psoriatic arthritis (6, 76, 300, 301). IL-22 mRNA and protein are increased in psoriatic plaques when compared to normal skin while levels in peripheral blood do not vary significantly (302). Increased lesional mRNA levels of IFN-γ, IL-17 and IL-22 in psoriasis lesions normalize to non-lesional levels following cyclosporine treatment (82), further highlighting the role these cytokines play in potentiating inflammation in the plaque. Interestingly, IL-22 has very little autocrine effect, as neither T cells nor NK cells possess the IL-22 receptor yet keratinocytes in psoriatic skin demonstrate increased levels of IL-22R (303). Expression of IL-22R can also be up-regulated by other pro-inflammatory cytokines elevated in psoriasis such
as IFN-γ (304). Murine psoriasis models have demonstrated epidermal hyperplasia mediated in part by IL-22/22R signaling (83), but the IL-22 dependency is not observed in human disease.

The Th17/IL-23 axis is crucial for maintaining the chronic inflammation characteristic of psoriasis (91). IL-23 is a key cytokine involved in autoimmunity that promotes the expansion and maintenance of Th17 cells, providing a link between innate and adaptive immunity in psoriasis (92, 93). TGF-β in combination with IL-6 and IL-21 signals through STAT-3 to direct Th17 differentiation from naïve cells in vitro, while IL-23 is required in vivo (305). TGF-β and IL-1, in addition to IL-6 or IL-21, act as differentiation factors for Th17 cells while IL-23 promotes growth and stabilization of Th17 cells. Although IL-23 promotes release of IL-17A, it does not appear to effect IL-22 production (77). Th17 cells express the receptor for IL-23 (5, 94, 95) and overexpression of IL-23 in psoriatic lesions promotes Th17 cell cytokine production, potentiating a cycle of inflammation between IL-23-producing DCs and IL-17-producing Th17 cells present in lesional psoriatic dermis (96, 97).

**IL-17A in human psoriasis**

As described above, involved psoriatic plaques contain increased numbers of Th17 cells (99, 306). Th17 cells are localized to the dermis of psoriasis patients and IL-17mRNA expression increases with disease development and severity (82). RT-PCR shows a relative increase in IL-17A mRNA in psoriatic lesions when compared to controls (94). While Th17 cells are the best characterized producers of IL-17A in psoriatic skin (5, 75, 101), other investigators have suggested that the major sources of IL-17 in human psoriatic skin may not be the classic Th17 cell, but other cells
present in the inflammatory cell milieu (80). These investigators show, in a small
cohort of 8 psoriasis patients, that the majority of IL-17A producing cells in active
plaques were neutrophils and mast cells while less than 10% of the dermal T cell
population expressed IL-17A by immunofluorescence microscopy (80). A second
study also identified mast cells as major producers of IL-17A in healthy skin as well as
psoriasis tissue as well as an enrichment of IL-17A producing neutrophils compared
to control subjects (127). Flow sorted neutrophils from these patients produced IL-
17A but not IL-17F as determined by Western blot.

Another potential source of IL-17A in the dermis of psoriatic skin may be γδ-T
cells, although in human psoriasis patients, a clear pathogenic role for γδ-T cells has
not been demonstrated. The frequency of γδ-T cells present in lesional skin of
psoriasis patients has been suggested to be between 1 (307) and 42 percent (98) of
the CD3+ T cell population in the skin. Moreover, of the γδ-T cells in the psoriatic
dermis of the latter study, only 15% of these cells produced IL-17A after stimulation
with IL-23 (98). An overall reduction in circulating numbers of γδ-T cells was seen in
the blood of psoriasis patients (2.16%) when compared to normal controls (4.21%)
and atopic dermatitis patients (5.18%) (307) suggesting that it is unlikely for γδ-T cells
to be the sole sources of pathogenic IL-17.

Finally, CD8+ cells isolated from psoriatic lesions produce both IL-22 and IL-17
(118, 308). An in vitro analysis of isolated T cells that spontaneously migrated from
cultured dermal skin fragments demonstrated that CD8+ T cells were capable of
producing IL-17A. The CD8+ population from psoriasis skin contained a significantly
higher percentage of IL-17A producing cells when compared to normal skin (3.39 and
0.35%, respectively) (80) suggesting a possible role for this additional T cell population in contributing to overall IL-17A production in psoriasis. These observations may provide a mechanistic explanation for the pathogenesis ascribed to CD8+ T cells in psoriasis.

**IL-17 targeted human therapeutics**

Recently, two clinical trials have demonstrated the importance of IL-17 and the IL-17 receptor (IL-17RA) as therapeutic targets for psoriasis. In the first clinical trial, ixekizumab (previously known as LY2439821), a humanized monoclonal antibody that binds to and neutralizes IL-17A was evaluated in 142 psoriasis patients treated with either ixekizumab (10, 25, 75, or 150mg at 0, 2, 4, 8, 12, and 16 weeks) or placebo (183). In patients with chronic moderate-to-severe plaque psoriasis, significant reductions in Psoriasis Area-and-Severity Index (PASI) scores, an assessment tool used to measure the severity of psoriasis based on the erythema, scaling, and induration of the lesions weighted by the overall area of involvement (24), occurred within the first week of treatment in the two highest dose groups when compared to placebo. As early as two weeks a 75% reduction in PASI was seen in the 150mg group. After 12 weeks, a reduction in PASI score by at least 75% over baseline in the 150mg, 75mg, and 25mg treatment groups was observed and sustained for ≥ 20 weeks. In both the 150mg and 75mg groups, a 100% reduction in PASI score (defined as complete clearance of plaque) was achieved in 40% of patients when compared to the placebo group after 12 weeks.

