

**Novel Mechanisms In Dendritic Cells That Promote Th2 and Th17
But Not Th1 Responses In The Lung**

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Novel mechanisms in dendritic cells that promote Th2 and Th17 but not Th1 responses in the lung

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University of Pittsburgh, 2008

Dendritic cells (DCs) are integral to differentiation of T helper cells into Th1, Th2 and Th17 subsets. We have dissected two novel pathways in DCs that specifically regulate CD4 T cell responses. The first is the role of the c-Kit-Phosphatidylinositol 3 kinase (PI3 kinase)-interleukin-6 (IL-6) axis and the second that of vascular endothelial growth factor (VEGF).

IL-6 plays a central role in regulating CD4 T cell immune responses by limiting a Th1 response and promoting Th2 and Th17 responses. We investigate pathways in DCs that promote IL-6 production and show that the allergen house dust mite or the mucosal adjuvant cholera toxin but not Th1-inducing adjuvant, CpG oligodeoxynucleotide (ODN) promote cell surface expression of c-kit and its ligand, stem cell factor (SCF), in DCs. This dual upregulation of c-kit and SCF results in sustained PI3-kinase signaling promoting IL-6 secretion. Intranasal administration of antigen into c-kit mutant mice or neutralization of IL-6 blunted Th2 and Th17 but promoted Th1 responses in lung-draining lymph nodes. DCs lacking functional c-kit elicit diminished allergic airway inflammation when adoptively transferred into mice. Expression of the Notch ligand, Jagged-2, which has been associated with Th2 differentiation, was reduced in DCs from c-kit mutant mice. DCs generated from mice expressing a catalytically inactive form of the p110 δ (p110D910A) subunit of PI3-kinase secrete lower levels of IL-6 upon stimulation with CT. These results collectively highlight the importance of the c-kit-PI3-kinase-IL-6 signaling axis in DCs in regulating T cell responses.

We also investigated mechanisms underlying the production of VEGF, which has been recently shown to be a Th2-skewing cytokine and to promote allergic asthma. We found that CT-stimulated DCs secrete high levels of VEGF while LPS induces minimal VEGF production. Activation of iNOS, NF- κ B and PI3 kinase enhanced production of VEGF in DCs whereas IL-

12, a Th1-skewing cytokine, inhibited VEGF production. This mechanism highlights a critical but previously unknown role for DC-derived VEGF.

Taken together, these findings broaden our understanding of diverse mechanisms in DCs that enable T cell polarization and offer novel targets for therapeutic interventions.

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1.0 Introduction

1.1 Dendritic cell mediated priming of CD4 T cell responses

Dendritic cells (DCs) are important antigen presenting cells (APC) that prime naïve CD4 towards different T cell lineage. Studies have shown that the nature of the antigen, its concentration, pattern recognition molecules and the cytokine milieu are key factors that determine the outcome of an immune response. DCs are derived from the bone marrow and migrate to tissues after development where they serve as immune sentinels. In steady state, DCs constantly sample antigens in their surroundings and migrate to the tissue-draining lymph nodes to present the antigen to naïve T cells and initiate the process of T cell priming (1).

The initial understanding of DC-mediated priming of T cells was based on a simple model involving two signals from DCs. Signal 1 is the T cell receptor (TCR) stimulation mediated by the binding of the TCR to the cognate peptide displayed by the major histocompatibility complex class II (MHCII). Adequate co-stimulation from DCs is also required, and this is provided by the interaction of CD28 molecule on T cells with CD80 or CD86 displayed on the surface of DCs. Although this model defines the requirements for T cell priming, it fails to explain how T cells are polarized towards a specific lineage. The antigen and its interaction with DCs was found to be an influential factor in driving the polarization of T cell by activating “Signal 3” in DCs (2).

Kalinski and colleagues introduced the concept of signal 3, first defined as the intrinsic ability of antigens to activate specific genes in DCs which in turn promoted the skewing of T cells towards a specific lineage (2). Suggested candidates for signal 3 included cytokines, pattern recognition molecules and signaling molecules specifically regulated by antigens. A study by De Jong *et al* confirmed that the outcome of an immune response was tailored by DCs to suit the nature of the specific pathogen. This finding further strengthened the concept of signal 3 and spurred numerous studies aimed at identifying novel candidates for “Signal 3.”(3)

1.1.1 T helper1 response

The development of Th1 requires high levels of IL-12 secreted by DCs. When primed with viral peptides, antigens from intracellular pathogens or LPS, DCs promote Th1 responses by an IL-12-dependent pathway(4). Th1 response is associated with high levels of Interferon- γ (IFN- γ) secretion, which also skews T cells towards a Th1 response. The ability of IL-12 in promoting IFN- γ production in T cells demonstrates its importance in bridging the innate and adaptive immune systems (5). IFN- γ produced by T cells alone or in synergy with microbial stimuli triggers the secretion of IL-12 from resident macrophages and DCs and this provides a positive feedback loop to amplify the Th1 response (6, 7). The transcription molecule associated with a Th1 response is T-box (T-bet)(8), which specifically activates IFN- γ production and upregulates expression of IL-12 β 2 on T cells. Mice with a deletion in the T-bet gene have abrogated Th1 responses, which renders them susceptible to several pathogens (9-11)}. Deficiency in either STAT-4, the downstream signaling target of IL-12 or in STAT-1, which is regulated by IFN- γ , result in defective Th1 signaling (12-14).

In addition to the production of IL-12, the upregulation of CD40 and toll like receptor 4 (TLR4) on DCs also serve as signal 3 in promoting a Th1 response and are critical for the production of IL-12 (15-17). DCs from CD40 knockout mice have reduced IL-12 production and are also defective in Th1 priming in response to intracellular pathogens (18, 19). TLR-4 is the pattern recognition molecule associated with LPS and deleting this gene in mice abrogates TLR-4-mediated signaling and renders them unresponsive to TLR-4 and resistant to LPS-induced sepsis (20).

The production of IFN- γ by CD4 T cells promotes CD8 T cell responses and mediates protection against viruses and intracellular pathogen. High levels of IFN- γ result in persistent inflammation and is responsible for autoimmune disorders like experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA) (21, 22). However, the immune pathology and disease progression of these diseases is also significantly affected by the recently discovered CD4 Th17 subset (described in detail 1.1.3).

CD4 Th1 cells restrict the development of both CD4 Th2 and Th17 responses, which can be therapeutic in the setting of asthma but damaging in autoimmune disorders.

1.1.2 T helper2 response

CD4 Th2 cells have an important role in humoral immunity that is required for protection against helminthic parasites. An overzealous CD4 Th2 response leads to allergies as well as asthma. (Discussed in detail in Chapter 1.2)

Low levels of IL-12 production by DCs strongly favor the establishment of Th2 response. Several Th2-skewing agents have selective mechanisms to inhibit IL-12 and unique pathways to promote Th2 response. For example, prostaglandin E2 (PGE2) and cholera toxin (CT) trigger elevated levels of cyclic AMP (cAMP) in DCs through the action of G-protein coupled receptors. CT also inhibits the expression of IL-12 β 2 on CD4 T cells (23). The downregulation of CD40 on DCs by PGE2 is another mechanism by which IL-12 is suppressed (24).

Whereas the mechanisms to subvert a Th1 response involve direct inhibition of IL-12 or its signaling molecule, the pathways that promote a Th2 response are very diverse. This diversity is an important reason that explains the challenge in developing therapeutic intervention in allergies or asthma.

Unlike IL-12 that strongly influences Th1 response, a counterpart cytokine that promotes Th2 responses has not been identified. IL-6 is produced by DCs in response to allergens or cholera toxin, which promote the development of Th2 response (3, 25). However, IL-6 is not produced in response to all Th2-skewing agents. IL-6 also has an important role in promoting Th17 responses, which has been discussed in much detail in chapters 1.3 and 2. IL-1 β is also secreted by DCs in response to Th2-skewing agents (3). Recent evidence also shows that IL-1 β is important for Th2 response *in vivo* and IL-1 β deficient mice succumbed to infection by *Trichuris muris*, which can be cleared by a Th2 response (26).

Several signaling molecules and pattern recognition molecules on DCs promote Th2 development. TLRs on DCs act as sensors to recognize conserved molecular structures of pathogens. The activation of TLRs 1, 3, 4, 7, 9 primarily induce a Th1 response. With the exception of TLR3, TLRs recruit the adaptor protein MyD88 for downstream signaling.

