Effect of Nitric Oxide on Myeloid Dendritic Cell Adhesion

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Effect of Nitric Oxide on Myeloid Dendritic Cell Adhesion

by

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ABSTRACT

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Effect of Nitric Oxide on Myeloid Dendritic Cell Adhesion

Director of Thesis: Fabian Benencia

Cell adhesion and motility are controlled by the interplay between the dynamics of actin cytoskeleton and the formation/disassembly of cell-matrix adhesion area. Dendritic cells (DCs), known as the initiators and modulators of innate and adaptive immunities, can migrate from peripheral tissues to lymph nodes (LN) after antigen challenge. However, the mechanism of how DCs manage to leave the tissue sites is not fully understood. Some previous *in vitro* studies on bone marrow-derived myeloid DCs suggest (1) in static cell culture systems, cellular production of inducible nitric oxide (NO) by mature DCs plays a role in decreasing adherence between these cells to extracellular matrix components (ECM); (2) NO inhibition restores adhesion events, especially on fibronectin. In this thesis, we try to uncover the way in which DCs decrease adhesive property to ECM after encountering foreign antigens. We found NO is involved in regulating the distribution of cytoskeleton and the expression protein kinases at the focal adhesion sites, suggesting the capability of NO in decreasing the adhesive properties of DCs.

Approved: _____

Fabian Benencia

Assistant Professor of Immunology

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INTRODUCTION

Biological properties of DCs

Growing evidence suggests DCs are potent immune regulators that could be applied to therapeutic usage (Palucka *et al*., 2011; Skalova *et al*., 2010). Edgar Engleman, in 1998, reported a study using DCs to create a cancer vaccine. On April 29, 2010, the Food and Drug Administration approved the first DC-based therapeutic vaccine, Sipuleucel-T, to treat hormone-refractory prostate cancer. To date, according to U.S. National Institutes of Health, there are more than 380 clinic trials focusing on the research of DC-based vaccines. To understand why DCs are widely studied, one must know their significant immunological roles.

DCs are professional antigen-presenting cells originating from hematopoietic bone marrow progenitor cells (Cunningham *et al*., 2010; Skalova *et al*., 2010). They are composed of two major subsets: plasmacytoid DCs (pDCs) and myeloid DCs (mDCs) (Skalova *et al*., 2010). pDCs arise from lymphoid progenitors, then circulate in blood and peripheral lymphoid organs (Mittelbrunn *et al*., 2009). They express TLR 7 and 9 at endosomal levels that recognize viral components, such as ssRNA and CpG DNA motifs (Skalova *et al*., 2010; Mittelbrunn *et al*., 2009). Activated pDCs can, in turn, secrete large amounts of type I (α/β) interferon (IFN) against viral infection (Mittelbrunn *et al*., 2009). mDCs arise from myeloid progenitors and are found in tissues, mucosal surfaces, secondary lymphoid organs and blood (Ueno *et al*., 2007). Langerhans cells (LCs) and interstitial DCs (intDCs) are the two best known subsets of mDCs (Mckenna *et al*.,

2005). LCs prime CD8+ T cells and polarize CD4+ T cells into Th1 or Th2, whereas intDCs promote naïve B cells into plasma cells (Colonna *et al*., 2004).

Once they leave the bone marrow, preDCs circulate through blood into tissue sites where they become immature DCs (Ueno *et al*., 2007; Murphy *et al*., 2008). Immature DCs persistently endocytose self and non-self-antigens into processing compartments (Steinman & Banchereau, 2007). Pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) are distinct pathogenic molecules recognized by DCs (Piccinini & Midwood, 2010). Through pattern-recognition receptors (PRR) such as toll-like receptors (TLR) and nucleotide oligomerization domains (NOD), they initiate signaling cascades so that DC "maturation" can occur (Piccinini & Midwood, 2010). Mature DCs express high levels of inflammatory chemokine receptor CCR7 (Murphy *et al*., 2008), which interacts with ligands expressed in the T-cell zones of secondary lymphoid organs, such as CCL19 (macrophage inflammatory protein-3β) and CCL21 (secondary lymphoid tissue chemokine) (Hansson *et al*., 2005). Thus, antigen-challenged DCs can migrate to the cortex of lymph nodes, where they acquire full maturation and present antigen to naïve T cells, a process known as antigen presentation (Granucci & Zanoni, 2009; Skalova *et al*., 2010).

Mature DCs display three major signals for antigen presentation to naïve T lymphocytes (Banchereau & Steinman, 1998; Murphy *et al*., 2008):

(a) *Signal for T cell activation*. This involves presentation of antigenic peptide-MHC complexes to T cell receptors.

(b) *Signal for T cell proliferation*. This involves maturation molecules CD40, B7.1 (CD80), and B7.2 (CD86) expressed on mature DCs, providing co-stimulation signals necessary for T cell growth. **Fig. 1**, an unpublished result from Dr. Benencia's laboratory, shows bone marrow-derived DCs acquire mature phenotype when treated with LPS and TNF *in vitro*.

(c) *Signal for T cell differentiation*. This requires cytokines secreted by DCs or tissue environment at the time of antigen recognition. Non-pathogenic antigens induce T cell tolerance or anergy, because they are not able to promote DC maturation or costimulatory molecule expression; otherwise, autoimmune disease may occur (Murphy *et al*., 2008).

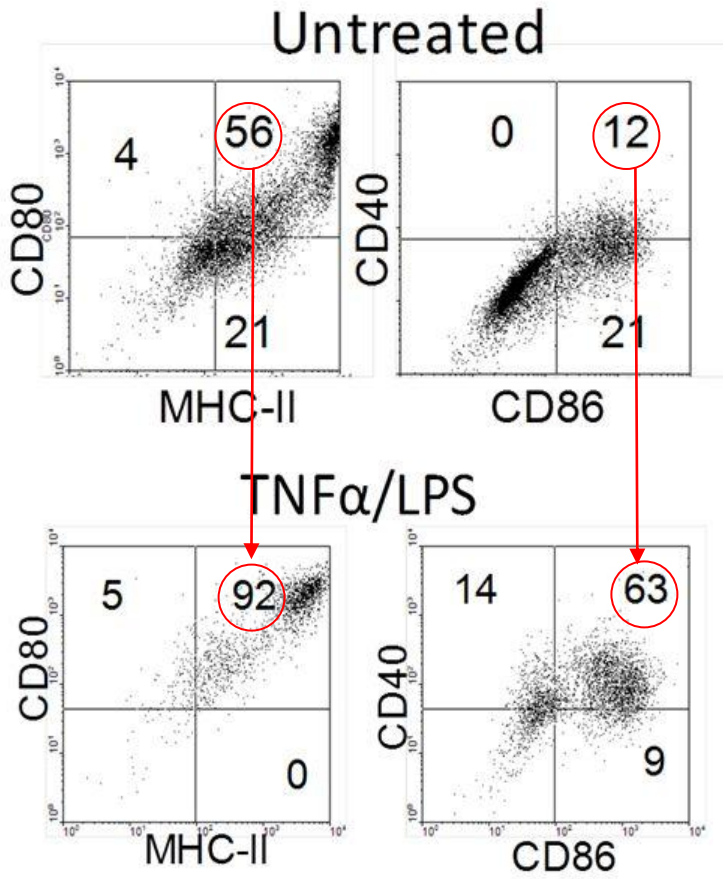

Figure 1. Flow cytometry analysis for MHC II and co-stimulatory molecules expressed on bone marrow-derived DCs. Upper levels represent immature DCs; lower levels represent DCs treated with TNF-α and LPS (mature). The highlighted area shows mature DCs increase the positive percentage of all molecules under analysis. (*Benencia et al., unpublished*)

Interaction between dendritic cells and extracellular matrix components

The ECM provides structural support and adhesion sites to surrounding cells (Alberts *et al*., 2002). Proteins of the integrin family play an important role in regulating

cell adhesion, spreading, migration, and structure by coordinating extracellular stimuli, such as ECM proteins, growth factors, shear stress as well as intracellular signals (Wozniak *et al*., 2004; Mitra *et al*., 2005; Acharya *et al*., 2010). Integrins are composed of two distinct chains, which are α and β subunits (Kinashi & Katagiri, 2005). In mammals, there are eighteen α and eight β subunits characterized, giving rise to twenty-four sets of integrin molecules (Janik *et al.*, 2010). Integrins have binding affinity to ECM. For example, in leukocytes, integrin α4β1 (CD49d/CD29) and α5β1 (CD49e/CD29) bind to fibronectin; α2β3 (CD41/CD61) bind to fibrinogen, fibronectin and vitronectin; αvβ3 (CD51/CD61) binds to fibrinogen, fibronectin, vitronectin, and osteopontin (Berlanga *et al*., 2005; Zeller *et al*., 1999).