A second clinical trial assessed the efficacy of brodalumab (previously known as AMG827), a monoclonal antibody that specifically blocks the IL-17 receptor A,
thereby neutralizing the effects of IL-17A, -17C, -17F, and 17E (184, 309, 310). In this trial, 198 psoriasis patients were treated with either placebo or brodalumab (70, 140, or 210mg at day 1 and weeks 1, 2, 4, 6, 8, and 10 or 280mg monthly). At the conclusion of the 12 week trial, the percentage improvement in PASI from baseline was above a mean percentage increase of 75% in the three highest dose groups, 45% in the 70mg group, and 16% in placebo. After 12 weeks, at least 90% improvement in PASI was seen in 72% of patients in the 140mg group and 75% in the 210mg group. Recently, Amgen has reported that patients taking brodalumab reported increases in suicidal ideation, leading the company to withdraw from its joint venture with Amgen in production and licensing of brodalumab (via the Amgen website: http://wwwext.amgen.com/media/media_pr_detail.jsp?releaseID=2052862).

Psoriasis and cardiovascular co-morbidities

A growing body of evidence suggests that psoriasis is associated with an increased risk of adverse cardiovascular events. Several studies have demonstrated that patients with psoriasis have an increased risk of numerous factors all associated with CVD (31, 38-41). More recently, it has been reported that patients with severe psoriasis have an increased risk of developing and dying of CVD independent of these risk factors (42, 44). Psoriasis patients have a higher prevalence of metabolic syndrome than the normal population (311) and this association increases with increased psoriasis disease severity (36, 37). Patients with mild to severe psoriasis have an increased risk of venous thromboembolism (49), hypertension (47), dyslipidaemia, myocardial infarction (46), and stroke (50, 51), while patients with
psoriasis >8 years have a higher prevalence of coronary artery disease (CAD) than patients without psoriasis (52).

A mechanism linking psoriasis pathogenesis and onset of CVD has not yet been elucidated, but a recent genome-wide association study (GWAS) found common genetic variants in psoriatic individuals that predispose them to increased risk of dyslipidemia, hypertension, and CAD, revealing an association between cardiovascular and metabolic disease genes with psoriasis (198). Additionally, the concept of a “psoriatic march” has been proposed where cardiovascular comorbidities and psoriasis are linked via a mechanism driven by the systemic inflammation observed in psoriasis (312, 313). This systemic inflammation is hypothesized to contribute to a state of insulin resistance, leading to endothelial cell dysfunction and resulting in atherosclerosis. Although epidemiological data has demonstrated that psoriasis patients are at risk of developing and dying of CVD (40, 42-46), the idea of causality cannot be definitively explored in humans as confounding factors are difficult to control for in the patient population.

We have recently shown using the skin-specific transgenic KC-Tie2 murine model of psoriasiform skin inflammation (190) that long term skin-specific inflammation may in fact have the capacity to cause systemic levels of inflammation sufficient to drive atherogenesis (193). KC-Tie2 psoriasiform mice develop skin lesions that are similar to human psoriasis, including areas of uninvolved and involved skin, increases in epidermal thickness (acanthosis), erythema, dermal angiogenesis, and increases in infiltrating T cells skewed towards the Th1/Th17 profile along with increases in DCs and macrophages and their derived cytokines, in particular IL-12/23
and TNF-α (190). These cutaneous characteristics of psoriasis were improved or reversed following gene repression, CsA administration (190), APC depletion using clodronate liposomes, TNF-α inhibition (223) and following chemical or surgical denervation (314, 315). Of particular relevance to cardiovascular comorbidities observed in the epidemiological human studies were our observations of elevated systemic inflammation, including increases in biomarkers associated with cardiovascular disease/risk including serum TNF-α, vascular endothelial growth factor (VEGF), IL-12, monocyte chemotactic protein-1 (MCP-1), S100A8/A9 and importantly systemic levels of circulating IL-17A (193). These increases occurred in the absence of hyperlipidemia or metabolic syndrome and preceded the spontaneous development of aortic root vascular lesions in 33% of the KC-Tie2 mice by one year of age. Moreover, KC-Tie2 mice were pro-thrombotic evidenced by arterial thrombosis clotting times being significantly shortened compared to controls. Of particular relevance to treating psoriasis patients, reversal of the skin disease in KC-Tie2 mice eliminated aortic root vascular lesions and returned thrombus formation to control mouse levels. This suggests that chronic sustained skin inflammation may be sufficient on its own, independent of hyperlipidemia or metabolic syndrome, to elicit levels of systemic inflammation sufficient to induce atherothrombosis. As a corollary to this observation, aggressive treatment of inflammatory skin disease may prevent cardiovascular disease co-morbidities. Recent results support this corollary in psoriasis patients (316), yet the specific mechanisms mediating this interaction have not yet been defined.
Th17 cytokines link psoriasis and CVD

The idea that Th17 cytokines may provide a link between psoriasis and CVD stems from independent literature reporting a pathogenic role of IL-17 in psoriasis (see above) and from murine and human studies examining the contribution of Th17 cells and their derived cytokines to atherosclerosis. Psoriasis patients with moderate to severe disease have significantly elevated serum IL-17A levels (99, 102). Patients with elevated IL-17A appear to be at the highest risk for developing and dying from cardiovascular complications including stroke and MI. With the advent of IL-17 biologics and their known efficacy in significantly improving psoriasis disease severity, time will tell whether this targeted inhibition of IL-17 will also lead to improved CVD outcomes in psoriasis patients.

In human atherosclerotic lesions, IL-17A/IL-17F are elevated at each stage of plaque development (317), are thought to be derived from mast cells and neutrophils, and increase in the late stage, more advanced plaque. CD4+ T cells also infiltrate athero-susceptible arteries and express both IFN-γ and IL-17 concomitantly (318); this combination of cytokines has been demonstrated to produce a synergistic effect on cultured human vascular smooth muscle cells (VSMC), leading to the secretion of IL-6, CXCL8, and CXCL10 which are pro-inflammatory cytokines and chemokines elevated in atherosclerosis. Moreover, patients with acute coronary syndrome (defined as acute myocardial infarction or unstable angina) show increased levels of Th17-related mediators such as circulating RORγt, plasma IL-17, IL-6, and IL-23 along with decreased levels of circulating Foxp3, plasma IL-10 and TGF-β, all associated with regulatory T cells (Treg) (319), demonstrating a cytokine and cellular milieu
reminiscent of psoriasis (Table 4.1). The profile of a proinflammatory cytokine milieu, increased monocyte/macrophage levels, and decreased Foxp3 in acute coronary syndrome is also reflected in psoriasis (112).
Table 4.1: Common cell types and mediators in psoriasis and atherosclerosis