Signaling through MyD88 was considered important for the induction of Th1 response (1, 27). However, the role for MyD88 in the induction of Th2 responses by allergens has also been established, indicating that activation of these signals is programmed by the antigen interacting with DCs (28, 29). Another example, which illustrates that DCs translate signals from pathogens is that LPS in high concentrations from *Escherichia coli* can activate the TLR4 pathway and induce a Th1 response, whereas LPS from *Porphyromonas gingivalis* activates the TLR2 pathway and promotes Th2 differentiation (30). Upregulation of CD86 and OX40 and ICOSL on DCs also promote Th2 responses (1). The role of ICOSL in promoting an exclusively Th2 response is controversial, since the involvement of ICOSL in EAE and other Th1 conditions has been shown (31, 32). It is interesting to note that statin, the cholesterol-lowering drug, which is also a Th2-skewing agent does not upregulate surface expression of known molecules on DCs or trigger the production of Th2-associated cytokine. Instead, the upregulation of a chitinase family member, YM1, in DCs following statin treatment is critical in inducing a Th2 response. DCs also utilize the notch pathway to instruct T cell differentiation (33). Upregulation of Jagged ligands (Jagged-1 and Jagged-2) in DCs upon stimulation with Th2-polarizing agents CT or PGE2 promote Th2 differentiation independent of IL-4. Delta-4 ligand on DCs is associated with the induction of a Th1 response following stimulation with CpG or LPS (34).

Following T cell differentiation, CD4 Th2 cells secrete the signature cytokines IL-4, IL-5, IL-13, IL-6 and IL-9. Although IL-4 is a potent Th2 skewing cytokine, it is not secreted by DCs but by mast cells, basophils and epithelial cells when stimulated antigen. IL-4 is critical for Th2 production, as demonstrated by the reduced Th2 responses of mice that are deficient in IL-4, IL-4 receptor or IL-4 activated transcription factor, STAT-6 (35-37). The binding of cytokine to their receptor activates the JAK/STAT pathway and mediates cytokine signaling. However, the suppressor of cytokine signaling (SOCS) molecules exert an inhibitory effect to ensure that cytokine signaling is not sustained. The SOCS molecule associated with IL-4 is SOCS-5. SOCS-5 binds to IL-4R α and turns off STAT-6 activation, and provides a mechanism by which IL-4 signaling and production is abolished in Th1 cells (38).

The signature transcription molecule associated with Th2 cells is GATA-3. Deletion of GATA-3 completely extinguishes Th2 responses and airway allergic inflammation (39-41). Other Th2-specific transcription molecules include STAT-6, c-maf and NFAT. GATA-3 is an essential transcription factor for Th2 differentiation, but is not required for IL-4 production in

differentiated CD4 Th2 cells. Deletion of GATA-3 in differentiated Th2 cells affects the production of IL-4 minimally, but affects IL-5 and IL-13 secretion significantly (41). Furthermore, IL-5 and IL-13 genes have functional GATA-3 binding sites, which necessitate the upregulation of GATA-3 for production of these cytokines (39). On the other hand, the transcription factor c-maf activates IL-4 and c-maf knockout mice have reduced IL-4 production, but they are not compromised in IL-13 and IL-5 induction (42, 43). Four members of the NF-AT family have been identified; NF-ATp (NF-AT1), NF-ATc (NF-AT2), NF-AT3 and NF-AT4. These molecules are present in the cytoplasm and upon TCR-stimulation become phosphorylated and translocate to the nucleus. NF-AT1 and NF-AT2 regulate Th1 and Th2 responses respectively (44). NF-AT also specifically activates IL-4 production (45). Deficiency of NF-AT2 inhibits IL-4 production and reduces overall Th2 responses (46, 47). GATA-3 cooperates with NFAT and c-maf to induce IL-4 production (43, 48, 49).

In conclusion, the significance of these several Th2 transcription factors implies that synergy between these molecules is important for triggering and reinforcement of Th2 responses, and their regulation is critical to prevent exaggerated Th2 responses. The different signature cytokines of Th2 coupled with the various transcription factors that promote a Th2 response highlight the fact the Th2 cells may not be a homogenous population of cells and a hierarchical usage of these transcription factors is critical to transmit the response.

1.1.3 T helper17 response

The discovery of the third CD4 T cell subset, Th17, has been truly valuable in understanding the mechanisms underlying autoimmune disorders and infectious disease models (50, 51). A Th17 response requires the involvement of several cytokines and transcription factors like the other CD4 T cell subsets. The IL-17 cytokine family includes six members: IL-17A, B, C, D, IL-17E (or IL-25) and IL-17F. While IL-25 is produced by Th2 cells, IL-17A and IL-17F are produced by Th17 cells along with cytokines IL-21, IL-22 (50, 51).

The initial discovery of CD4 Th17 came from a set of studies investigating the reason why deficiency in IL-12p19 and IL-12p40 protected mice against inflammatory diseases like experimental autoimmune encephalitis (EAE), whereas the lack of IL-12p35 and IFN- γ rendered the mice susceptible. It is known that the heterodimeric cytokine IL-23, which belongs to the IL-

12 family of cytokines, requires IL-12p40 and p19 for its signaling. In contrast, IL-12 signals through IL-12p40 and p35 (52). This suggested the development of a novel inflammatory CD4 T cell subset dependent on IL-23 but negatively regulated by IFN- γ . The discovery of CD4 Th17 established a role for IL-23 in sustaining the Th17 phenotype, rather than being involved in its development (53). Th17 requires the dual combination of IL-6 and TGF- β for the development of CD4 Th17 in mice, and IL-1 β along with IL-6 promotes the development of Th17 in human CD4 T cells (54-57). Recently, IL-21 in the absence of IL-6 has been shown to cooperate with TGF- β to promote Th17 responses and IL-21 receptor deficient mice have reduced Th17 cells. This illustrates an alternate mechanism for Th17 induction (58).

Th17 cells are important in controlling several pathogenic infections like *Klebsiella pneumoniae*, *Mycobacterium tuberculosis* and *Borrelia burgdorferi* by recruiting neutrophils (59). In addition, CD4 Th17 cells secrete chemokines CXCL9, CXCL10 and CXCL11, which attract Th1 cells. Production of IFN- γ by Th1 cells can offer protection by enabling clearance of the pathogen (60).

CD4 Th17 has been implicated in several autoimmune disorders like EAE, CIA and experimental autoimmune uveitis (EAU), which are promoted in its presence. In addition, Th17 cell differentiation and proliferation is inhibited by the presence of IFN- γ or the cytokine IL-27, which promotes IFN- γ production (61, 62). IL-2 is a key cytokine that regulates Th1 and Th2 responses. Both these subsets are dependent on IL-2 for proliferation. However, IL-2 has been shown to constrain expansion of Th17 cells: deleting IL-2 in mice results in lymphoproliferative disorder, which is now understood to be mediated by overexpansion of Th17 cells. The same phenotypic effect is seen with the deletion of STAT-5, which is the downstream target of IL-2 (63, 64).

Th17 development is programmed by specific cytokines and transcription factors. Th17 cells specifically express and require the presence of the transcription factors belonging to the family of orphan nuclear receptors; ROR- γ t and ROR- α (65, 66). The requirement for IL-6 in the development of Th17 subset implicates a direct role for STAT-3 in regulating the TH17 response (67). Both Th1 and Th2 subsets antagonize the Th17 response. The presence of IFN- γ significantly reduced Th17 development and IFN- γ knockout mice exhibit severe EAE, due to

uncontrolled Th17 cell expansion. Although, IL-25 is a member of Th17 family, it is produced by Th2 cells and antagonizes the development of Th17.

The identification of Th17 subset has certainly enhanced our understanding of host protection and autoimmune disorders; however it has also added a layer of complexity in establishing regulatory networks that promote or antagonize the different CD4 T cell subsets.

1.2 Asthma

Asthma is a debilitating inflammatory disease of the lower airways with clinical manifestation characterized by recurrent episodes of wheezing, coughing and breathlessness. From an immunological viewpoint, it is an inflammatory disease due to an exaggerated CD4 T Th2 immune response. Asthma is also marked by the overexpression of the Th2 pro-inflammatory cytokines IL-5, IL-13, and IL-4. Several studies in animals and humans have confirmed definitive roles for CD4 Th2-specific cytokines in the initiation and propagation of the inflammatory cascade. Hallmark features of asthma are the recruitment of eosinophils and mast cells, airway hyper-responsiveness to irritant and excess mucus secretion and high Immunoglobulin E (IgE) titers in the serum. Various cells belonging to both innate and adaptive immune system participate in the disease pathogenesis and this section focuses on role of the individual cell types and their role in asthma (68).