Integins' specific binding affinity to ECM can be modified by intracellular signals in an "inside-out" manner, contributing to the first step of cell migration (Janik *et al.*, 2010). Then, activated integrins orchestrate proteins at cell-substratum to produce intracellular signals in a so called "outside-in" manner, continuing the cell migration process (Janik *et al.*, 2010). At this point, the actin cytoskeleton can exert force to the surroundings via integrin (Janik *et al.*, 2010). At last, the migration cycle can be recovered in a migrating cell by forming new bind sites in its front and taking away the bind at the rear (Janik *et al.*, 2010). However, if the binding affinity is very high, a cell could firmly attach to its surroundings, and migration reduces (Janik *et al.*, 2010). On the the other hand, *in vitro* study suggests random cell motion occurs when binding affinity is relatively low (Janik *et al.*, 2010).

Focal adhesions are large protein complexes that serve as the linkages in the dynamic formation/disassembly process between the actin cytoskeleton and ECM (Mitra *et al*., 2005; Bolos *et al*., 2010). Focal adhesion kinase (FAK) and the proto-oncogene tyrosine-protein kinase, called steroid receptor co-activator (SRC), are important nonreceptor intracellular tyrosine kinases that connect cytoskeleton and cell surface integins (Bolos *et al*., 2010). **Fig.2** shows SRC-dependent FAK phosphorylation is required for focal adhesion assembly/turnover and cell migration. FAK contains an erythrocyte band four 1-ezrin-radixin-moesin (FERM) domain at the N-terminal that functions in kinase activity and a focal adhesion targeting (FAT) domain at the C-terminal (Bolos *et al*., 2010). The clustering of integrins causes autophosphorylation of Y397 residue at FERM that gives rise to a binding site for SRC. SRC can in turn phosphorylate FAK at Y576 and Y577 residues, rendering full catalytic activity of FAK. Further, FAK phosphorylation at Y925 creates binding sites to integrin-binding proteins, such as paxillin (PAX) and talin, which are capable of interacting with actin skeleton and controlling the binding affinity for the cells (Mitra *et al*., 2005).

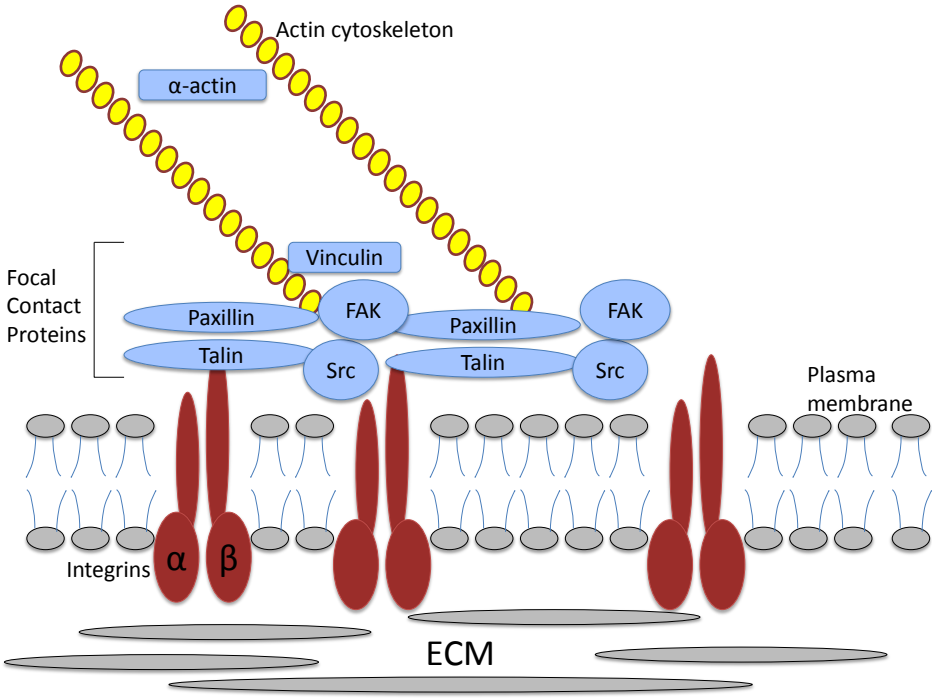

Figure 2. An oversimplified sketch of focal adhesion site. (Adapted from Mitra *et al.*, 2005)

In focal adhesion contacts, integrins connect with the cytoskeleton via intracellular focal adhesion anchor proteins that promote cell adhesion and migration (Alberts *et al*., 2008). Immature DCs may rely on the interaction with ECM for sampling antigens in the local tissue; however, it is suggested by some studies that *in vivo* rapid migration of DCs to LN is integrin independent (Lämmermann *et al*., 2008; Spurrell *et al*., 2009). This could be explained by cytoskeleton remodeling, integrin and focal adhesion contact protein redistribution occurs in maturation DCs that could potentially lower the binding force between DCs and ECM (Verdijk *et al*., 2004).

NO decreases DC-ECM adherence

We have recently shown that murine myeloid DCs are able to interact with different ECM (Sprague *et al*. 2011). **Fig. 3** depicts unpublished results from our lab showing differential adhesion of murine immature myeloid DCs to ECM-coated surfaces.

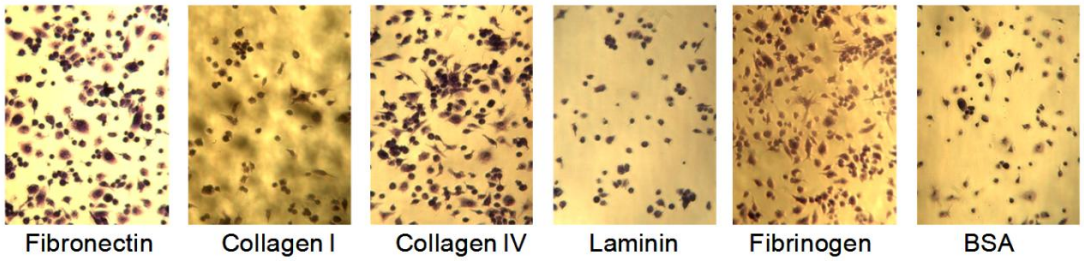

Figure 3. Adhesion of immature DCs to ECM components. Immature DCs were plated onto different ECM-coated plates for 3 h. Then, non-attached cells were washed and attached cells stained and photographed. BSA is a control for non-specific attachment. DCs have some degree of attachment to inert surfaces being called "adherent cells," together with macrophages and neutrophils. (*Benencia et al., unpublished*)

Different results were obtained when mature DCs were used in the same setting (**Fig. 4**). In particular, we were able to detect a dramatic decrease in the adhesion of these cells to ECM components. This could be caused by a decrease in the expression of integrins on the surface of DCs, but also it could be caused by soluble products generated by the same cells. In particular it has been shown by Goligorsky *et al*. that NO was able to interfere with endothelial cells in the context of focal adhesions. They concluded that

NO constrains the formation of cytoskeleton, inhibits the phosphorylation of FAK, and blocks paxillin to the focal adhesion sites, all resulting in the reduced adhesive property of endothelial cells (Goligorsky *et al*., 1999). NO is a cellular gaseous molecule responsible for regulating multiple cellular functions, including the relaxation of smooth muscle cells, neurotransmission, cell proliferation, and apoptosis (Yao *et al*., 1998). With respect to immune cells, NO can be produced in response to antigen challenge and inflammation stimuli (Qureshi *et al*., 1996; Adler *et al*., 2010), so that the antimicrobial activity against microorganism can occur (Groote & Fang, 1995).

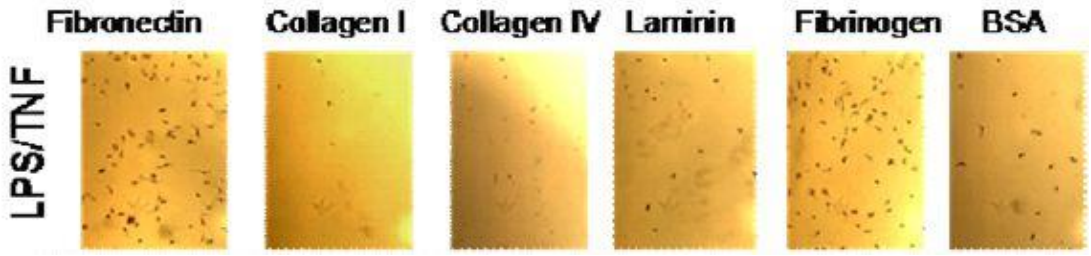

Figure 4. Adhesion of mature DCs to ECM components. Mature DCs were plated onto different ECM-coated plates after 3 days in culture with LPS/TNF. Then, non-attached cells were washed and attached cells stained and photographed. The amount of dye associated with attached cells was quantified. BSA is a control for non-specific attachment. (*Benencia et al., unpublished*)

The cellular production of NO requires oxidation of L-arginine at the oxygenase domain and electrons supplemented by oxidation of NADPH reductase (Garvey *et al*., 1997; Alderton *et al*., 2001). The process also requires coenzymes, such as

protoporphyrin IX haem, FMN, FAD and BH4 (Knowles & Moncada, 1994). There are three NOS isoenzymes: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS/NOS II) (Knowles & Moncada, 1994; Kroncke *et al*., 2001; Adler *et al*., 2010). Both nNOS and eNOS produce NO constitutively in low quantities, but NOS II can be activated by histoincompatible cells, LPS/IFNγ, and inflammatory cytokines, such as IL-1 and TNFα, all resulting in large quantities of NO production (Knowles & Moncada, 1994). Because nitrite is a stable and nonvolatile breakdown product of NO, its presence is investigated in culture media as an indication of NO production. Previous unpublished studies from our lab show that DCs are able to generate NO in response to inflammatory stimuli (**Fig. 5**). This is regulated by the inducible NOS since NOS II inhibitors such as aminoguanidine (AMG) and N-[3-(Aminomethyl)benzyl]acetamidine, commercially known as 1400W, impair this effect (**Fig. 5**). Also, myeloid DCs generated from NOS II knock out (KO) mice are not able to generate NO in response to inflammatory stimulation. (**Fig. 5**).