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T cells have also been demonstrated to play a critical role in atherosclerotic plaque formation in murine models. ApoE\(^{-/-}\) mice fed a high fat diet express higher levels of IL-17 and IFN-\(\gamma\) in CD4\(^+\) T cells and macrophages that infiltrate the atherosclerotic plaque in the carotid vascular wall during early (16wk) and late (24wk) atherosclerosis (320) and demonstrate increases in circulating IL-17A levels (321). Splenic ROR\(\gamma\)t and IL-17A levels are also increased in apoE\(^{-/-}\) mice with the greatest differences observed early in the onset of atherogenesis suggesting a pathogenic role for the increase (322). Aortic CD4\(^+\) T cells and \(\gamma\delta\)^+ T cells from apoE\(^{-/-}\) mice produce increased amounts of IL-17A when fed a high-fat diet (321). The strongest evidence for a role for IL-17A in atherogenesis is the observation that functional inhibition of IL-17A using monoclonal antibodies (139) or adenovirus delivered fusion blocking protein (323) in apoe/-/- mice fed a high fat high cholesterol diet leads to reduced atherosclerotic lesional area, as well as a decrease in the number of aortic root plaque infiltrating CD3\(^+\) T cells (324) and decreases in serum IL-6, G-CSF, CXCL1 and infiltrating aortic macrophages (138). Moreover, apoE/-/- animals back crossed to IL-17/-/- mice develop significantly less atherosclerotic plaque, containing less macrophage infiltrate and have reduced levels of MCP-1, IL-1\(\beta\), IL-6, IFN-\(\gamma\), and IL-12p40 (323).

**Conclusion**

Psoriasis presents the opportunity to examine and isolate various pathologic cell subsets including IL-17-producing Th17 cells. The ability to obtain these cells makes psoriasis a unique disease that is amenable to isolation of primary cells that are rarely available from other diseases such as atherosclerosis. Because many key
immunologic mechanisms are shared between psoriasis and atherosclerosis, lessons learned from Th17 cells isolated from psoriatic tissue regarding Th17 cell development and activity, Th1-Th17 interplay, monocyte/macrophage interaction with pathogenic T helper cell subsets, etc, may provide key insight into pathological processes occurring in atherogenesis as well. Indeed, it is possible that patients with co-morbid diseases such as psoriasis and atherosclerosis may be at risk for cellular mediators from one disease site exacerbating the co-morbid condition. For instance, skin inflammation associated with increased levels of Th17 cells and their derived cytokines may release pro-inflammatory mediators or even cells themselves that may initiate or participate in other co-morbid conditions such as atherosclerosis, metabolic syndrome or inflammatory bowel disease. If the development of co-morbid conditions is precipitated by inflammatory mediators that initiate with skin disease (in the case of psoriasis) then it is logical to predict that aggressive treatment of the initiating factor may also mediate resolution of the co-morbidities associated with disease. Thus it will be of great interest to follow psoriasis patients treated with current therapeutic regimens, especially the new class of IL-17 inhibitors to determine if a concurrent decrease in co-morbid pathologies is also decreased.
CHAPTER 5

ONGOING WORK AND FUTURE DIRECTIONS
In order to extend our observation that psoriasis patients have elevated percentages of intermediate monocytes (see Chapter 2, Figure 2.2) and increased percentages of circulating monocyte-monocyte doublets (see Chapter 2, Figure 2.3), we designed a cross-sectional human study to observe the effects of standard-of-care psoriasis systemic treatments on biomarkers of cardiovascular disease, serum markers, and circulating immune cell populations.

A secondary goal of the study is to determine if improvement in psoriasis severity, as measured by PASI and/or BSA (Body Surface Area), correlates with decreases in these related biomarkers. Additionally, we sought to determine if these decreases correlated with decreases in coronary artery inflammation, as visualized by positron emission tomography-magnetic resonance imaging (PET/MRI) with fluorodeoxyglucose (FDG), and carotid artery calcium scoring (CACS) following one year of therapy.

To assess alterations in the overall monocyte expression profile of these psoriasis patients, we monitored patients at baseline and at various time points during one year of standard therapies (see Chapter 1, pages 34-36), which included some patients on biologic therapies. Blood and serum samples were taken at 4, 12, 24, 36, and 52 weeks post-treatment. PET-MRI/FDG and CACS plaques measurements were taken at the baseline and 52-week visits to determine if arterial inflammation had decreased. Additionally, we wanted to determine if any change in arterial inflammation correlated with biomarker fluctuation.
Modulation of monocyte phenotype, including surface expression profiles, was especially interesting as we wanted to determine whether intermediate monocytes or myeloid derived suppressor cell (MDSC) populations were altered following standard-of-care therapy, possibly implicating a role for them in potentiation of disease and response to therapy. MDSCs, an immature monocyte population, were included as another outcome variable for this study.

The first year of the two year study has been completed with the recruitment of 22 patients and active enrollment of 19 patients (see Table 5.1 for demographics). Patient median PASI was 22.3 (ranging from 10.2-46.7), indicating that moderate to severe plaque psoriasis patients were predominantly enrolled. These patients had a median disease duration of 13.5 years (with a range of 0-48 years; several patients were newly diagnosed, with recent onset) and a median age of 39 (range of 19-65). The current cohort is 70% male and 30% female, with 80% identifying as Caucasian and 20% identifying as African American. Forty five percent are smokers. Within the current cohort, we have assessed HLA-Cw6 expression (see Chapter 1, page 22) in 10/19 individuals and interestingly, only 50% are positive for the mutation. Using body mass index (BMI) guidelines set by the NIH (http://www.nhlbi.nih.gov/health/educational/lose_wt/risk.htm), 20% of the patients were in the normal weight range (BMI: 18.5-25); 30% of the patients were overweight (BMI: 25-29.9); and 50% of the patients were considered obese (BMI >30).