1.2.1 Dendritic cells in asthma

The ability of our immune system to mount an appropriate response while maintaining tolerance to non-pathogenic organisms as well as self antigens is an essential principle in immunology. Dissecting the immunopathology of asthma allows us to gain understanding of the intricate details of this process. Because the lung faces constant provocation from innocuous particles and pathogenic organisms, the immune system must be both tightly regulated and finely tuned. Dendritic cells (DCs) are important players in the innate, as well as adaptive immune system. Upon antigen encounter, DCs initiate either a tolerogenic or immunogenic immune response depending on the nature of antigen as well as the context in which it was encountered.

While tolerance was once thought to be a passive ignorance of an antigen, recent studies indicate that several pathways operate to ensure the establishment of tolerance by DCs (69, 70).

Murine models of tolerance are achieved through repeated exposures to antigen in the absence of adjuvant. In the airways, mucosal DCs, which reside below the epithelial cells act as sentinels and continually sample the external environment by projecting their dendrites. The interstitial mucosal DCs are CD11c⁺ and reveal an immature state evident from their high endocytic capacity and low expression of costimulatory molecules (69). DCs repeatedly exposed to antigen fail to mature and often trigger the production of the immunosuppressive cytokines IL-10 and TGF- β . These dual cytokines greatly influence the development of naïve T cells into adaptive regulatory T cells (Tregs), crucial for dampening the immune response and inducing tolerance (71, 72). The induction of adaptive Tregs is coupled with the failure of immature DCs to induce naïve CD4 T cell proliferation. T cell receptor stimulation in the absence of co-stimulation from DCs induces apoptosis and all these factors combined lead to respiratory tolerance.

Pulmonary tolerance is also dictated by the subsets of DCs interacting with the antigen. Myeloid DCs (CD11c⁺, CD11b⁺, B220⁻) are primarily responsible for inflammatory immune responses whereas plasmacytoid DCs (CD11c⁺, CD11b⁻, B220⁺) are more tolerogenic in nature. Both myeloid and plasmacytoid DCs uptake antigen and upregulate the expression of CCR7, which ensures the migration of the DCs to the lung-draining lymph nodes. Our group as well as others have shown the important role of plasmacytoid DCs in maintaining tolerance. In mice tolerized with OVA, the number of plasmacytoid DCs dramatically increased in the lung-draining lymph nodes, dominating the myeloid DCs. This was in contrast to the inflammatory model in which the myeloid DCs were the dominant subset in the lung-draining lymph nodes. Lambrecht and colleagues found that depletion of plasmacytoid DCs resulted in an exacerbated Th2 response even upon exposure to harmless antigens and showed that these DCs promote tolerance via the generation of regulatory T cells. Collectively, these studies corroborate the importance of plasmacytoid DCs in the maintenance of tolerance in the airways (73, 74).

Under normal conditions, the usual outcome to harmless antigens is unresponsiveness or tolerance. However, there is constant breach of tolerance that occurs at the mucosal surface, which can trigger an allergic response. Again, the DCs are the key players in initiating this allergic response, triggering the development of Th2 cells and re-activating the effector Th2 cells

in the lungs. In doing so, DCs create a sustained Th2 environment leading to allergic asthma. Induction of a Th2 response in the lung mediated by DCs is inherently tied to the nature of the allergen. Consider the non-enzymatic compound phytoprostane derived from pollen grains of Birch (*Betula alba*). Phytoprostane bears functional and structural homology to prostaglandin E2, which is known to prime DCs towards a Th2 polarizing capacity. This priming occurs through upregulation of CD86 expression and inhibition of IL-12 production (75). One of the immunodominant antigens from house dust mite, Derp1, subverts a Th1 response by cleaving CD40 from DCs. CD40 expression on DCs specifically promotes Th1 response by upregulating the production of IL-12 (76). Furthermore, the promotion of Th2 response by Derp1 has been attributed to the release of extracellular ATP triggered by the allergen, which activates the purinergic receptors on DCs (77). This example illustrates the two-fold nature of the Th2 response. While it is necessary that a Th2-skewing molecule is upregulated in DCs, it is equally important that a Th1 response must be suppressed. Allergens may also utilize the TLR pathway to establish a Th2 response. Low level LPS contamination seen in most allergens facilitates the promotion of Th2 by activating the TLR-4 pathway in DCs. In contrast, high level of LPS contamination in allergens switches the response to a dominant Th1 response and this is associated with elevated levels of IL-12 (28). These studies exemplify that antigenic proteins from allergens endow DCs with a capacity to induce a Th2 response through a gamut of sophisticated mechanisms.

1.2.2 CD4 T cells in asthma

Several studies conducted in the last decade have collectively established the importance of Th2 cells in asthma. The use of genetically modified mice has helped in our understanding of cytokine network, which drives the pathogenesis, demonstrating that multiple Th2 cytokines have the potential to modulate airway inflammation. The signature Th2 cytokines IL-4, IL-5 and IL-13 have been found to be elevated in the BAL of asthmatics (78).

Studies in animal models of asthma provide convincing evidence that IL-13 plays a critical role and can recapitulate the hallmarks of allergic asthma independent of other Th2 cytokines (79). The role of IL-13 in asthma emerged from research that focused on the role of IL-4 in allergic responses. In the Th2 arena, IL-4 occupies an important role as a potent Th2-

polarizing cytokine, which directly affects allergic responses. Although studies with IL-4-deficient mice demonstrated the role for IL-4 in inducing the asthma-like phenotype, priming with the allergen followed by administration of neutralizing IL-4 antibody did not protect the mice from the pathology mediated by the Th2 response. These studies not only reinforced the importance of IL-4 in the priming and proliferation of naïve CD4 T cells towards a Th2 phenotype, but also strongly suggested that alternate ligand activation of the IL-4 α receptor could sustain the Th2 response during the effector phase (80-83). The specific role of IL-13 in regulating mucus production is a critical function despite the fact its deletion significantly constrains the features of asthma. Blocking IL-13 reduced mucus-containing cells in the airways and the converse was seen in transgenic mice overexpressing IL-13 in lung epithelial cells (79, 84).

IL-5 plays a pivotal role in the recruitment, priming and survival of eosinophil and the expression of the IL-5 receptor on human and murine eosinophils reflects this influence. Studies in IL-5 deficient mice have revealed that this cytokine is not responsible for the development and maturation of eosinophils under homeostatic conditions but contributes significantly in reducing eosinophilia during allergen-induced inflammation (85, 86). Deletion of the IL-5 dependent transcription factor GATA-1 results in the ablation of eosinophilia in during allergic inflammation (87). In contrast to the role of IL-13, which regulates production of mucus and other components of the inflammatory response, deletion of IL-5 specifically alters eosinophilia in the lung. These studies have collectively helped us to understand the distinguished roles of these cytokines in coordinating the inflammatory pathway associated with asthma.

The Th2 specific transcription factors also regulate allergic inflammation. Studies from our lab and others have contributed significantly towards deciphering the molecular programming of CD4 Th2 cells. The discovery of GATA-3 as the Th2-specific transcription factor prompted further investigation into its role in regulating allergic responses. Because deletion of GATA-3 in mice results in embryonic lethality a dominant negative mutant of GATA-3 expressed under the influence of CD4 promoter was created. These mutant mice demonstrated a significant reduction in Th2-specific cytokines and displayed attenuated features of asthma upon allergen sensitization and challenge. The central role of GATA-3 in Th2 differentiation both *in vitro* and *in vivo* underscores the importance of this transcription molecule in allergic responses (88).

The signal transducer and activator of transcription (STAT) proteins are critical regulators of cytokine production. The downstream signaling of IL-4 results in the phosphorylation of STAT-6 and its translocation to the nucleus. Similar to IL-4^{-/-} mice, STAT-6^{-/-} mice also displayed reduced allergic inflammation, thus demonstrating the co-operative roles exerted by cytokines and transcription factors (89, 90). The effect of the transcription factor NF-κB which modulates the function of many cytokines is dominant in developing Th2 rather than Th1 cells, and using p50^{-/-} mice to inhibit this transcription factor significantly reduced airway inflammation. The lack of NF-κB mediated transcriptional activity specifically impaired the expression of GATA-3 in developing Th2 cells without compromising the expression of the Th1-specific transcription factor, T-bet or the production of IFN-γ in Th1 cells (91). Similarly inhibition of NFAT prevents allergic inflammation (92). These findings exemplify the intricate synergy mediated by transcriptional factors in promoting one specific arm of the immune response while inhibiting the other.