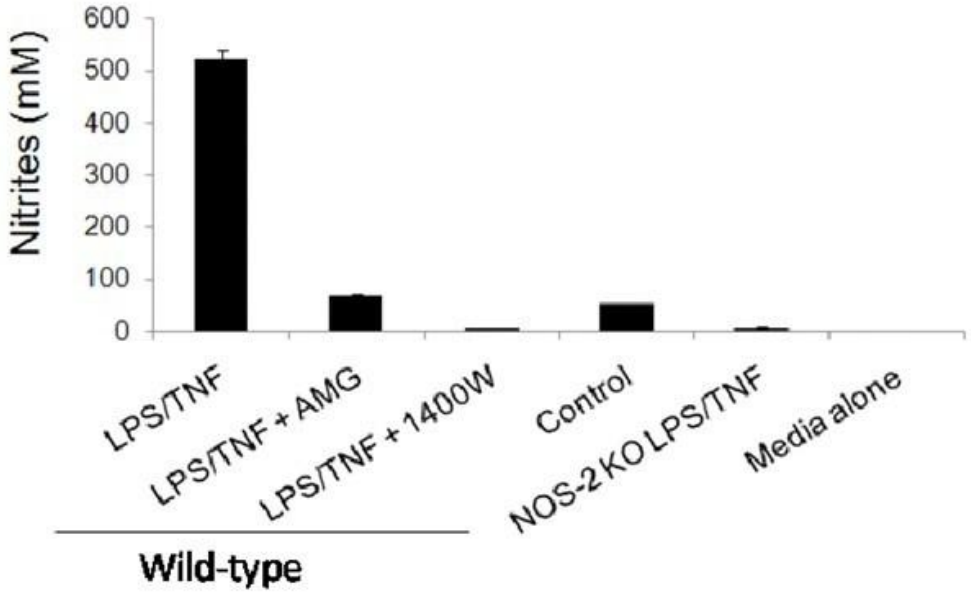

Figure 5. Production of nitrites by DCs. Myeloid DCs were stimulated with inflammatory factors in the presence or absence of different NOS II inhibitors (AMG and 1400W) for 48 h. Presence of nitrites in the cell culture supernatants were determined by the Griess assay. (*Benencia et al., unpublished*)

Additionally, for the last two decades NO has been known to decrease leukocyte adhesive properties (Kubes *et al*., 1991). For example, NO decreases intercellular platelet adhesion and aggregation (Radomski *et al*., 1990). Inhibiting NO production from endothelium promotes adherence of DCs to endothelial cells (Zhu *et al*., 2009). Further, NO increases the migration capability of macrophages, a close relative to DCs (Maa *et al*., 2008). Our unpublished data show that DCs treated with inflammatory factors for 72 h in the presence of NOS-II inhibitors increase their adhesion to some ECM components (**Fig. 6**).

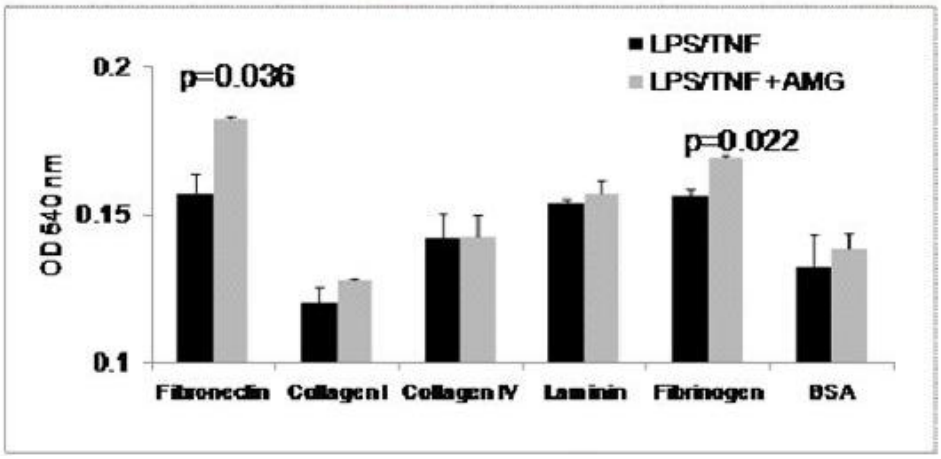

Figure 6. Adhesion of mature DCs to ECM components. Mature DCs were plated onto different ECM-coated plates after 3 days in culture with LPS/TNF in the presence or absence of AMG, a NOS II inhibitor. Then, non-attached cells were washed and attached cells stained. The amount of dye associated with attached cells was quantified. BSA is a control for non-specific attachment. Data were analyzed by ANOVA followed by Bonferroni post-comparison tests. Significance was considered at $p<0.05$. (*Benencia et al., unpublished*)

Since it was observed that AMG was effective in increasing DCs-ECM adhesion, especially to fibronectin-coated surfaces, we investigated this in more detail. As shown in **Fig. 7**, similar results were obtained with another NOS II inhibitor or when NOS II KO DCs were used for these studies.

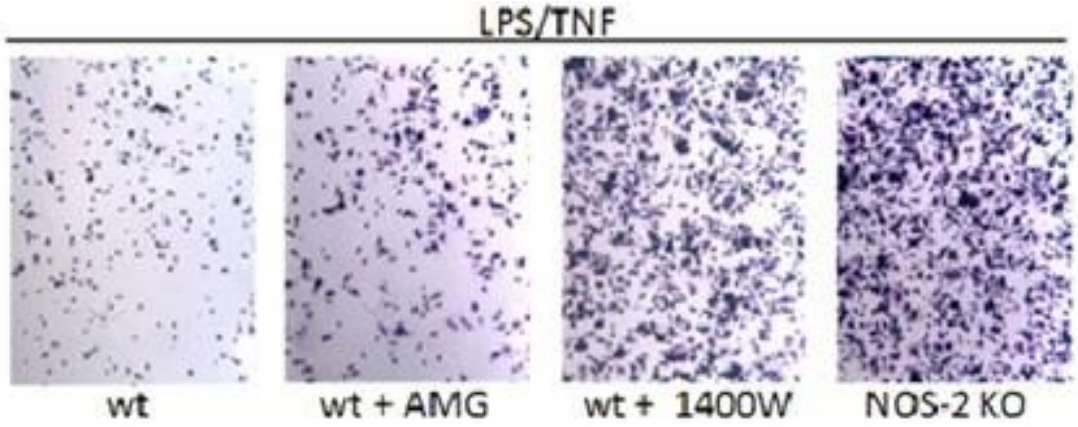

Figure 7. Adhesion of mature DCs to fibronectin. After 3 days of maturation with inflammatory factors in the presence or absence of AMG or 1400W, DCs were recovered and plated on fibronectin plates for 3h. Non-attached cells were washed and attached cells stained. An experimental group was also set up with NOS II KO. (*Benencia et al., unpublished*)

A summary of previous work performed in our lab follows: Overall, (1) We were able to prepare mature DCs by administering inflammatory stimuli to immature cells. (2) Immature DCs show differential adhesive properties to ECM-coated surfaces. Further, we could observe dramatic decrease in adhesion between mature DCs and ECM-coated surfaces in the same experimental setting. (3) Cellular production of NO by DCs is regulated mostly by NOS II, since we observed that DCs increased the production of NO by tenfold when treated with imflammatory stimuli, and that NO production decreased dramatically when NOS II inhibitors were administered. (4) Adhesion of mature DCs to fibronectin increased in the presence of different NOS II inhibitors, or when cells from NOS II KO mice.