Among these patients, we have confirmed associations reported in our original psoriasis cohort (Chapter 2), including a positive correlation between PASI and doublet percentages at the baseline visit, before undergoing treatment (Fig 5.1A).
This correlation is no longer significant after one month (Fig 5.1B), 3 months (Fig 5.1C), or 6 months of treatment (Fig 5.1D), indicating that the systemic treatments may alter monocyte aggregates which are present in the monocyte population of untreated psoriasis patients.
Table 5.1: Patient demographics of human comparative effectiveness research

<table>
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<tr>
<th>Patient</th>
<th>Age</th>
<th>Disease Duration</th>
<th>PASI</th>
<th>BSA</th>
<th>Gender</th>
<th>Race</th>
<th>Blood Pressure</th>
<th>BMI</th>
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<td>W</td>
<td>122/81 Hyp</td>
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<td>125/81 Hyp</td>
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<td>W</td>
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<td>36.7</td>
<td>N/A</td>
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Figure 5.1: Correlations of doublet percentage and psoriasis disease severity as measured by PASI.

(A) There is a statistically significant ($p=0.023$), positive correlation between disease severity as measured by PASI and doublet percentage at baseline. (B) The correlation is no longer statistically significant after 1 month of therapeutic treatment, (C) 3 months of treatment, or (D) 6 months of treatment.
After one month of systemic psoriasis treatment, PASI and intermediate monocyte percentage decrease from baseline levels (as shown in the top row of Figure 5.2). The PASI scores continue to trend downward through 9 months of treatment for most patients (Figure 5.2, first column) while classical monocyte populations do not change significantly over time (Figure 5.2, middle column).

A statistically significant negative correlation was observed between classical monocytes (CD14++CD16\textsuperscript{neg}) and MDSC percentage at baseline (Figure 5.3A), while a statistically significant positive correlation was observed between intermediate monocytes (CD14++CD16\textsuperscript{+}) and MDSCs (Figure 5.3B). This positive correlation however, is not sustained at 3 months or 6 months post-treatment, much like the doublet and PASI relationship observed in Figure 5.1, suggesting that systemic medications alter the monocyte phenotype observed at baseline in psoriasis patients and that myeloid cells respond to therapy.
Figure 5.2: Time course of changes in PASI and monocyte populations (intermediate and classical) from baseline following systemic therapy at subsequent time points.

**Top row:** Change from Baseline to 1 month (n=11). **Second row:** Change from 1 month to 3 months (n=8). **Third row:** Change from 3 months to 6 months (n=7). **Bottom row:** Change from 6 months to 9 months (n=4).
Figure 5.3: Correlations at baseline of circulating blood monocyte populations with MDSC percentage.

Percentages are among total live PBMCs. (A) Classical monocytes and (B) intermediate monocytes at baseline demonstrate significant correlations with MDSC % at baseline ($r=-0.559$, $p=0.038$ and $r=0.632$, $p=0.015$, respectively). Time course of correlation between intermediate monocytes and MDSC % at (C) 3 months and (D) 6 months post-treatment. The correlations in C and D are not statistically significant, although the number of observations is decreased compared to baseline.
Once we finish enrollment and compile the data over multiple time points, we can begin to stratify the patients based on type of medication, age, gender, smoking status, hypertension, etc. Baseline PET/MRI results suggest that measurement of FDG uptake in aorta is more reliable versus carotid. Therefore, we have adjusted our analyses to include this modality. Patients will have a second PET/MRI at one year post-treatment, and from this data we will be able to assess if decreases in monocyte biomarkers correlate with decreases in aortic inflammation.

Furthermore, we will determine if changes in monocyte phenotype (biomarkers) are able to predict whether or not individuals were responsive to particular therapeutics. The concept of change in monocyte phenotype as a predictive biomarker for clinical response has been recently demonstrated in the literature in other immune-centric diseases. One group reported change in absolute numbers of total monocytes as well as numbers within the monocyte subsets to predict whether RA patients would respond to standard methotrexate therapy (325). Another group reported that specific increases in the classical monocyte subset alone accurately indicated chronic myelomonocytic leukemia (CMML) in patient cohorts (326). Initial statistical analysis of our preliminary results has identified nominal significant differences in several phenotypic combinations in our cohort which are currently being investigated, and lead to the promise of using myeloid response as a biomarker to identify psoriasis patients responding to systemic treatments. It may be possible to also use these myeloid responses to predict whether a patient will respond to any particular type of therapy better than alternative therapeutics. Finally, alterations in the myeloid population may also be predictive of therapies that, in addition to
improving skin phenotype, also improve associated risk outcomes for psoriasis patients, including potential cardiovascular complications.

**Murine Studies**

The murine psoriasiform model, KC-Tie2, exhibits increased vascular thrombosis, monocytosis, and increased monocyte doublets (as discussed in Chapter 1, pages 36-38 and as shown in Figure 2.11) when compared to WT controls, indicating the presence of a skin-driven inflammatory component in these mice. We used this model to determine potential links between CVD and psoriasis as we can modulate mechanisms in the mouse model that we are not able to modify in humans.

To begin to study the potential interactions between psoriasis and cardiovascular disease, we first looked at S100A8/9 (also known as MRP8/14 or calprotectin), a complex of S100A8 and S100A9, two small calcium-binding proteins and damage associated molecular pattern molecules (DAMP) involved in neutrophil chemotaxis and adhesion to fibrinogen (327, 328). S100A8/9 is elevated in human psoriasis patients and is predictive of potential development of CVD (328-330). Furthermore, S100A8/9 genes are located within the psoriasis PSORS4 susceptibility region (331), and serum and skin S100A8/9 levels are upregulated in psoriasis patients (332). S100A8/9 molecules can also participate in a pro-inflammatory feedback loop, as introduced in Chapter 1, page 34. However, the significance of S100A8/9 protein in psoriasis pathogenesis remains unclear.