The scaffold for CD4 T cell phenotypes was restricted to Th1 and Th2 until the recent discovery of Th17 cells, which secrete copious amounts of IL-17 and IL-22. Cytokines IL-6 and TGF-β are critical in inducing this phenotype in mice, whereas the combination of IL-1β and IL-6 primes CD4 T cells towards the Th17 phenotype in humans (55, 56, 93). The expansion of the CD4 T cells to include Th17 cells has spurred several investigators to uncover the role of IL-17 in allergic inflammation. Increased levels of IL-17 found in BAL fluids of asthmatics suggest that it may play a role in promoting allergy and asthma. It has been found that the principal role of IL-17 during the initial allergen trigger is to recruit neutrophils into the lungs (94, 95). Neutrophils are the first cellular infiltrate in the lung in response to allergen, where it is possible that they mount an unsuccessful antimicrobial response in an attempt to eliminate the allergen. Progression of airway inflammation results in neutrophils being replaced by other asthma-associated cell types such as eosinophils, mast cells and CD4 Th2 cells (59). On the other hand, the elevated levels of IL-17 in asthmatics during the peak of airway inflammation strongly suggest another possible unknown role beyond neutrophil recruitment (94). However, this has not been adequately explored and further study is warranted.

Th2 cells amplify the allergic inflammation whereas Tregs dampen it during allergic responses. The suppression mechanism mediated by Tregs is diverse and tightly regulated. Naturally occurring Tregs responsible for governing central tolerance, phenotypically express the

activated IL-2 receptor (CD25) and the transcription factor forkhead box protein FOXP3. Thymically derived, naturally occurring Tregs suppress inflammation via the expression of membrane-bound TGF- β and CTLA-4 (96). The suppressive function in these cells is activated only upon T-cell receptor stimulation and exposure to IL-2. During inflammation or allergic responses, these cells are recruited to the site of inflammation and exercise their suppressive function. Adaptive Tregs generated in response to antigens are phenotypically indistinguishable from the naturally occurring Tregs (70). Studies have shown that depleting Tregs or adoptively transferring them into mice can swing the immune response from exacerbated allergic inflammation to tolerance, underscoring the importance of these cells in suppressing inflammation (97, 98). The suppressive effect mediated by these cells is contact dependent involving membrane-bound TGF- β (72). Another cytokine association with inducing tolerance is IL-10. An important subset of Tregs cells is the IL-10-producing type 1 regulatory T cells (Tr1), which protect against airway hyper-activity. Tr1 cells express FOXP3 and produce high levels of IL-10, TGF- β , IL-5, low amounts of IFN- γ and IL-2, but no IL-4. An obligatory role for IL-10 in the development of this subset of Tregs has been shown. More importantly, IL-10-derived from CD8 α DCs in the lung induce this specialized Treg phenotype (99).

In summary, these studies have shown that different CD4 T cell subsets in the lung have specialized functions that amplify or inhibit allergic inflammation.

1.2.3 Macrophages in asthma

Macrophages are important players in asthma. Alveolar macrophages (AM) are immune effector cells resident in alveolar spaces and conducting airways. The strategic location of these macrophages, coupled with the expression of different pattern recognition receptors, equips them to identify and eliminate danger signals. The secretion of various soluble and diffusible mediators is also critical in their innate immune function. These macrophages function as a double-edged sword, both suppressing and promoting DC function (100). The depletion of resident AMs with clodronate prior to allergen challenge amplifies airway inflammation. This illustrates the role of AMs in contributing to the Th1/Th2 balance (101). Macrophages secrete high levels of IL-12p70 and IFN- γ in response to mycobacterial peptides, LPS and viral antigens. Together, these are effective in counter-regulating an amplified Th2 response. In addition, the

production of nitric oxide and other free radicals in response to LPS has been shown to promote the suppressive function of macrophages (102). AMs are also the most important source of the regulatory cytokine IL-10. Adoptive transfer of macrophages from Sprague Dawley rats, which are resistant to allergic inflammation into susceptible Brown Norway rats further demonstrates the importance of macrophages in subduing inflammatory responses in the lungs (103). This illustrates the importance of AMs in maintaining a balance between inflammation and suppression.

1.2.4 Epithelial cells in asthma

Prior to interaction with DCs, allergens are faced with the challenge of breaching the physical barrier of epithelial cells. A variety of proteases secreted by allergens equip them to disrupt the epithelial-cell barrier, resulting in the secretion of various compounds, some of which influence DCs polarization towards a Th2 phenotype (104). The epithelial barrier is no longer regarded as a passive molecular sieve. We now recognize its important function as a link between the innate and adaptive immune system.

Several recent studies have provided interesting insight into this emerging concept. The cockroach allergen triggers the secretion of VEGF from epithelial cells resulting in increased vascular permeability. Interestingly, the role of VEGF as a Th2 skewing factor has also found support (105). Thymic stromal lymphopoietin (TSLP), an epithelial cell-derived cytokine that bears strong homology to IL-7 is secreted in response to allergen provocation. DCs are unable to secrete TSLP, but they preferentially upregulate the TSLP receptor, which is composed of the IL-7 receptor and TSLP receptor. A TSLP induced Th2 response by DCs is mediated through the upregulation of OX40L expression. In response to TSLP, resident DCs amplify the Th2 response by secreting chemokines IL-8 and eotaxin-2, which in turn attract neutrophils, eosinophils and CD4 Th2 cells (106, 107). TSLP typifies the relay of events initiated at the surface and when transmitted to various cells this eventually translates into an inflammatory cascade. Similar to DCs, epithelial cells also express a diverse range of receptors, which prove pivotal in inducing a Th2 response upon activation. Several studies have documented the expression of toll like receptors (TLRs) on human primary airway as well as alveolar type II epithelial cells. The secretion of IL-8 and IL-6 from epithelial cells following TLR4 stimulation with LPS has been

reported. The expression of the pattern recognition receptors on epithelial cells opens a new interpretation for this cell type as an important bridge between innate and adaptive immunity (108). Studies have also shown that sensitization with house dust mite triggers a strong Th2 response elicited by the release of the proinflammatory cytokine GM-CSF. This release occurs through the activation of protease-activated receptors (PARs) on the respiratory epithelium (104, 109). Airway epithelial cells are important, not only as the initiators of the Th2 immune response but also as key players in airway remodeling. For example, the γ -Aminobutyric acid (GABA) receptor is upregulated on epithelial cells upon exposure to allergen. Blocking the signaling of this receptor with pharmacological inhibitors diminished two essential features of asthma; airway goblet cell hyperplasia and overproduction of mucus (110). The surprising, yet significant finding that epithelial cells express acidic mammalian chitinase that trigger Th2 response and is elevated in asthmatics, underlines the diversity in protein repertoire that enable Th2 response (111).

1.2.5 Mast cells and basophils in asthma

Mast cells (MCs) reside in mucosal tissues where they actively participate in immune surveillance and host defense. MCs are discussed in conjunction with basophils since they bear strong resemblance both functionally and phenotypically. A striking difference between these cell types is that mast cells are derived from a myeloid lineage and express an array of receptors. These receptors enhance the ability of MCs to respond to a variety of stimuli resulting in secretion of several potent and biologically active mediators (112). The major growth factor of mast cells is stem cell factor, which activates the c-kit receptor expressed on mast cells. The role of MCs in immune response has been dissected by utilizing MC-deficient mice, which have decreased c-kit signaling. Increased numbers of MCs in the airways of asthmatic patients underline its prominent role in the pathology of asthma. In allergic responses MCs were initially thought to be restricted to the activation of the cells through the Fc ϵ R1, the high-affinity receptor for IgE. Mast cells express a variety of receptors including toll-like and complement receptors (113, 114). These receptors are activated by different stimuli resulting in the de-granulation of MCs, illustrating an immunoregulatory role for mast cells. MCs promote and amplify Th2 responses in part through the release of Th2-skewing cytokines, IL-4 and IL-6. Also, de-

granulation of mast cells in the lung releases IL-16, which is a chemoattractant for Th2 cells and elevated levels of IL-16 have been detected in bronchoalveolar lavage fluid from asthmatics (115). Mast cell-derived mediators influence survival, migration and polarization of DCs. Histamines, released in response to allergen-activated mast cells elevate intracellular cyclic AMP (cAMP) in DCs, which is a potent inducer of Th2 responses (116). It is also interesting to speculate whether mast cell-derived IL-1 β and TNF- α may act in concert with histamines to influence DC function and phenotype. The extremely low frequency of MCs coupled with the inability to present antigen has diminished their significance in orchestrating the allergic inflammatory response. Additionally, conflicting reports evaluating the role of MCs in allergic responses also lessens their importance as initiators of the immune response (112). In conclusion, mast cells products contribute to tissue remodeling during allergic responses and amplify the ongoing inflammation.