HYPOTHESIS AND SPECIFIC AIMS

Since NO has been shown to decrease the adhesive properties for lymphocytes to extracellular matrix components (Yao et al., 1998), and NO increases the migration capability of macrophages (Maa et al., 2008), based on the previous work by our lab and others, we hypothesize that NO is a potent gaseous molecule produced by mature DCs that function in decreasing the adhesion of these cells to ECM.

Next, we try to uncover the mechanisms by which our hypothesis could be validated. Specifically, we aim to find if (1) NO could inhibit surface adhesion molecules expressed on DCs or (2) NO could modify components recruited at focal adhesion sites that reduce the cell adhesion property.

EXPERIMENTAL DESIGN

Several techniques were used to study in detail the adhesion of DCs to ECM under flow condition, expression of surface adhesion molecules, and modification of proteins and the actin cytoskeleton at focal adhesion sites. **Fig. 8** shows experiments that will be discussed in this thesis.

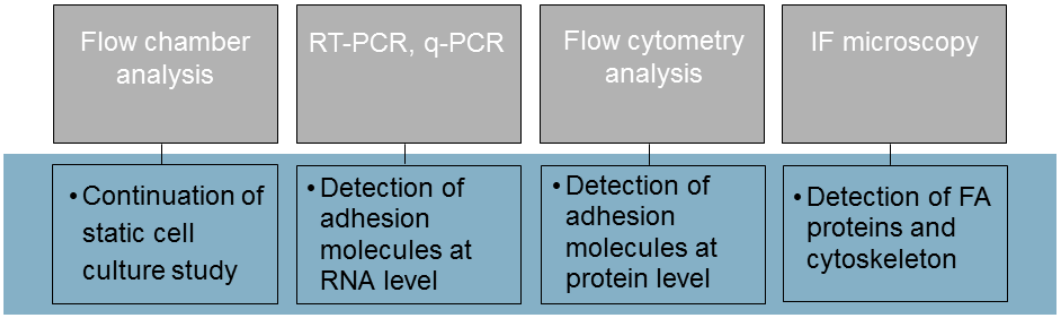

Figure 8. Experimental design.

MATERIALS AND METHODS

Cell lines and treatment

Bone marrow-derived DCs were prepared by Dr. Benencia as described in detail elswhere (Muccioli *et al.*, 2011). DCs were maintained in RPMI media (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS, Sigma, St Louis, MO) (Muccioli *et al.*, 2011). Cells were matured in the presence of 5 ng/ml GM-CSF (Peprotech Inc., Rocky Hill, NJ) supplemented with 1 μg/ml lipopolysaccharide (LPS, SIGMA) and 10 ng/ml tumor necrosis factor-alpha (TNF-α, 315-01A, Peprotech) as described (Muccioli *et al.*, 2011). In order to inhibit NOS-II, DCs were treated during maturation with aminoguanidine (AMG, 1 mM, Sigma) or N-(3-[Aminomethyl]benzyl)acetamidine (1400w, 400 μM, Calbiochem, Gibbstown, NJ) (Benencia *et al.*, 2001; Benencia *et al.*, 2003). These NOS II inhibitors are structurally similar to L-arginine that competitively bind to and inhibit NOS II (Matta *et al*., 2009; Garvey *et al*., 1997). JAWS II (ATCC, Manassas, VA) is an immortalized and established murine immature DC cell line (Mendoza *et al*., 2003). JAWS II cells were matured in the presence of 1 μg/ml LPS and 10 ng/ml of TNF-α. AMG (1 mM, Peprotech) and 1400w (400 μM) were also added as NOS-II inhibitors. RAW is an established murine macrophage cell line known to express SRC (Khadaroo *et al*., 2003). They acquire DCs morphology by challenging with 1 μg/ml LPS (Saxena *et al*., 2003).

Flow cytometry

Cells were subjected to three-color flow cytometry on a FACSort flow cytometer using CellQuest 3.2.1f1 software (Becton Dickinson, San Jose, CA) as we recently described (Sprague *et al.,* 2011). We collected 10,000 events per sample. Non-specific staining was blocked with Fc block with a solution consisting of PBS 2% FBS plus 0.05% sodium azide (FACS buffer) (Sprague *et al.,* 2011). Fluorescein isothiocyanate (FITC) conjugated monoclonal antibodies against CD41 (eBioscience, San Diego, CA) and CD49d (eBioscience, San Diego, CA) were used at 1/50 dilution; phycoerythrin (PE) conjugated monoclonal antibodies that recognize CD51 (eBioscience, San Diego, CA) and CD61 (eBioscience, San Diego, CA) were used at 1/50 dilution. Isotypes (BD Pharmingen, San Diego, CA) were used at 1/100 dilution to eliminate non-specific staining.

Griess reagent system

Supernatants obtained from 72h DC cultures (wild type untreated DCs [control]), wild type DCs treated with LPS (1μg/ml) and TNF-α (20 ng/ml), additional AMG (1mM) and 1400W (400 μM) treated DCs, and NOS II KO LPS/TNF-α treated DCs and RAW culture mediums (RAW control, RAW LPS, RAW LPS/AMG) were analyzed by the Griess reagent system (Promega Corporation, WI) as recently described (Muccioli *et al*., 2011). This system detects nitrite ($NO2^-$), which is a stable product of NO in liquid matrices.

RT-PCR

RNA extracted from DCs was isolated by TRIzol (Invitrogen, Carlsbad, CA), then reverse transcribed into cDNA by the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) and analyzed for the expression of several adhesion molecules by means of the polymerase chain reaction (PCR) as we recently described (Sprague *et al.,* 2011). The cDNA samples were evaluated for the expression of CD49d, CD49e, CD41, CD51, and CD61. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a housekeeping gene used as a control for DNA load. In order to eliminate possible contaminating genomic DNA, all RNA samples were treated with DNAse (Invitrogen, Carlsbad, CA) (Sprague *et al.,* 2011). The PCR cycling was conducted with Taq polymerase at 94°C for 3 min, 57°C for 30 min, and 72°C for 70 seconds, in a total of forty cycles (Sprague *et al.,* 2011).

Parallel-plate flow chamber experiments

Cells were cultured in Dulbecco's Modification of Eagle's Medium (Invitrogen, CA). Sample concentration was measured by a hemocytometer and was adjusted to 2 x 10^6 cells/ml (Ng and Swartz, 2006; Tal *et al*., 2011). Then, DCs samples were subjected to a series of parallel-plate flow chamber experiments on untreated or fibronectin-coated 3.5cm petri dishes. The experimental settings were discussed in Wiese *et al.,* 2009.

The flow conditions were set at the wall shear stress at 0.5 and 1 dyn/cm^2. These values were chosen according to the lymphatic flow condition (in a range of 0.7-1 dyn/cm^2) (Ando *et al.*, 1994). In addition, we focused on low flow levels so as to mimic

the venous flow condition (~1.5 dyn/cm^2) (Ando *et al.*, 1994; Burns *et al.,* 2007) where the DCs will mature upon contact with the antigen (Ng and Swartz, 2006).

The wall shear stress τ_w can be calculated as:

$$\tau_w = \frac{6\mu Q}{bh^2}$$

μ = media viscosity (μ = 0.01P for experiment medium at room temperature)

(1P = 1 dyn/cm^2)

h = channel height (the height of the gasket is 0.0254 cm)

b = channel width (the width of the gasket is 0.5 cm)

Q = volumetric flow rate

From the given wall shear stress, we calculated the respecting volumetric flow rate at Q= 0.161 and 0.323 ml/min. Immature DCs treated with S-Nitroso-N-Acetyl-D, L-Penicillamine (SNAP) (0.5mM) were studied at these flow rates. DCs without treatment were controls. Cells were studied immediately, 0.5h, or 1h after treating with SNAP. The flow chamber was recorded during experiments, and the attached cells were counted from the recordings.

Immunofluorescence microscopy

Immunofluorescence microscopy was used to study the distribution of FAK, paxillin, SRC, and actin cytoskeleton in RAW cells and DCs. Control, LPS, and LPS/AMG were cultured for different times on culture slides, fixed in acetone for 10 minutes and rinsed 5 minutes in PBS twice. Anti-FAK/PAX/SRC (rabbit) antibodies (4μg/ml) were added as the primary antibodies against their respecting proteins. Anti-rabbit AF488 (4μg/ml) (R&D Systems, Minneapolis, MO) against primary antibodies was used as a secondary antibody. Phalloidin was used for cytoskeleton staining. Finally, slides were counterstained with 4',6'-diamidino-2-phenylindole hydrochloride (DAPI) (Vector Laboratories, Inc. Burlingame, CA) in order to visualize nuclei as previously described (Muccioli *et al*, 2011). Pictures from each sample were collected under fluorescent microscopy at 400X or 1000X magnification.