S100A9-deficient (-/-) mice develop less atherosclerosis and are protected against thrombosis (333) whereas KC-Tie2 mice have elevated skin and serum S100A9 in addition to being pro-thrombotic (190, 193). We therefore hypothesized
that genetic deletion of S100A9 on a KC-Tie2 genetic background strain would improve psoriasiform skin disease and rescue the pro-thrombotic phenotype.

Unexpectedly, KC-Tie2 mice backcrossed to S100A9-/- mice had sustained skin inflammation and did not demonstrate improvements in skin disease as KC-Tie2xS100A9-/- mice demonstrate significantly increased epidermal thickness (acanthosis) when compared to WT littermate controls and S100A9-/- mice. No improvement in gross pathology (Figure 5.4A) or acanthosis (Figure 5.4B) was apparent in KC-Tie2 x S100A9-/- skin vs. KC-Tie2 mouse skin.

Furthermore, KC-Tie2 x S100A9-/- mice are pro-thrombotic and do not have prolonged clotting times when compared to KC-Tie2 mice (Figure 5.4C), as determined by the Rose-Bengal occlusive thrombosis assay (for an explanation of methods, please see Chapter 3, pages 89-90). This indicates that the deletion of S100A9, a molecule known to be involved in CVD, is not sufficient to rescue the pro-thrombotic phenotype of the KC-Tie2 mouse.

As previously indicated, increased levels of monocytosis correlate with presence of skin disease in our second model of chronic psoriatic disease, K5-IL-17C, and in the IMQ-induced acute psoriasiform model (see Figure 3.2A, 3.2B, and 3.3). We now demonstrate increased CD11b$^+$ Ly6C$^{high}$ monocytes in LNs of KC-Tie2 mice on the C57Bl/6 background when compared to WT C57Bl/6 controls as shown in Figure 5.5B, (65.1% ± 13.7; n=19; solid triangles versus 18.3% ± 5.6; n=7; solid circles; p<0.0001). These cells remain elevated in KC-Tie2 x S100A9-/- animals when compared to WT controls, also shown in Figure 5.5B, (58.2% ± 7.4; n=3; solid diamonds; p=0.017). There is no significant difference between KC-Tie2 and KC-Tie2
x S100A9/- mice. Representative flow data is shown in Figure 5.5A; for gating strategy, see Figure 3.1.

We also observed increased CD11b$^+$ Ly6C$^{\text{high}}$ monocytes in spleens of KC-Tie2 mice when compared to WT controls as shown in Figure 5.5C, (66.0% ± 19.8; n=7; open triangles) versus 18.6% ± 7.3; n=9; open circles; p=0.0002). These splenic cells remain elevated in KC-Tie2 x S100A9/- animals when compared to WT controls, as shown in Figure 5.5C, (63.5% ± 15.1; n=3; open diamonds; p=0.009). As observed in LNs, there is no significant difference in splenic CD11b$^+$ Ly6C$^{\text{high}}$ monocytes between KC-Tie2 and KC-Tie2 x S100A9/- mice. It is worth noting that more replicates of the S100A9/- mice are necessary; we are currently breeding in anticipation of this and will add more to our numbers.

The increases in CD11b$^+$ Ly6C$^{\text{high}}$ monocytes in these mice correlate with observed increases in skin disease as measured by epidermal thickness, indicating that increased circulating immune cells correlate with an overall level of increased inflammation. The S100A9 deletion in KC-Tie2 mice fails to improve skin disease, decrease circulating levels of pro-inflammatory monocytes, or rescue prolonged thrombosis clotting times.
Figure 5.4: KC-Tie2 and KC-Tie2 x S100A9/- mice exhibit sustained skin inflammation

(A) KC-Tie2 mice on a C57Bl/6 background were mated with S100A9-deficient (-/-) mice (also on a C57Bl/6 background). Adult KC-Tie2 and KC-Tie2 x S100A9/- mice appear similar in terms of gross phenotype. (B) Representative H&E-stained dorsal skin sections from control, S100A9/-, KC-Tie2, and KC-Tie2 x S100A9/- mice. Quantification is shown below the H&E images. (C) S100A9/- mice demonstrate longer times to occlusive thrombus formation compared to non-transgenic littermate controls, suggesting that S100A9 is protective. KC-Tie2 mice had decreased clotting times, however KC-Tie2 x S100A9/- also showed significantly shorter clotting times vs. control mice. *P<0.05 vs. littermate controls. n = 9-12/group (B) and n = 14-20/group (C).

The data in this figure was completed in collaboration with members of the laboratories of Drs. Nicole Ward and Daniel Simon.
Figure 5.5: CD11b$^+$Ly6C$^{\text{high}}$ monocytes are increased in KC-Tie2 mice and remain elevated in KC-Tie2 x S100A9$^-$/- animals

(A) Representative flow cytometry of CD11b$^+$Ly6C$^{\text{high}}$ cells isolated from control, KC-Tie2, S100A9$^-$/-, and KC-Tie2 x S100A9$^-$/- skin draining lymph nodes (LN). (B) Quantification of CD11b$^+$Ly6C$^{\text{high}}$ monocytes in LN from WT control, KC-Tie2, S100A9$^-$/- and KC-Tie2 x S100A9$^-$/- animals. A significant increase is observed in KC-Tie2 mice (p<0.0001; n=19) and KC-Tie2 x S100A9$^-$/- (p=0.017; n=3) animals compared to controls (n=7). (C) Similar findings are observed in the spleen; a significant increase is observed in KC-Tie2 mice (p=0.0002) and KC-Tie2 x S100A9$^-$/- (p=0.009; n=3) animals compared to controls.
Interestingly, PCR analysis of dorsal skin of KC-Tie2 x S100A9/-/- mice revealed increases in several pro-inflammatory cytokines at the RNA level such as IL-17A and IFN-γ (Figure 5.6A and B, respectively). IL-6, a cytokine known to play a role in psoriasis and cardiovascular disease, was increased at both the RNA and protein level (protein determined by ELISA; Figure 5.6C and D, respectively).