Whereas the importance of mast cells in inflammation has been extensively investigated, the role of basophils is less well understood due to the lack of a good genetic model. Unlike mast cells, basophils from circulation are recruited to tissues during inflammation. Similar to mast cells, basophils secrete a range of soluble mediators including IL-4 and IL-13, which are important for Th2 response. Basophils' importance in allergic response has received confirmation with recent evidence showing basophil migration to lung-draining lymph nodes in response to proteases from allergens and their ability to induce a Th2 response via production of TSLP (117). The broad immunoregulatory functions that basophils and mast cells exert through the action of their mediators have often resulted therapeutic interventions for asthma targeting these cell types.

1.2.6 Murine models of asthma

The ability to perform BAL and bronchoprovocation in asthmatics has greatly enriched our knowledge of asthma pathogenesis. However, animal models of asthma have proved fundamental in dissecting important specifics of asthma. A main advantage is the fact that mice efficiently recapitulate the cardinal features of asthma, making it a valuable tool in furthering the science of asthma. An important question asked by researchers is which animal model of asthma

is appropriate to answer their research hypothesis. Although there are no definite answers to this question, exploring the different models of asthma will help in determining which model may be the best fit to research a specific hypothesis.

Traditionally, allergic airway inflammation has been generated by the intraperitoneal administration of OVA coupled with alum. Alum, a chemical adjuvant, generates robust Th2 priming and results in the recruitment of the CD4 Th2 cells into the lungs upon challenge with aerosolized OVA. The mechanism of the adjuvant property of alum has confounded researchers and earned it the reputation of being the “dirty little secret” of immunologists. Evidence from a recent study suggests we have now taken a step towards unraveling this secret. Lambrecht and colleagues show that alum induces the migration of monocytes and DCs into the peritoneum(118). The elevation of uric acid induced by alum in the peritoneum acts an endogenous danger signal in activating the DCs. However, the exact mechanism by which uric acid induces Th2 polarization in DCs needs to be elucidated. The major criticism of using this animal model of asthma is that it deviates from the natural course of exposure to allergens and that the peritoneal cavity represents an immune milieu strikingly different from the lung environment.

The launch of cholera toxin as a mucosal adjuvant prompted investigations utilizing it in allergic inflammation. The viscous nature of alum prevents its delivery via the intratracheal or intranasal route. By contrast, OVA coupled with cholera toxin (CT) can be delivered via the intranasal route, which helps better understand the initiating events involved in the priming of T cells in lung-draining lymph nodes. It has been shown that CT elicits a dominant population of myeloid DCs in the lung-draining lymph nodes effectively inducing Th2 priming. The airway inflammation induced by OVA/CT mirrors all the features of asthma (73).

Several studies have also standardized protocols for utilizing aeroallergens like ragweed or the allergens derived from house dust mite and cockroach, which have been implicated as causative agents in human asthma (109, 119, 120). The use of “real” allergens in murine models rather than an “innocuous” protein like OVA helps to better characterize the features of human asthma. However, repeated administration of the allergens to induce airway inflammation combined with the tediousness of removing LPS from the allergen preparations often proves a deterrent to using these models. Another advantage of the OVA model of allergic inflammation is the ability to use a tolerance model in parallel induced through the repeated exposure to OVA,

which closely mimics the inhalation of innocuous particles. The simultaneous comparison of inflammation and tolerance in response to the same antigen offers great insight into the function of specific genes in asthma. These factors in combination, along with the availability of the DO11.10 transgenic mice with TCR specific for the OVA peptide, which facilitates adoptive transfer studies, makes the OVA model a common choice in studying airway inflammation. A disadvantage of the murine model of asthma is that mice often exhibit remission of disease in chronic situations. However, comparison of murine data obtained using different models with human disease can expand the understanding of allergic inflammation significantly.

1.3 Biology of c-kit and its ligand SCF

The discovery and identification of c-kit proto-oncogene marked an important milestone in understanding the biology of this receptor that is widely expressed in hematopoietic stem cells, myeloid progenitor cells, DCs, mast cell and pro-B and pro-T cells (121). In many cell types, like the B and T cells, the expression of c-kit is lost upon cell differentiation. However, mast cells, natural killer (NK) cells and DCs of the immune system retain their expression of c-kit, suggesting an important role for this molecule in these cell types (122, 123). Certain lineage of cells that express c-kit also produce its ligand, stem cell factor (SCF), indicating a self-regulated feedback to enhance receptor expression. c-kit signaling has profound effects in various biological functions such as spermatogenesis, melanin pigment formation and erythropoiesis (123, 124). Mutations in c-kit results in the development of various tumors due to aberrant signaling of the receptor, which necessitates a complete understanding of c-kit structure, the initiation of signaling events as wells as the downstream targets of the receptor (125, 126).

1.3.1 Structure and signaling mediated by c-kit

c-kit is a type III, tyrosine kinase receptor (127) which shares strong homology and function to platelet-derived growth factor receptor, macrophage colony stimulating factor receptor, and the F1 cytokine receptors. All type III receptors are characterized by the five immunoglobulin like domains in the extracellular region, followed by 70-100 a kinase domain (residues 70-100) in the cytoplasm. Similar to most tyrosine kinase receptors, the extracellular

domain facilitates the binding of the ligand and the cytoplasmic domain serves to transduce the signal (128). Alternate splicing of murine c-kit mRNA results in two isoforms characterized by the presence or absence of a GNNK (glycine-asparagine-asparagine-lysine; codons 510-513) tetrapeptide in the juxtamembrane region of the extracellular domain. In humans, the expression of similar splice variants has been documented. These isoforms of c-kit are expressed in different ratios in various cell types and also differ in their signaling capabilities (129). The binding of the SCF induces the homodimerization of the c-kit receptor resulting in the phosphorylation of selective tyrosine residues on the c-kit, thereby unmasking docking sites for the Src-homology2 (SH2)-containing signal transducers (130). Site-specific mutagenesis studies have revealed a hierarchical importance in the phosphorylation of tyrosine residues. Some mutations can completely abrogate c-kit signaling, while others only significantly dampen the overall signaling (131, 132). The downstream signaling pathways of c-kit are mediated primarily via the phosphatidylinositol –3 (PI3) kinase(133). Other pathways closely associated with c-kit signaling include the JAK/STAT pathway and src-kinase pathways.

1.3.2 PI3-kinase pathway in c-kit signaling

The involvement of PI3-kinase in c-kit signaling has been extensively characterized. A combination of molecular mutagenesis studies and biochemical analyzes have shed light on the relationship between c-kit and PI3-kinase. The p85 regulatory subunit of PI3-kinase specifically associates with phosphorylated tyrosine residue 721 of c-kit, resulting in the recruitment and phosphorylation of protein kinase B (AKT) (133). Transgenic mice harboring a point mutation in the tyrosine residue have revealed the physiological importance of this residue (134). Substitution of tyrosine with phenylalanine resulted in both reduced spermatogenesis and impaired follicular development. Phosphorylated-AKT enhances the survival and proliferation of the primordial cells and specifically mediates several downstream functions through NF- κ B signaling as well as phosphorylation of the pro-apoptotic molecule BAD (135). Phosphorylation of BAD inhibits the pro-apoptotic function of the molecule, which is one of the reasons why impaired c-kit signaling results in reduced proliferation and survival of several cell types. The c-kit gene maps to the dominant white spotting (W) locus in mice (136). Furthermore, mutations in the W locus have shown to cause deficiency of melanocytes as well as reduced PI-3 kinase

activity (137). It is interesting to note that extracellular c-kit mutations results in the hyperactivation of c-kit, marked by prolonged PI3-kinase activation and enhanced cell survival and proliferation. However, PI3-kinase mediated signaling was not required for the internalization of c-kit (138). This indicates that mechanisms which downregulate c-kit signaling pathways are also operative in cells to prevent sustained signaling.