Real-Time PCR

The expression of adhesion molecules and SRC were analyzed at the mRNA level by Real-Time PCR (qRT-PCR). qRT-PCR was performed by the comparative threshold cycle (ΔC_T) method and normalized to GAPDH as we recently described (Sprague *et al.,* 2011). In this assay we used SYBR Green (Quantas Biosciences, CA) for detection of PCR reaction. cDNA samples of immature DCs, DCs cultured with LPS/TNF in the presence or absence of AMG/1400W, and NOS II KO DCs were prepared by Dr. Benencia. Primers of CD49d, CD49e, CD41, CD51, CD61, and SRC (Integrated DNA

Technologies, CA) were used to amplify their relating cDNA and generated by using the Primer3 web program as we recently described (Sprague *et al.,* 2011).

Statistical analysis

The one-way ANOVA method was to determine the differences between DCs vs. DCs SNAP on normal 3.5cm petri dish; DCs vs. DCs SNAP on fibronectin-coated surface in flow chamber analysis. The same method was used to determine the significance of adhesion molecules expressed in immature DCs vs. mature DCs in qPCR analysis. Similarly, ANOVA was used to determine the significance of nitrites production in mature RAW cells vs. other groups in the Griess reagent system. Statistical significance was defined as a p value < 0.05. Error bars represent mean + SD.

RESULTS

Previous unpublished data from the Benencia lab showed that NOS inhibitors inhibit the production of NO by mature DCs and that these cells show more adhesion events to different surfaces in static systems. In order to study the behavior of these cells in a biologically relevant system, we performed a series of studies using a parallel flow chamber assay.

First we investigated the capability of NO to decrease adhesion of DCs to inert surfaces (polystyrene) under flow. It is noteworthy to comment that DCs, as monocyte/macrophages, are adherent cells and as such can attach in a non-specific way to this type of surface. For these studies we used SNAP, a well-described NO donor (Monastyrskaya, *et al*., 2002).This molecule breaks down in solution, generating large amounts of NO.

The rationale for using SNAP is that we could treat all the cells at the same time with a similar concentration of SNAP and thus we did not introduce, for these studies, individual variation due to some cells producing different amounts of NO in response to maturation stimuli. As shown in **Fig. 9**, pre-treatment of DC for 1h with SNAP (0.5mM) (Monastyrskaya *et al.,* 2002) impaired the capability of the cells to adhere to plastic surfaces as determined using two different flow conditions.

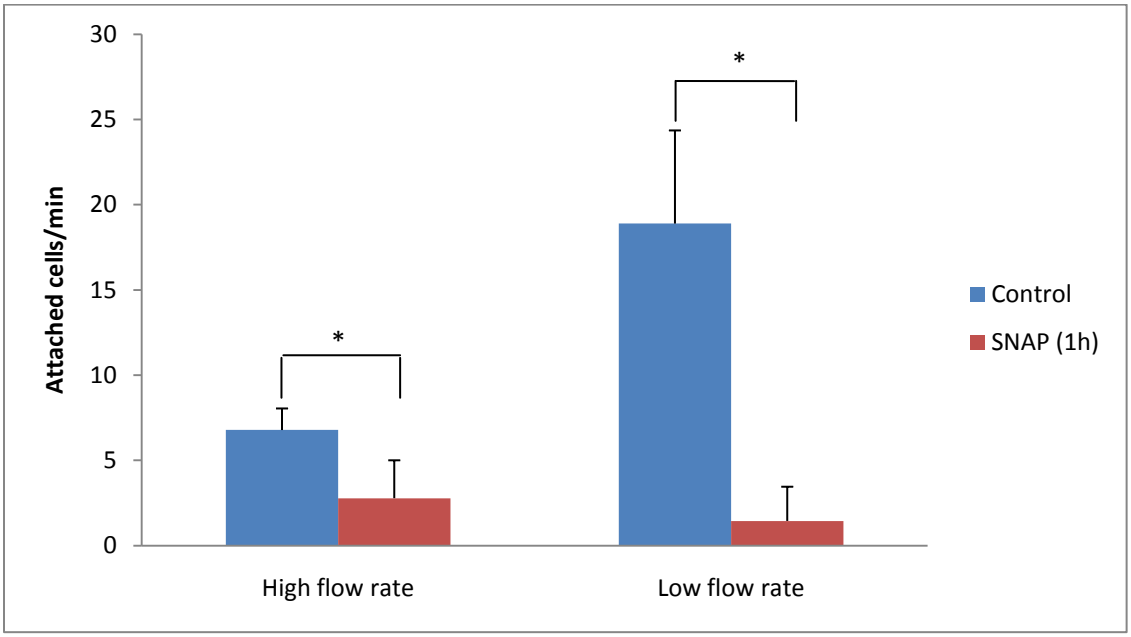

Figure 9. New attached DCs/min to polystyrene surfaces in flow chamber assay. In both cases when high flow rate (0.323 ml/min) and low flow rate (0.161 ml/min) were studied, SNAP-treated DCs decreased adherence events when compared with DCs without treatment. In each column, the data are acquired by counting the attached cells from 3 separate 3-minute-long video records, repeated twice. Errors are reported as mean + SD. *ANOVA variance analysis was conducted to determine the significance of DCs vs. DCs SNAP at the same flow rate $p<0.001$.

In a complementary series of experiments we decided to investigate the attachment of DCs to fibronectin, a typical extracellular matrix component (**Fig. 10)** (Alberts *et al*., 2008). In addition, the effect of NO on DCs seemed to greatly impact their capability to attach to fibronectin and fibrinogen. When we performed this experiment in similar conditions as those described in the previous experiment, we were able to observe that the adhesion to fibronectin was also impaired by pre-incubation of the cells in a SNAP containing solution for 0 to 1h.

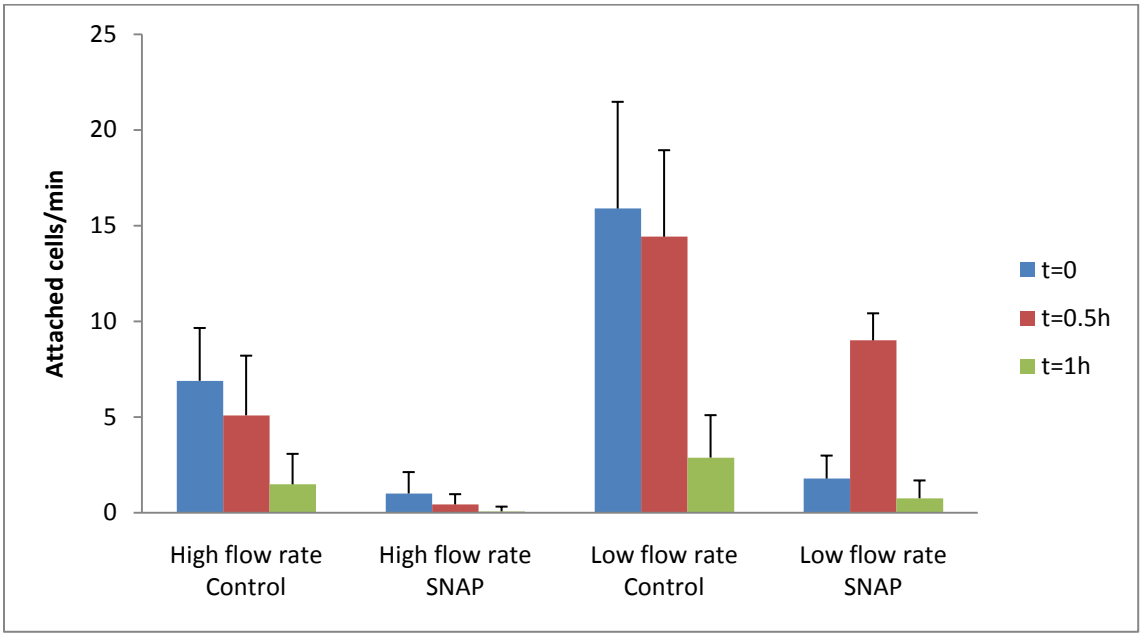

Figure 10. New attached DCs/min to fibronectin surfaces in flow chamber assay. In both cases when high flow rate (0.323 ml/min) and low flow rate (0.161 ml/min) were studied, SNAP treated DCs decreased adherence events when compared with DCs without treatment. In each column, the data are acquired by counting the attached cells from 4 separate 3-minute-long video records, repeated twice. Errors are reported as mean + SD. ANOVA variance analysis was conducted to determine the significance of DCs vs. DCs SNAP at the same flow rate and time $p<0.001$.

The use of extracellular matrix components such as fibronectin helps to better reproduce an in vivo situation. Taking into account the observed effects of NO on DC adhesion to fibronectin, we decided to study the expression of integrins by our cells upon maturation in the presence or absence of NO inhibitors. As described above, integrins on the surface of the cells are responsible for attachment to different ECM molecules. As we have recently described, DCs show an array of different integrins that allow the cells to attach to different ECM components (Sprague *et al*. 2011).