In humans, increased IL-6 expression is observed in psoriatic dermis, contributing to downstream T cell dysfunction characterized by improper Treg suppression of Teff cell mechanisms (113, 115). Additionally, keratinocytes participate in a pro-inflammatory auto-feedback loop where they sense increased secretion of keratinocyte-derived S100A8/9 and begin to upregulate production of IL-6 (165). IL-6 can also serve as a differentiation factor for Th17 polarization (as discussed in Chapter 4, page 110). Finally, IL-6 associates with cardiovascular disease (334, 335). More specifically, increases in serum concentrations of IL-6 correlate with an increased risk of developing a CVD event in both men and women (336, 337).

Therefore, we hypothesized that IL-6 was playing a role in sustaining the prolonged thrombosis clotting times observed in the KC-Tie2 x S100A9/-/- mice. Subsequently, we backcrossed the KC-Tie2 mice with IL-6 deficient (-/-) mice in an effort to improve skin disease and thrombosis outcomes.
Figure 5.6: KC-Tie2 and KC-Tie2 x S100A9-/- mice demonstrate increases in IL-6 at the RNA and protein level

(A) IL-17A and (B) IFN-γ are increased in KC-Tie2 x S100A9-/- mice at the RNA level when compared to WT controls. IL-6, a cytokine known to be important in psoriasis and chronic inflammation, is also increased at the RNA level (C) and the protein level (D).

This work was completed in collaboration with members of the laboratory of Dr. Nicole Ward.
The KC-Tie2 x IL-6-/- mice improved (resembling WT controls) at the gross pathology level (Figure 5.7A). However, KC-Tie2 x IL-6-/- mice did not demonstrate statistically improved skin inflammation, as measured by epidermal thickness, when compared to KC-Tie2 mice (30.4μm ± 14.5; n=22 versus 35.5μm ± 16.0; n=10, respectively; Figure 5.7B and quantification in D), but it worth noting that acanthosis measurements did trend downward toward control (7.3μm ± 0.73; n=4) levels.

This same trend is mirrored in quantification of immune cells from H&E sections. KC-Tie2 mice, when compared to WT controls, have statistically increased numbers of CD11b (140.4 ± 34.3; n=7 versus 59.8 ± 13.9; n=4, respectively; p=0.006, Figure 5.8B), CD8 (14.6 ± 9.7; n=7 versus 2.6 ± 2.1; n=4, respectively; p=0.006, Figure 5.8C), and F4/80 (125.6 ± 36.4; n=7 versus 76.7 ± 7.3; n=4, respectively; p=0.042, Figure 5.8D), yet CD4 counts do not demonstrate the same increase. These immune cells counts do not statistically decrease in KC-Tie2 x IL-6-/- mice when compared to KC-Tie2 animals, but they do trend downward. CD4 counts (Figure 5.8A), however, increase in KC-Tie2 x IL-6-/- when compared to KC-Tie2 (17.3 ± 7.6; n=8 versus 15.2 ± 7.7; n=7, respectively).

Importantly, KC-Tie2 mice backcrossed with IL-6-/- mice did exhibit prolonged thrombosis clotting times when compared to KC-Tie2 mice (30.0 min ± 10.8; n=13 versus 15.8 min ± 7.6; n=20, respectively, p=0.0001; Figure 5.7C, dark grey bar versus white bar) and WT controls (28.5 min ± 12.4, n=19; p=0.0003; Figure 5.7C, black bar), indicating that the deletion of IL-6 improved thrombosis outcomes. This suggests that IL-6 may be sufficient for promotion of thrombosis in this particular murine model.
Figure 5.7: KC-Tie2 mice backcrossed to IL-6 knockout mice have sustained skin inflammation but are not pro-thrombotic

(A) KC-Tie2 x IL-6-/- mice resemble more closely WT controls than age-matched KC-Tie2 mice at the gross phenotype level; however at the histological level shown in (B), KC-Tie2 and KC-Tie2 x IL-6-/- mice have similar levels of acanthosis. (C) Consistent with our prior reports, KC-Tie2 mice have a shortened time to occlusive thrombus formation (p=.0003). KC-Tie2 x IL-6-/- mice, despite having sustained skin inflammation, exhibit clotting times resembling control mouse levels; n = 13-20/group. (D) Quantification of epidermal thickness measures as shown in (B). KC-Tie2-IL-6-/- mice demonstrate decreased acanthosis when compared to KC-Tie2 mice, although not statistically significant.

The thrombosis data in this figure (panel A) was completed in collaboration with members of the laboratories of Drs. Nicole Ward and Daniel Simon.
Figure 5.8: Immune cell counts in KC-Tie2-IL-6-/- mice

KC-Tie2 x IL-6/- mice resemble more closely WT controls than age-matched KC-Tie2 mice at the gross phenotype level; however, KC-Tie2 and KC-Tie2 x IL-6/- mice have similar levels of immune cell infiltrate as shown for (A) CD4, (B) CD11b, (C) CD8, and (D) F4/80; n = 4-8/group.
As shown in Figure 5.5, KC-Tie2 mice have increased lymph node-derived and splenic CD11b⁺Ly6C<sup>high</sup> monocytes compared to controls as measured by flow cytometry (Figure 5.5B and C; triangles versus circles), suggesting that chronic skin inflammation may generate Ly6C<sup>high</sup> cells which mediate vascular inflammation. Using the KC-Tie2 x IL-6<sup>−/−</sup> model where thrombosis is protected, we monitored percentages of CD11b⁺Ly6C<sup>high</sup> monocytes in both the spleen and lymph nodes. Eliminating IL-6 in the KC-Tie2 model significantly decreased CD11b⁺Ly6C<sup>high</sup> monocytes in LNs of KC-Tie2 x IL-6<sup>−/−</sup> when compared to KC-Tie2 mice as shown in Figure 5.9B, (55.0% ± 13.8; n=19; plus signs versus 65.1% ± 13.7; n=19; solid triangles; p=0.014). Crossing IL-6<sup>−/−</sup> mice with KC-Tie2 decreases the lymph node-derived CD11b⁺Ly6C<sup>high</sup> monocytes but it does not decrease it in the spleen (Figure 5.9C, X signs versus open triangles). Representative flow data is shown in Figure 5.9A; for gating strategy see Figure 3.1.