Protein kinase C, a known negative regulator of PI3 kinase pathways phosphorylates residues S741 and S746 downregulates c-kit signaling (139, 140). This is evident from mutational studies, where substitution of these serine residues to alanine resulted in prolonged c-kit signaling. SOCS1 and SOCS8 bind to c-kit and dampen downstream signaling of the receptor (141, 142). The deregulation of the activation or inhibitory pathways of c-kit has adverse effects, often resulting in tumor formations.

c-kit signaling has been shown to regulate the production of various cytokines and growth factors. Of specific interest to our study is the regulation of IL-6 production mediated by c-kit, which has been documented in mast cells and as we have reported, also in DCs. Several adapter proteins have been associated with PI3-kinase signaling and a significant body of research has focused on adapter molecule, Gab2. Gab2 is an important activator of PI3-kinase and Gab2 deficient mice show reduced airway inflammation, decreased IL-6 production and a reduction in mast cells. This study connects the importance of c-kit mediated signaling via PI3-kinase and Gab-2 in regulating mast cell function and IL-6 production (143).

1.3.3 Other pathways involved in c-kit signaling

Addition of SCF has shown to activate the Jak/STAT pathway and Jak2 specifically associates with c-kit and results in the specific activation of STAT-1, STAT-3 and STAT-5. The involvement of these STAT pathways also directly implicates a role for c-kit in the development of pro-B and pro-T cells as well as mast cells. SCF-induced Jak/STAT pathway is critical for the development and differentiation of fetal liver cells Furthermore, a synergistic cooperation between the PI3-kinase pathway and Src kinase has also shown to be important for c-kit signaling (136).

1.3.4 Biology of Stem cell factor (SCF)

SCF, the ligand of c-kit is encoded by the Steel (Sl) locus on chromosome 12 in humans and chromosome 10 in mice (144, 145) Like c-kit, SCF also exhibits two distinct isoforms that arise from alternative splicing of exon 6 of the mRNA. The 248 amino acid isoform of SCF is cleaved in the domain encoded by exon 6 resulting in the soluble form of SCF, comprising of 165 amino acid residues. In contrast, membrane-bound SCF lacks the proteolytic cleavage site encoded by exon 6, which results in anchoring of the protein to the membrane. Alternatively, the membrane-bound form may also produce a soluble form from proteolytic cleavage (146, 147).

Work from the Leder laboratory elegantly showed the presence the membrane-bound form of SCF, even before the mechanism behind this process was understood. Unlike other molecules, where the discovery of the membrane-bound followed the identification of the soluble form, SCF proved to be the opposite. Mast cells expressing c-kit can proliferate in the presence of 3T3 fibroblasts. This first suggested that fibroblasts cells had the capacity to produce the unknown ligand, SCF. The supernatants from the fibroblast culture could not induce the proliferation of mast cells, which provided further evidence that a membrane-bound ligand was secreted by the fibroblasts. Membrane-bound SCF has signaling properties, distinct to that of the soluble form and this results in varied biological functions mediated by the two isoforms (148, 149).

A number of proteases have been implicated in cleaving membrane-bound SCF to produce the soluble form. In mice, A desintegrin and metalloprotease19 (ADAM 19) and ADAM33 have been shown to induce soluble SCF through proteolytic cleavage (150). Matrix metalloproteinase-9 (MMP-9) is responsible for the active recruitment of hematopoietic stem cells (HSC) during infection as well as other stress-induced responses. This is mediated through the cleavage of the membrane-bound SCF from stromal cells to release the soluble form. Soluble SCF plays a critical role in the proliferation, differentiation and survival of HSC. In humans,

mast cell chymase is the enzyme that cleaves membrane-bound SCF and the soluble form produced also contributes to mast cell proliferation (151-154).

A great amount of research has contributed towards understanding the functional and biological differences contributed by two isoforms.

1.3.5 Role of c-kit and SCF in immune cells

Most immunological work relating to c-kit, except in the field of mast cells has primarily focused on the phenotypic expression of the receptor rather than the signaling axis upon ligation with its ligand. c-kit or CD117 has most commonly been associated with the development and function of mast cells. Mutations in c-kit can severely impair mast cell function and in fact, completely abrogate mast cell development *in vivo*. Bone marrow- derived mast cells from c-kit mutant mice show no phenotypic difference when compared to the WT mice. However, functions of mast cell- derived mediators from c-kit mutant mice were found to be severely compromised (112). These included the production of various soluble mediators like IL-6 and TNF- α and these studies collectively show that the expression and the signaling of c-kit can prove pivotal in dictating immune response outcomes. In addition, the deficiency of c-kit results in embryonic lethality in mice, which is not surprising considering the important role it plays in HSC development and differentiation. However, this often prevents an accurate assessment of the role of c-kit. The use of bone marrow –chimeras and the sorting of cells based on c-kit expression are complement approaches used to dissect the exact function of c-kit.

Natural killer (NK) cells are important cells of the innate immune system, that offer vital protection against invading pathogens and tumors through the release of cytolytic granules, which directly lyse cells or via the production of IFN- γ , which can restrict the viral infection, prior to activation of CD8 T cells (155). NK cell differentiation from HSC cells is a complex and multistep process tightly regulated by several enzymes and signaling molecules. The expression of c-kit on HSC assigns a pivotal function for the receptor in the development and maturation of NK cells. Administration of exogenous SCF to humans or mice results in an increase in NK cell number and promotes the cytolytic activity, further strengthening the role for c-kit and its ligand in regulating NK cell activity (156) . Reconstituting NK cell deficient mice with HSC deficient in c-kit by utilizing the technique of bone marrow-chimeras has convincingly showed that c-kit

expression and signaling is critical for NK cell activation. This study was the first to dissect the importance of this phenomenon (157).

Considering that Pulendran and colleagues documented the expression of c-kit on spleen DCs in 1997, it is surprising to note the paucity of research in addressing the role of c-kit in DCs (158). c-kit on DCs has long been ignored because the *in vitro* development of bone marrow – derived DCs from progenitor cells stamped c-kit as a stem cell specific marker. In fact several studies correlated a decrease in c-kit expression with the DCs development and maturation (159, 160). Borg *et al* for the first time assigned an important immunoregulatory function to c-kit on DCs. This study showed that c-kit signaling in DCs negatively regulates the ability of DCs to activate NK cells. DCs from c-kit mutant mice or the treatment with tyrosine kinase inhibitor, Gleevec, triggered the upregulation of a cell-associated molecule in DCs, which greatly enhanced their cytolytic activity and also IFN- γ secretion from NK cells. This study not only implicated an important role for c-kit on DCs, but also helped better understand the therapeutic role of Gleevec in treating tumors like gastrointestinal stromal tumors (161). The recently discovered interferon-producing killer dendritic cells (IKDCs), endowed with its ability to kill tumor cells and produce high amounts of IFN- γ , were produced in response to chemotherapy with Gleevec. The IKDCs exhibited several NK cell-specific markers, including c-kit (162).

Both membrane-bound and soluble SCF are expressed on mast cells and SCF is critical in inducing mast cell development and proliferation. Mice with specific mutations in c-kit or SCF exhibit absence of mast cells in addition to other defects (112). Human peripheral blood eosinophils were shown to express membrane-bound and soluble SCF, which offers a plausible mechanism by which eosinophils can influence allergic responses (163).

These studies have opened the doors to further unraveling the function of c-kit on DCs in response to various stimuli. Our study, described in detail in chapter 2 has also significantly contributed in understanding the mechanisms by which DCs from c-kit mutant mice prime naïve T cells towards a Th1 response and deviate from a Th2 response, even when stimulated with potent Th2-inducers.

1.4 Biology of IL-6

The pleiotropic cytokine interleukin-6 (IL-6) has several functions in both innate and adaptive immunity. In spite of the fact that IL-6 was one of the earliest discovered cytokines, the exact nature of its function is still poorly understood. IL-6 is produced by a wide range of cell types and exerts its influence on many different cell types including immune as well as non-immune cells. IL-6 is an important mediator of the acute phase response, which is triggered by infection and inflammation, and it also plays multiple roles during the later adaptive immune response. These include mediating protection against incoming pathogens, stimulating antibody (Ab) production by B cells, regulating macrophage and dendritic cell differentiation, and modulating the response of regulatory T cells during microbial infection. On the other hand, the role of IL-6 in inhibiting neutrophil migration during acute inflammation in the lungs illustrates its anti-inflammatory property. This dual function of IL-6 as both a pro and anti-inflammatory cytokine highlights its importance in the regulation of immune responses (164).