In a first series of studies we decided to investigate the expression of some of these molecules on DCs by qualitative PCR analysis. In particular, we investigated the expression of CD49d, CD49e, CD41, CD51, and CD61, which can direct cell adhesion to fibronectin (Berlanga *et al*., 2005; Zeller *et al*., 1999). To accomplish this, DCs were treated for 48 h with a typical inflammatory cocktail containing LPS and TNF-α as we recently described (Sprague *et al*. 2011). Some experimental groups were also treated with 1400W, a specific NOS-II inhibitor as described above. As shown in **Fig. 11,** we did not observe by this qualitative experiment any decrease in the expression of integrins in NOS-II inhibited cells.

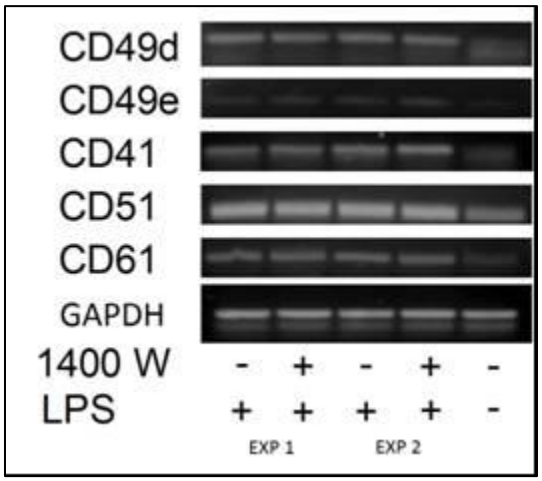

Figure 11. Detection of adhesion molecules at the levels of RNA by RT-PCR. Mature DCs treated without or with 1400W were analyzed in both experiments 1 and 2. LPS and 1400W negative group represents immature DCs. GAPDH is the housekeeping gene used as a control for cDNA load.

In order to investigate this in detail, we performed a quantitative real-time PCR. This technique allows us to investigate in a quantitative way the levels of expression of any molecule at the level of RNA as we have recently described (Sprague *et al*. 2011). As seen in **Fig. 12**, we were able to see upregulation of all molecules in mature cells (treated with LPS) with respect to immature cells (not LPS-treated) at the level of RNA, even though they do not attach more to fibronectin.

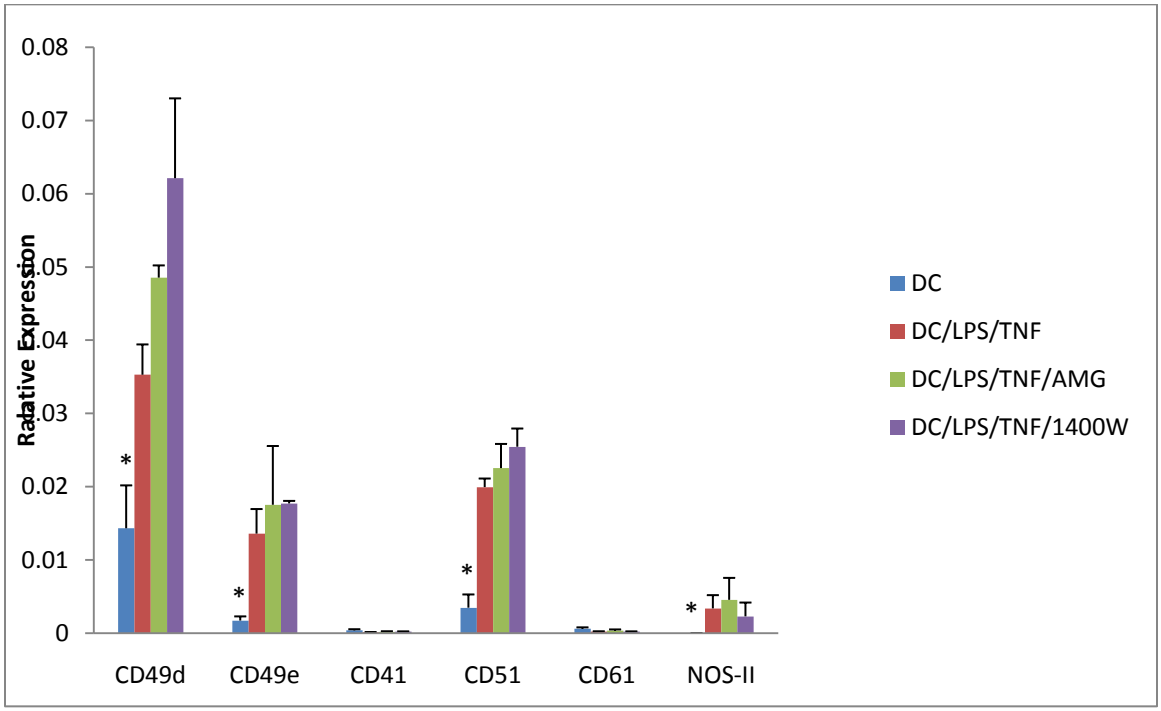

Figure 12. Expression of adhesion molecules mRNA in BMDC is shown in relation to the housekeeping gene GAPDH. In this Real-Time PCR analysis, samples were run in duplicate and represent 2 independent experiments. Errors are reported as mean + SD. *ANOVA variance analysis was conducted to determine the significance of adhesion molecules expressed in immature DCs vs. mature DCs, $p<0.05$.

The expression of CD49d, CD51, CD41, and CD61 at the protein level was investigated by flow cytometry analysis. To accomplish this, cells were cultured in the tissue culture flask with the presence of LPS (an inflammatory molecule that induces maturation of DCs) for three days. Some cells were treated with additional NOS-II inhibitors: AMG and 1400W. Cells that attached to the flask surface and cells that flowed in the culture medium were recovered separately and stained for integrin expression as recently described by the Benencia laboratory (Sprague *et al*. 2011). In particular, CD41 and CD49d were stained with specific monoclonal antibodies conjugated with FITC, and CD51 and CD61 were stained with PE-conjugated antibodies.

As mentioned previously, mature DCs decrease their adhesion to different surfaces, and this effect was abrogated in the presence of NOS II inhibitors. As shown in **Fig. 13**, NOS II inhibitors did not modify in a similar way the levels of adhesion molecules expressed on the surface of DCs. In some cases, the molecules were upregulated and in others, they were not modified. Thus, these data make difficult to interpret our previous unpublished observations indicating that untreated groups show more cells attached to the surface.

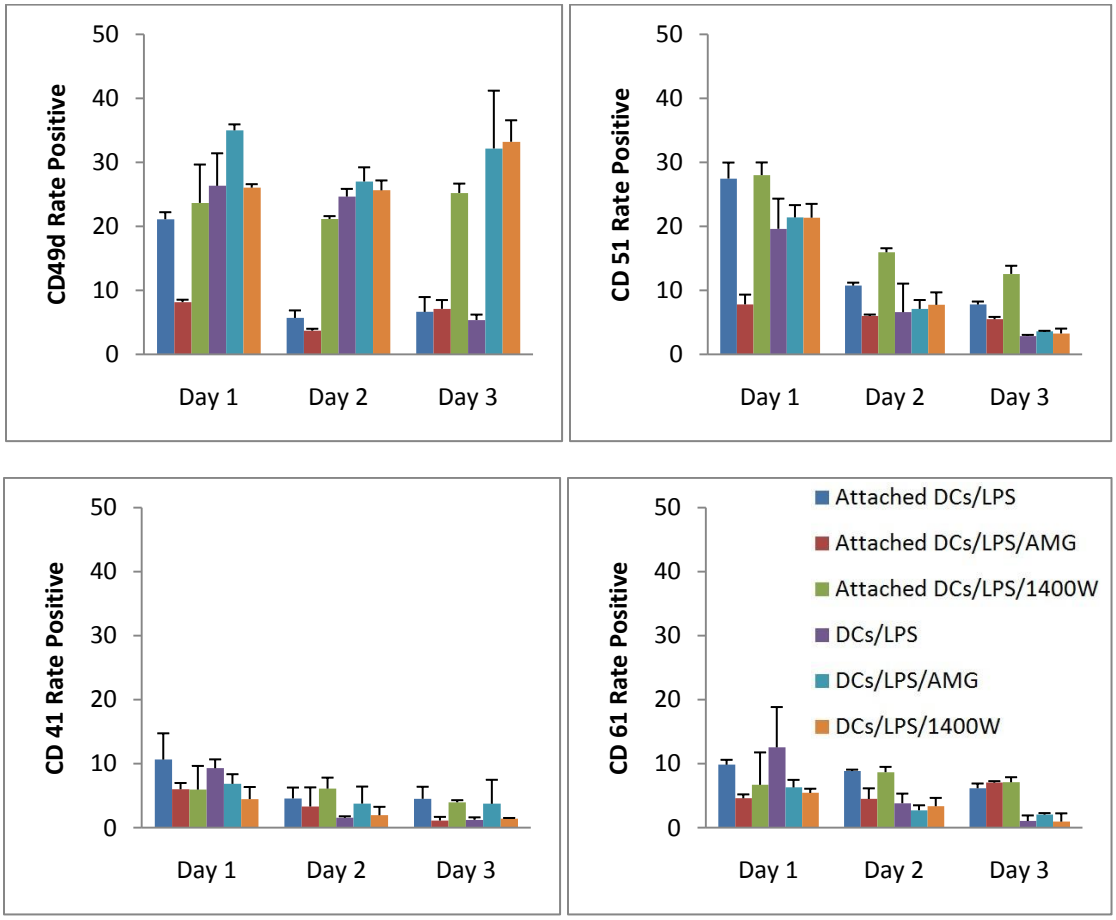

Figure 13. Flow cytometry analysis for some adhesion molecules on DCs. Mature DCs were cultured without or with NOS II inhibitors for 72h and recovered each day. The attached and non-attached DCs were studied separately. The experiment was repeated two times with similar results.