Backcrossing KC-Tie2 with IL-6<sup>−/−</sup> mice did rescue the pro-thrombotic phenotype when compared to controls (Figure 5.7C) and decreased skin-draining LN-derived monocytes compared to KC-Tie2 mice (Figure 5.9B), but did not decrease skin-resident immune cells (Figure 5.8) or significantly improve skin disease as measured epidermal thickness (Figure 5.7B and D). This suggests that the promotion of thrombosis may occur independently of skin inflammation but may still be linked to monocytosis in KC-Tie2 mice, and suggests a role for skin derived-IL-6 in promotion of thrombosis. This also suggests that mediating levels of IL-6 expression in humans could potentially decrease levels of CVD events and/or symptoms of psoriatic disease.
Figure 5.9: CD11b+Ly6C<sup>high</sup> monocytes decrease in the lymph nodes, but not spleens, of KC-Tie2 x IL-6<sup>-/-</sup> mice

(A) Representative flow cytometry of CD11b<sup>+</sup> Ly6C<sup>high</sup> monocytes isolated from the skin draining LNs of IL-6 and KC-Tie2 x IL-6<sup>-/-</sup> mice. (B) Quantification of LN biological replicates comparing control, KC-Tie2, IL-6<sup>-/-</sup> and KC-Tie2 x IL-6<sup>-/-</sup> animals. WT and KC-Tie2 percentages are reported in Figure 5.5. CD11b<sup>+</sup>Ly6C<sup>high</sup> cells in LNs reveal significant increases in pro-inflammatory monocytes in KC-Tie2 vs. control mice. These levels decrease in the LN yet remain increased in KC-Tie2 x IL-6<sup>-/-</sup> mice in the spleen (C), despite a return of thrombosis clotting times to control mouse levels (see Figure 5.7).
Conclusions and Future Directions

Psoriasis is an immune-mediated disease of the skin characterized by red, raised, scaly plaques that is accompanied by several co-morbidities such as psoriatic arthritis, obesity, and metabolic syndrome (9, 338, 339). One of the most recently well-defined co-morbidities of psoriasis is CVD. Many epidemiological studies have demonstrated that patients with moderate to severe psoriasis have a greater risk of developing CVD (42, 44, 46, 50, 53, 54, 202, 205, 249, 279, 290, 316). In individuals with CVD, increases in pro-inflammatory monocytes (both classical and intermediate) have been reported to correlate with and predict an increased risk of developing disease (144, 212). Our psoriasis cohort demonstrated increased percentages of these circulating intermediate monocytes, indicating the presence of systemic inflammation and suggesting a potential role for these cells in psoriasis pathology. As intermediate monocytes are also known to play an important role in CVD, we hypothesized the potential presence of a common immune mechanism mediating both psoriasis and CVD.

In addition to increased circulating intermediate monocytes, we report that our cohort of psoriasis patients demonstrate increased circulating monocyte aggregates. The discovery of a monocyte-monocyte aggregate in the absence of platelets is novel, and the observed increased adhesion phenotype could be involved in mediating reported cardiovascular comorbidities. Increases in intermediate monocytes and monocyte-monocyte doublets both correlate with disease severity as measured by PASI, and the correlations are perturbed when patients begin therapeutic treatments, suggesting that there is an underlying monocyte dysfunction present in psoriasis.
patients. This monocyte dysfunction could, perhaps, be indicative of the corresponding CVD co-morbidities.

Further research is needed to characterize the phenotype of the monocyte doublet pairs and better determine the mechanism driving their adhesion. Additionally, characterization of the pairs monocytes bound to other monocytes (monocyte:monocyte) versus monocytes bound to T cells (monocyte:T cell) will be informative. Monocyte:monocyte doublet pairs may utilize a completely different mechanism of adhesion compared to monocyte:T cell doublet pairs. Furthermore, the mechanism of doublet formation and/or breaking apart of the doublet pairs must be assessed. One experimental approach to address this question would be the use of blocking antibodies targeted at integrins and actin, in an attempt to characterize the conjugation that is forming at the interface of the two bound monocytes. Once we understand what triggers and mediates the doublet formation, we can develop targeted therapies that will block this interaction and decrease the numbers of circulating doublets in human psoriasis patients.

Another way to visualize doublet interactions could be to use confocal imaging and staining of focal adhesion kinase and/or integrins. The concern with staining for integrins is that they will be in an open, or active, conformation and therefore not be amenable to staining by antibodies that might recognize the bent, or inactive, conformations. Function-blocking antibodies that are aimed at interrupting the active conformation may be interesting in that they may provide a mechanism to inhibit the doublet formation if we can pinpoint which integrin(s) is/are responsible for the adhesive interactions. Either way, a more comprehensive characterization of these
sub-populations, perhaps including the use of deep sequencing of the monocyte:monocyte versus monocyte:T cell pairs and/or visualization of the doublet pair interactions, will lead to an improved understanding of cellular interactions in the blood of psoriasis patients which could potentially participate in psoriatic and/or CVD plaque formation.

Once we can determine what is causing aggregates to form, we can begin to parse out a mechanism of doublet formation and downstream significance. Once such potential mechanism is shown in Figure 5.10. Here, we hypothesize that the activated endothelium of psoriasis patients releases pro-inflammatory cytokines such as IL-6, TNF-α, and IL-17A resulting in platelet activation (Figure 5.10A). Circulating, patrolling non-classical monocytes (CD14\textsuperscript{neg}CD16\textsuperscript{++}) encounter the activated endothelium and potentially become activated, maybe by upregulating surface integrins and/or binding to platelets. The activated non-classical cell then encounters a circulating classical monocyte in the bloodstream, and doublet formation occurs, with or without platelets (Figure 5.10B and C). Communication and/or physical contact between the cells activates the classical monocytes, which then differentiate to an intermediate phenotype (Figure 5.10D). Increases in the intermediate monocyte population and monocyte doublets lead to the chronic inflammation observed in psoriasis (Figure 5.10E).