1.4.1 IL-6 signaling

The IL-6 family of cytokines includes IL-6, IL-11, LIF (leukemia inhibitory factor), OSM (oncostatin M), CNTF (ciliary neurotrophic factor), CT-1 (cardiotrophin-1) and CLC (cardiotrophin-like cytokine), all of which utilize the IL-6 receptor complexes to signal. The receptors employed by the IL-6 cytokine family are composed of the non-signaling receptors (IL-6R α , IL-11R α and CNTFR α) and the signal transducing receptors (gp130, LIFR and OSMR). The binding of cytokine to the receptor results in receptor dimerization and subsequent recruitment of the Janus Kinase (Jak) tyrosine kinase family members. The Jaks can autophosphorylate or phosphorylate each other triggering docking of the STAT molecule. The STATs dimerize and translocate to the nucleus to induce downstream gene transcription or repression. IL-6 cytokine signaling is tightly regulated at various levels to prevent unnecessary inflammation. As one example, although the expression of gp130 is ubiquitous, the signaling of IL-6 is restricted by the presence of the α -receptors (165).

The presence of soluble IL-6R adds an additional layer of complexity in understanding the biology of this cytokine. The soluble form of the receptor is produced as a variant by alternative splicing or through proteolytic cleavage of the membrane-bound receptor. The molecular mechanisms underlying the generation of the soluble receptor are poorly understood.

The soluble IL-6 receptor when bound by IL-6 can directly activate gp130, bypassing the need for the functional IL-6 α subunit. Although most soluble receptors act as antagonists, this is a rare example of a soluble receptor acting agonistically and amplifying rather than inhibiting the immune response. The elevated levels of the soluble IL-6R documented in several inflammatory disorders has made it an obvious choice for therapeutic intervention (166). The increase of soluble IL-6R in the BAL of asthmatics and in murine models of allergic inflammation has been documented and the administration of neutralizing antibodies against the soluble IL-6R prior to allergen challenge greatly ameliorates airway inflammation (167).

Signaling via gp130 results in the activation of Jak-1, Jak-2 and Jak-3. The important signal transducer for gp130 is Jak-1. Jak-1 deficiency severely compromises IL-6 signaling. The IL-6- family of cytokines primarily activate STAT3 in most signaling cascades and to a lesser degree, can also induce STAT1 activation (165). The dual activity of STATs mediated by IL-6 is a mechanism by which it exerts its proinflammatory vs. anti-inflammatory role. The tyrosine phosphorylation of STATs results in their nuclear translocation and studies have also shown that the dephosphorylation of STAT leads to its export from the nucleus. Interestingly, this constant shuttling of STAT-3 can proceed independently of receptor phosphorylation and signaling, resulting in prolonged STAT-3 signaling (168).

Although, prolonged STAT-3 signaling has been reported in certain pathological conditions, the negative regulators of IL-6 dampen IL-6 signaling. Negative regulation is primarily executed by SOCS-3 but the protein inhibitor of activated STAT-3 (PIAS) also plays a role (165). SOCS3 is significantly upregulated in macrophages and dendritic cells following TLR stimulation and it negatively regulates IL-6 signaling. The binding of SOCS3 to the IL-6R subunit gp130 selectively inhibits IL-6 allowing for the signaling of IL-10 to remain unaffected. Macrophages from SOCS-3 conditional knockout mice also show prolonged STAT-3 activation. Counter to expectation, IL-6 signaling in these cells resulted in an anti-inflammatory response mediated by the upregulation of IFN- γ -specific genes, similar to those mediated by IL-10. The specific role of SOCS3 in DC function and development has also been examined. SOCS3-transduced DCs are potent inducers of Th2-cell differentiation, both *in vitro* and *in vivo* and were also effective in suppressing experimental autoimmune encephalitis (EAE) by inhibiting the Th1 and Th17 arms of the immune response. Furthermore, the level of SOCS3 expression was increased in T cells from patients with asthma. This increase of SOCS3 in asthmatics correlates

with the documented increase in IL-6 from the BAL of asthmatics. The important role of SOCS3 in regulating IL-6 signaling has resulted in this molecule being targeted by several therapeutic studies (169).

PIAS family members regulate the transcriptional activity of many genes. PIAS1 and PIAS3 inhibit STAT1 and STAT3 DNA-binding activities respectively. IL-6-induced activation of STAT3 is blocked in myeloma cells via the up-regulation of PIAS3 synthesis (170). A recent study has shown the mechanism of PIAS-mediated inhibition of STAT-3 function to be mediated by association with SMAD-3 upon TGF- β stimulation (171). The study offers interesting insight into molecular mechanisms that regulate IL-6 inhibition mediated by TGF-beta signaling. In summary, the complexity of the biology of IL-6 stems from the diverse functions mediated by this cytokine and the varied nature of the molecules that regulate its signaling.

1.4.2 IL-6 in T cell responses

IL-6 is the only cytokine to be implicated in Th1, Th2 and Th17 responses (50). As a central regulator of the T cell response, it is able to activate and inhibit various molecules in a concerted manner. Rincon and colleagues initially reported the role of IL-6 in modulating T cell responses, and IL-6 secreted by antigen presenting cells was shown to be important in skewing naïve T cells towards a Th2 response (172). This study resolved the unanswered question as to how antigen presenting cells (APCs), which do not secrete the potent Th2 skewing cytokine IL-4, were able to prime T cells towards a Th2 response. IL-6 not only promotes the Th2 arm of the immune response, but concomitantly inhibits the Th1 responses. IL-6 inhibition of Th1 responses is mediated by direct suppression of IL-12 secretion by DCs and through the upregulation of IL-10. IL-6 also cripples the Th1 response in T cells by inhibiting the production of IFN- γ via the upregulation of SOCS-1. SOCS-1 deficient mice show overproduction of IFN- γ , defining the role of SOCS-1 as a negative regulator of IFN- γ (173). IL-6 promotes Th2 responses by upregulating the Th2-specific transcription factors GATA-3, c-maf and NFAT-2. The inability of GATA-3 to activate the IL-4 promoter suggests that IL-6 may also activate other Th2 transcription factors. The activation of GATA-3 and c-maf by IL-6 is dependent on endogenous IL-4 (174). However, IL-6 mediated transcriptional activity of NFAT is independent of IL-4. CD4 T cells from transgenic mice expressing a dominant negative mutant of NFAT were

handicapped in their ability to induce the production of IL-4 and this prevented IL-6 from inducing a Th2 phenotype (46). These studies collectively indicate that IL-6 can skew T cells towards a Th2 response independent of IL-4 but the presence of IL-4 further helps to amplify the response.

Our understanding of how IL-6 helps to maintain the balance between Th1 and Th2 responses also comes from various infectious disease models. Interleukin-6-deficient mice infected with *Borrelia burgdorferi*, which induces a Th1 response, have decreased Th2 responses and increased Lyme arthritis mediated by the amplified Th1 response (175). IL-6 deficient mice infected with helminthic eggs from *Schistosoma mansoni* initially show a lag in IL-4 production when compared to the WT mice (176). However, the overall Th2 responses showed no significant difference, but the level of IFN- γ in the IL-6 deficient mice was increased. Balb/c mice susceptible to *Leishmania major* infection due to the inability to mount a robust Th1 response when treated with anti-IL-6 antibody following infection had decreased IL-4 secretion and increased IFN- γ , but no overall difference in the disease outcome (177). These studies collectively show that IL-6 limits the production of IFN- γ while promoting the production of IL-4.

IL-6 also plays an important role in asthma. Increased levels of IL-6 have been detected in the BAL fluids of asthmatics (178). The inhibitory role of IL-6 in blocking aeroallergen-induced responses in the lung has also been reported. These studies reinforce both pro and anti-inflammatory roles for IL-6, and show that the deficiency of IL-6 can in fact be therapeutic (179). The opposing roles of IL-6 documented in asthma can be attributed to different models that were tested. In chronic models involving administration of OVA coupled to alum, it is possible that IL-4 production is triggered independently of IL-6, leading to inflammation whereas in acute models of inflammation, the proliferation of T cells is more dependent on IL-6.