Taking into account that NO can participate in decreasing adhesion without affecting integrin expression, and as seen in the flow chamber experiments, in a rapid way, we decided to investigate the effect of NO generation on molecules that regulate the interaction of integrins with the cytoskeleton. In particular, we analyzed the cytoskeleton

structure and the expression of FAK and paxillin by fluorescence microscopy, because previous works suggest that these molecules could control cell adhesion (Puklin-Faucher & Sheetz, 2009).

Fig. 14 shows the distribution of the cytoskeleton in DCs, DCs treated with LPS, and DCs treated with LPS and AMG. Actin filaments were visualized by staining with phalloidin, a typical fluorescence reagent that interacts with actin cytoskeleton (Lavi *et al.,* 2012). It can be seen that the actin filaments in immature DCs and mature DCs treated with NOS II inhibitor spread to a larger extent than that in mature DCs, creating more area of contacts with the surroundings.

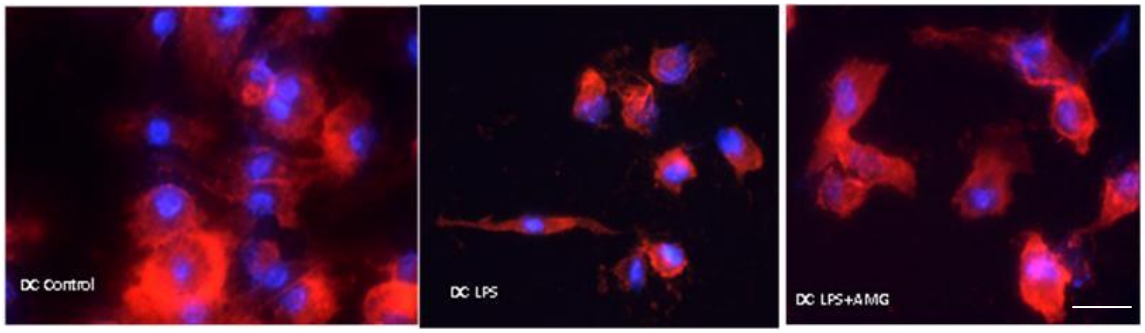

Figure 14. Immunofluorescence staining of actin cytoskeleton in bone marrow-derived DCs. DCs were cultured on fibronectin and treated with LPS/TNF in the presence or absence of AMG for 48h. Then, cells were stained with phalloidin (red staining). Nuclei were counterstained with DAPI (blue staining). Control represents immature DCs. (400X). Scale bar = 10 µm.

It has been shown that macrophages decrease their adhesion to substrates in the presence of NO (Maa *et al.,* 2008). In order to determine if these cells, which share with DCs the property of phagocytosis, antigen presentation, and migration, behave in a similar fashion to our cells, we performed a series of experiments with RAW cells, a macrophage cell line (Saxena *et al*., 2003; Khadaroo *et al*., 2003). As shown in **Fig. 15,** similarly to what happens with DCs, upon stimulation with LPS, RAW cells generate large amounts of NO as determined by a nitrite from the cell supernatants. Nitrite is a stable and nonvolatile breakdown product of NO, thus, the value is representative of the production of NO by RAW. In addition, this effect was abrogated when cells were stimulated with inflammatory factors in the presence of a NOS-II inhibitor such as AMG.

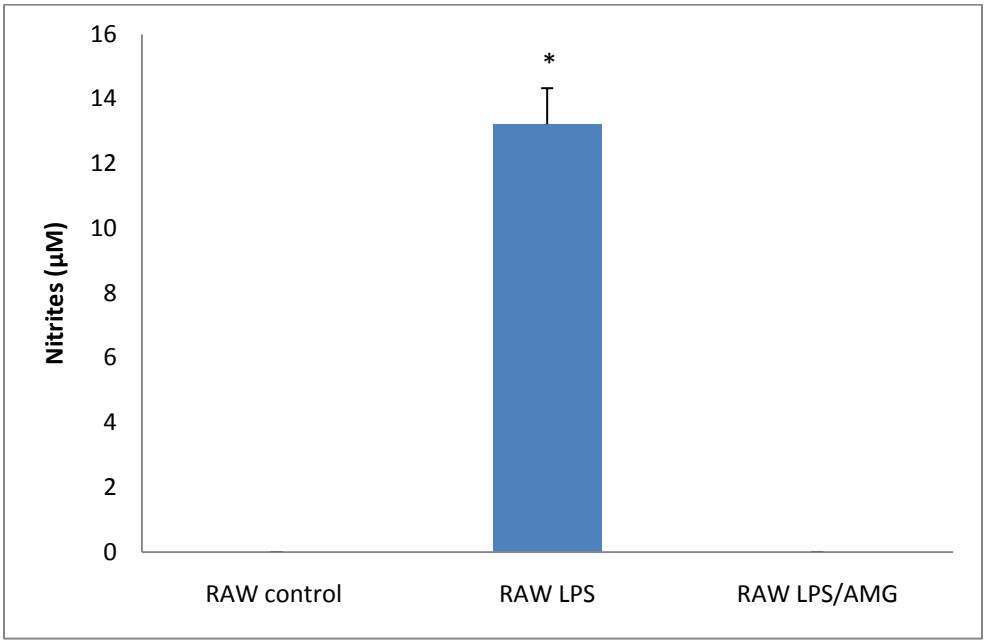

Figure 15. Production of nitrites in RAW supernatants. A nitrite standard reference curve (1.56, 3.13, 6.25, 12.5, 25, 50, and 100 µM) was prepared (50 µl/well) in triplicate in a 96-well plate. In separate wells, sample supernatants were loaded (50 µl/well) in triplicate. Sulfanilamide solution (50 µl) and N-1-napthylethyenediamine dihydrochloride (NED) (50 µl) were loaded onto all wells. The colorimetric values were determined in a spectrophotometer and the concentration of nitrites was calculated. Error is reported as mean + SD. *ANOVA variance analysis was conducted to the determine significance of nitrites production in mature RAW cells vs. other groups, P < 0.0001.

Next, we analyzed the cytoskeleton distribution in these cells by phalloidin staining. We observed, similar to what happened to DCs, a decrease in the contact surface of the cells upon stimulation with inflammatory factors (**Fig. 16**). Thus, this might be a general mechanism by which antigen-presenting cells decrease their adhesion to substrate upon contact with antigens that induce maturation. Further, under a greater magnification, we observed the actin cytoskeleton in immature DCs tended to cluster at the edge of the

cells, presumably at the focal adhesion area (see white arrows in RAW control). However, attenuated staining of actin cytoskeleton could be observed under the cell membrane in mature DCs (see white arrows in RAW LPS). This leads us to investigate some focal adhesion anchor proteins that function in connecting the cytoskeleton under cell membrane and controlling cell adhesion properties (Mitra *et al.,* 2005).

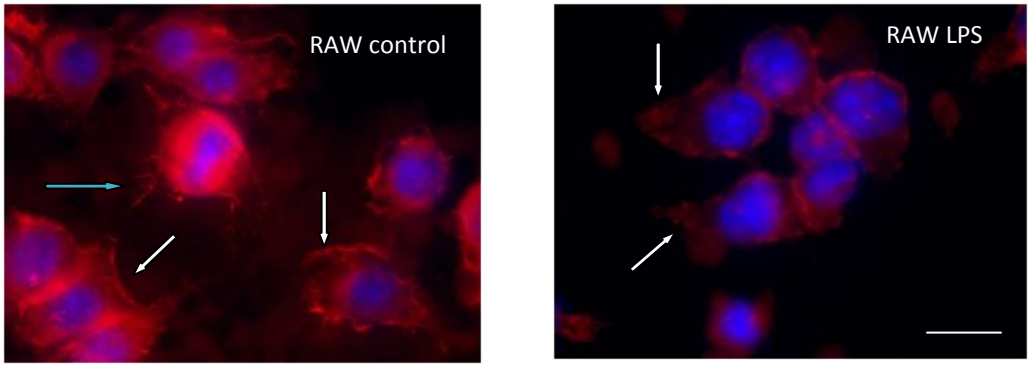

Figure 16. Immunofluorescence staining of cytoskeleton on RAW cells. RAW cells were cultured on fibronectin in the presence or absence of LPS for 48h. Then, cells were stained with phalloidin (red staining) and DAPI (blue staining). (1000X). Scale bar = 2.5 µm.