This hypothesis would require that the doublets seem to “dis-aggregate” on their own by an unknown mechanism in order for the classical monocyte to differentiate into an intermediate monocyte. However, the overall idea of doublets
being a mechanism for monocytes to transmit a differentiation signal to one another is interesting. We did not see any differences in the patrolling monocyte populations between psoriasis and control cohorts, but we did see that the majority of doublets in the intermediate gate were not intermediate monocytes bound to other intermediate monocytes, but non-classical bound to classical monocytes. As the observation of the presence of an abnormal intermediate doublet population is specific to the psoriasis cohort, it seems as though these doublets are playing a role in the disease pathology, and potentially participate in the overall activation of the singlet intermediate population increase.

While we do not have evidence that this is the only mechanism of doublet formation and cell-cell signaling, once we understand the interaction we can more specifically target it and determine if the classical monocytes directly differentiate into intermediate monocytes.
Figure 5.10: Hypothesized mechanism of doublet formation and monocyte differentiation in humans

(A) Activated endothelium releases pro-inflammatory cytokines such as IL-6, TNF-α, and IL-17A and stimulates platelet activation. Patrolling non-classical monocytes (CD14negCD16++) encounter the activated endothelium and potentially upregulate surface integrins and/or bind to platelets. (B and C) Doublet formation occurs between the activated non-classical monocyte and a classical monocyte, with or without platelets. (D) Communication between the cells activates the classical monocytes, which then differentiate to an intermediate phenotype. (E) Increases in the intermediate monocytes and doublets lead to the chronic inflammation observed in psoriasis.
Another potentially interesting set of experiments could be designed to answer the question of whether or not increased myeloid/monocyte precursors are emerging from the bone marrow and where/when the CD14 and CD16 differentiation occurs. This transition has been better defined in mouse than in human, but one report of a human cohort indicates that the myeloid precursors in the bone marrow most closely resemble intermediate monocytes when surface expression and functionality is considered (340). When only surface expression of CD14 and CD16 is considered, the cells more closely resemble the classical CD14⁺CD16⁻ population, indicating that interactions in circulation may influence cell differentiation. These samples were not taken from psoriasis patients, so it would be very interesting to compare psoriasis bone marrow samples to controls and determine if there is a different starting population of cells that egress from the marrow, or if the local environment of the blood vessels influences their differentiation.

Still, the question remains as to what role both CD14 and CD16 play in monocyte differentiation and/or function. What happens if you stimulate CD14 - could this lead to doublet formation? Our research so far does not indicate a functional role for CD14 in psoriasis monocytes, but stimulation with an endogenous ligand such as LPS, fibrinogen, or S100A8/9, might answer the question more completely. Additionally, CD16 is an Fc complex receptor. It is possible that there are Fc complexes circulating in psoriasis that these receptors are responding to, but this is not a well-characterized phenomenon of psoriasis. Furthermore, as we observe increases in the CD16⁺ populations following culture of monocytes on tissue culture plastic, there seems to be an alternative, unidentified mechanism of upregulation of
CD16 expression. However, this does indicate that CD16 plays some unidentified functional differentiation role of classical monocytes to intermediate monocytes.

There is a lot of remaining analysis regarding the correlation of clinical data with monocyte biomarkers. Questions remain such as: Does the monocyte transition in the blood measure or correlate with inflammatory changes in the skin? What comes first – does psoriasis cause its associated comorbidities or do these comorbidities lead to an overall chronic inflammation that initiates psoriasis? Future studies should focus on stratifying the psoriasis population by disease severity, duration, or perhaps doublet formation, and attempt to identify if any of these measures are driving the increase in observed intermediate populations.

As immune depletion studies are not feasible in humans, we utilized murine models to study the effects of monocytes and cardiovascular disease. The KC-Tie2 psoriasiform model demonstrates characteristic hallmarks of psoriasis such as plaque formation, increased dermal immune cell infiltrate, and increased levels of serum pro-inflammatory cytokines. Interestingly, these mice also develop aortic inflammation and are pro-thrombotic. Finally, these mice recapitulated the monocyte doublet formation that we observed in our human cohorts.

Visualization of the doublet pairs was informative in human patients, indicating that while platelets did participate in monocyte-monocyte doublet formation, the doublet pairs could occur in their absence as well. To confirm that the doublet formation is indeed occurring in the same manner in the murine model, future experiments should target visualization of these monocyte pairs in KC-Tie2 mice. Once the monocyte-monocyte interactions are confirmed, along with the necessity of
platelets for these interactions, then a mechanism of doublet formation can begin to be assessed. Furthermore, the monocyte-platelet aggregates present in these mice should be quantified.

In an attempt to improve the CVD phenotype of these mice, we backcrossed KC-Tie2 mice with two mouse strains deficient in molecules important in human psoriasis: S100A9<sup>-/-</sup> and IL-6<sup>-/-</sup>. While deletion of S100A9 in KC-Tie2 was not sufficient to rescue the pro-thrombotic phenotype, deletion of IL-6 did result in prolonged thrombosis times and improved the CVD outcome. Furthermore, circulating pro-inflammatory monocyte (CD11b<sup>+</sup>Ly6C<sup>high</sup>) levels in the KC-Tie2 x IL-6<sup>-/-</sup> mice were decreased from levels seen in KC-Tie2 mice, suggesting that perhaps the monocytes participated in mediating the thrombosis.

While this is encouraging as a potential link between psoriasis and CVD events, the observed correlation with monocytosis and thrombosis outcomes was not replicated in a separate model of chronic psoriasis, K5-IL-17C, or in an acute IMQ-induced model. Here, CD11b<sup>+</sup>Ly6C<sup>high</sup> monocytes remained elevated independent of thrombosis outcome. This indicates that different approaches to identifying the thrombotic and CVD mediators are necessary to completely resolve the CVD phenotype across all models. These studies would ideally lead us to a candidate molecule or gene that could be targeted in humans to simultaneously improve disease in psoriasis patients and their associated CVD events.
August 10, 2015

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