Finally, we decided to investigate the expression of FAK 925 by DCs. To accomplish this, anti-FAK 925 (rabbit) antibodies (4µg/ml) were added as the primary antibodies, then, anti-rabbit AF488 (4µg/ml) were used as secondary antibodies for staining. As **Fig. 17** suggests, attenuation of FAK 925 staining could be observed in mature DCs without NOS II inhibitor treatment. Since we have previously discussed that

phosphorylation of FAK at the amino acid 925 controls the recruitment of integrin-binding proteins, such as paxillin and talin, which are capable of interacting with actin skeleton and controlling the binding affinity for the cells to substrates (Mitra *et al*., 2005), we suggest a possible way in which, upon contact with antigens, DCs decrease adhesion properties by decreasing adaptor proteins that connect integrin and actin cytoskeleton.

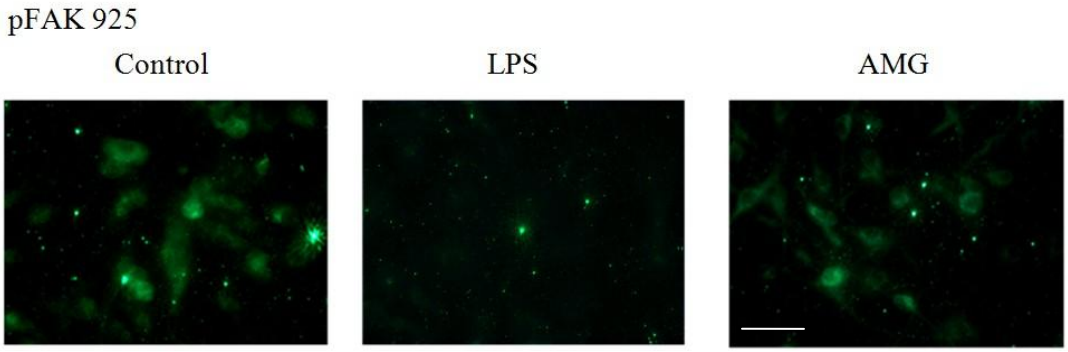

Figure 17. Immunofluorescence staining of pFAK 925 in BMDC. BMDC were cultured on fibronectin and treated with inflammatory factors in the presence or absence of AMG for 48 h. Then, cells were treated with anti-FAK 925 (rabbit) antibody and stained anti-rabbit AF488 (green staining). Control represents immature DCs. (400X). Scale bar = 10 µm.

To validate our assumption, a similar result could be observed in **Fig. 18**, in which paxillin was stained by anti-paxillin (rabbit) (4µg/ml) and anti-rabbit AF488 (4µg/ml).

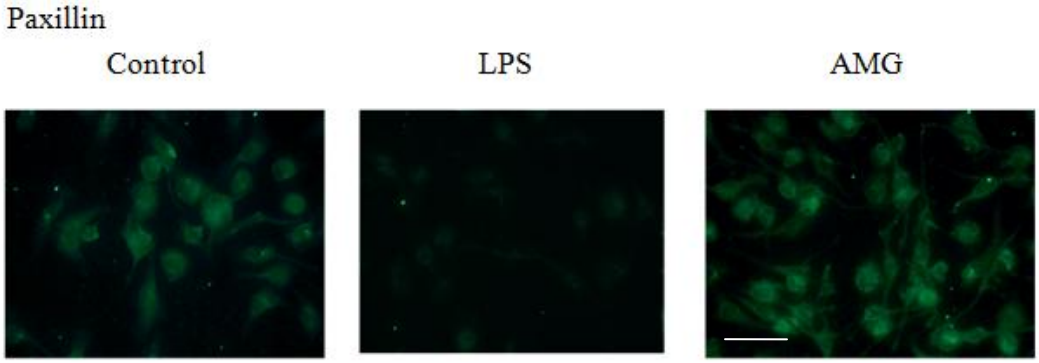

Figure 18. Immunofluorescence staining of Paxillin in BMDC. BMDC were cultured on fibronectin and treated with inflammatory factors in the presence or absence of AMG for 48h. Control represents immature DCs. (400X). Scale bar = 10μm.

CONCLUSION AND FUTURE WORK

We were able to show soluble factors and ECM components are able to determine particular DC profiles (Sprague *et al*., 2011). Flow cytometry analysis indicates that DCs upregulate maturation molecules upon LPS challenge (**Fig. 1**), while unpublished data show mature DCs lost their phenotype after NOS II inhibition. This could be due to the role played by NO during DC maturation. Data obtained by using the Griess reagent system suggests NOS II inhibitors are able to attenuate NO production from DCs and RAW cells (**Fig. 5 and 15**). To this extent, we study the effect of NO in regulating adhesive properties of DCs to ECM components. In our lab's previous data, we are able to show NO is involved in decreasing the adhesion of these cells to ECM in static culture systems (**Fig. 3, 4, 6, and 7**). This is supported by flow chamber analysis, in which SNAP, a NO donor, decreases adhesion events on both polystyrene- and fibronectin-coated surfaces (**Fig. 9 and 10**). However, when we try to determine whether a downregulation of surface adhesion molecules could occur in mature DCs, **Fig. 11 and 13** show that the integrin subunits that we are interested in will not be regulated by NO in a consistent way, but DCs downregulate adhesion molecules as maturation proceeds, which could partially explain the decreased adhesive properties in mature DCs.

Alternatively, NO may be involved in rearranging the components distributed under the cell membrane, such that the connection between cytoskeleton and surface integrin is weakened by the disappearance of focal adhesion anchor proteins, and eventually, down-modulated affinity between cells and substrates could occur (Chigev *et al.,* 2011). This idea is supported by immunofluorescence microscopy (**Fig. 14, 16-18**), in

which (1) clustering of actin cytoskeleton could be observed under the cell membrane in immature APCs but not in mature cells; (2) downregulation of paxillin and FAK in mature DCs, which could be recovered by NOS II inhibition. Thus, we suggest a possible mechanism by which APCs decrease adhesion to substrate upon contact with antigens and migrate to LN driven by chemoattractant cytokines (Murphy *et al*., 2008). This idea could explain some work by others in which integrin activity is not involved in rapid migration of leukocytes and similar migration rate could be observed in both control and integrin negative cells in 3D ECM matrices (Lammermann *et al.,* 2008). Further, in order to thoroughly investigate the interaction under focal adhesion sites, future work could focus on the expression of additional integrin-binding proteins, such as talin and vinculin, that are typically recruited at that area. Also, it would be interesting to investigate the phosphorylation process (Mitra *et al.,* 2005) at the focal adhesion site, and compare the effect of NO in regulating these protein kinases. Finally, DCs subjected to different treatments could also be investigated by using a flow chamber detachment assay. This could help determine if mature cells decrease their ability to interact with ECM and if cells under NO inhibition could recover their adhesion properties.

Another possible explanation for the decreased adhesion property in mature DCs could be the morphological change upon DC activation. Some studies suggest the microvilli structure displayed by immature DCs helps better antigen scanning at tissue sites. On the other hand, the veils surrounded structure in mature DCs favors antigen presenting toward LN (Verdijk *et al.,* 2004). Since DCs need to be fully matured at the time of antigen presentation, they must manage properly the homing to LN in a time-

dependent manner. The morphological change may help the cells to be less adhesive and allow the cells to perform random contacts with cell substrates in a rapid way, such that DCs could manage the arrival at LN in the appropriate time (Spurrell *et al.,* 2009). In fact, we could observe from the flow chamber assay that few DCs attach to the surface followed by quick departure. Since the morphological change is very much related to our previous discussion of the rearrangement in cytoskeleton and focal adhesion proteins, they could be regarded as the similar ideas supporting each other. Finally, our work in **Fig. 16** (blue arrow) suggests a microvilli structure in immature DCs. Although **Fig. 16** could not validate the veils surrounded structure in mature cells, we could tell that differences in morphology between immature and mature DCs exist. Thus, future work could focus on the determination of whether NOS II inhibited APCs recover the immature cell morphology and maintain relatively strong adhesion to ECM.

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