Revival of interest in IL-6 biology was seen with the discovery of Th17 cells. The initial discovery of Th17, which came from a collection of studies carried out on IL-12p40, IL-12p19 and IFN- γ knockout mice, did not suggest a role for IL-6 in mediating Th17 responses, but suggested a role for IL-23. However, the combination of IL-6 plus TGF- β is now known to be responsible for the Th17 phenotype in mice and the fact that IL-6 in concert with IL-1 β induces the same phenotype in humans underscores the importance of IL-6 in Th17 responses (50, 55). Furthermore, the requirement of STAT3 in the development of Th17 corroborates the importance

of IL-6. IL-23 is not critical for the development of Th17 subset but is required for the selective expansion of these cells. Inhibition of Th17 development by IFN- γ is mediated by the Th1-specific transcription factor T-bet. Initially, ROR- γ was designated as the Th-17-specific transcription factor; however, a recent study has shown that ROR- α is required for the differentiation of Th17 cells. The role of ROR- α as a Th17-specific transcription factor now explains the residual population of Th17 cells seen in ROR- γ deficient mice. ROR- α is upregulated in response to IL-6 and TGF- β and the dual orphan nuclear receptors synergistically cooperate to induce Th17 responses (65, 66).

Several autoimmune and inflammatory disorders are characterized by overzealous CD4 T cell responses combined with failed regulatory mechanisms exerted by CD4 Tregs. The role of IL-6 in regulating Tregs was established by a study that showed that the production of IL-6 from APCs via TLR activation enabled effector T cells to overcome the suppressive function of Tregs (180). It is interesting to note that Treg-mediated suppression was inhibited by IL-6 even though the proliferation of Tregs was stimulated by IL-6. Recent studies have shown that IL-6 in conjunction with IL-2 can actually promote the suppressive activity of Tregs, which is switched off in co-cultures with effector CD4 T cells and Tregs. The suppressor of cytokine signaling-3 (SOCS-3) specifically inhibits IL-6 signaling. Low levels of SOCS-3 expression enhance the suppressor function of Tregs whereas retroviral overexpression of SOCS-3 abrogates Tregs' suppressive function (181). Although innate and acquired immunity have been considered as independent systems, recent advances in IL-6 biology have shown the importance of this cytokine in the role of an immunological switch.

1.5 Biology of VEGF

Vascular endothelial growth factor (VEGF) is one of the most potent mediators of angiogenesis and is a critical regulator of vascular permeability to water and proteins. Since the development of vascular supply is fundamental to most biological processes, the production of VEGF in various organs is indispensable. Hence, it is not surprising that the deletion of VEGF results in embryonic lethality. The human VEGF gene is localized in chromosome 6p21.3 and several isoforms of VEGF arise from alternate splicing. VEGF protein, commonly referred to as VEGF-A, is a 45 KDa glycoprotein (182).

1.5.1 VEGF receptor signaling

VEGF signaling is mediated by two tyrosine kinase receptors. The fms-like-tyrosine kinase (Flt-1 or VEGFR-1) and kinase domain region (KDR) receptors bind VEGF with high affinity. Fetal liver kinase-1 (Flk-1 or VEGFR-2) is the murine homolog of KDR receptor, has high homology sequence to the human KDR receptor. The VEGF receptors belong to the type II tyrosine kinase receptors, characterized by the seven-immunoglobulin-like domains in the extracellular region, a single transmembrane region and a tyrosine kinase domain. The affinities of the receptors for VEGF have shown to vary greatly and the VEGFR-1 receptor has the highest affinity for VEGF. An alternatively spliced soluble form of VEGFR-1, lacking the transmembrane sequence and the cytoplasmic domain has been identified in several organs. This soluble receptor binds VEGF, functioning as a manacle to trap free VEGF. The role of the soluble receptor as an antagonist of VEGF signaling has been reported (182).

The major difficulty in understanding signaling pathways of the VEGF receptor is due to the crossregulation that operates between the receptors. There are conflicting findings regarding the signaling pathway for VEGFR-1. Some studies have shown that the VEGFR-1 may function in sequestering VEGF and prevent its binding to VEGFR-2. Alternatively, it has been proposed that VEGFR-1 via the PI3-kinase pathway activation results in inhibition of VEGFR-2 (183). VEGFR-1^{-/-} mice die *in utero* due to overgrowth of blood vessels and not due to defective vasculargenesis, thus implicating a negative role for this receptor in signal transduction.

The soluble and membrane-bound forms of the receptor have distinct biological functions. For example, the soluble receptor is expressed in high levels in the cornea and the receptor acts as a sink for free VEGF. The over-expression of the soluble receptor in the cornea maintains the avascularity of the tissue, which is responsible for the optical clarity and vision (184). The signaling components mediated by VEGFR-1 are poorly understood due to weak signaling responses seen in cell culture systems. Also, Tyrosine residue 1169 is weakly phosphorylated in response to VEGF and this induces a mild and transient activation of the MAP-kinase (182).

On the other hand, VEGFR-2 induces strong signaling. VEGFR-2 upon stimulation with VEGF is autophosphorylated. Using biochemical analysis, studies have identified several

tyrosine Phosphorylated residues 951, 1054, 1059, 1173, and 1214. Phosphorylation of residue 1175 results in the binding of phospholipase C- γ (PLC- γ), via the SH-2 domain. Activated PLC- γ activates the downstream target MAP-kinase. Recruitment of the SH-2 domain also results in the binding of the regulatory PI3-kinase subunit, p85. This results in the activation of AKT (182). Transgenic mice that harbor a point mutation in tyrosine 1173 residue die *in utero* and multiple organs show poor vasculargenesis (185). Although, both VEGFR-1 and VEGFR-2 knockout mice die on or around day 9 (186) following embryonic development, these mice have very different phenotypes. These studies emphasize the different signaling roles of the two receptors.

1.5.2 Pathways in the lung regulating VEGF production

The lung is a highly vascularized organ and exhibits high levels of VEGF. VEGF receptor has been detected on alveolar type II cells, epithelial cells, vascular smooth muscles, macrophages as well as neutrophils. The expression of VEGF protein is more restricted to epithelial cells.

Hypoxia-inducible factor-1 α (HIF-1 α) is a potent inducer of VEGF production and the hypoxic environment in a developing lung favors production of VEGF (187). The importance of this transcription molecule, HIF-1 α , in regulating VEGF production is demonstrated from lung biopsies of infants with bronchopulmonary dysplasia (188). Premature infants receive respiratory support from mechanical ventilators, which deliver a high concentration of oxygen producing a hyperoxic environment. This environment results in the inhibition of HIF-1 α and thus suppresses VEGF production. Transgenic mice overexpressing VEGF were found to be protected from hyperoxic lung injury when compared to the WT control (189, 190). Our lab has also shown that activated AKT is protective against hyperoxia-mediated lung injury (191, 192). This directly correlates with the fact that VEGF-mediated signaling activates PI3-kinase pathway and induces phosphorylation of AKT activation. On the other hand, asthma, emphysema and chronic obstructive pulmonary disorder (COPD) are characterized by elevated levels of VEGF. High levels of VEGF have been reported in BAL fluids of asthmatics. The presence of elevated levels of VEGF in asthmatics has been postulated to contribute significantly to tissue edema (193). However, a novel role for VEGF as a Th2 skewing factor has been recently identified and CD4

Th2 cells also secrete copious amounts of VEGF (194). We have also dissected a critical role for DCs secreting VEGF in promoting Th2 responses (discussed in detail in chapter 3).

Another molecule closely associated with VEGF and asthma is nitric oxide (NO). Nitric oxide (NO) is an essential gaseous regulator and also an important second messenger exerting a range of physiological functions. Elevated levels of exhaled NO from asthmatics and increased levels of nitric oxide synthase (NOS) in lung biopsies from asthma patients implicate an important role for this molecule in regulating the pathophysiology of asthma (195). VEGF is an important mediator of NO production and several pulmonary alterations are contributed by VEGF in a NO-dependent mechanism. In VEGF transgenic mice, pharmacological inhibition of NO results in diminished features of asthma suggesting the importance of VEGF-NO signaling axis in regulation pulmonary inflammation (196).

Molecules inhibiting VEGF production or signaling have not been studied in great detail. Studies have shown that IL-12 is a potent inhibitor of VEGF production and inhibition of IL-12 or its deficiency promotes tumor formation (197-199). PI3-kinase is a potent inhibitor of IL-12 production and promotes VEGF production (200). This implicates a direct role for the molecule in VEGF production. Genetic studies in zebrafish showed for the first time VEGF mediates notch-specific signals. VEGF promotes the expression of delta-4 ligand in human endothelial cells. Although, delta-4 ligand expression is dependent on VEGF, the molecule exerts an inhibitory role by suppressing VEGF production. Delta-4 ligand promotes the expression of the soluble VEGFR1, which is known to inhibit VEGF production and signaling (201). Since, VEGF and the notch pathway are important in development the interactions between the pathways though obvious are poorly characterized. It is interesting to speculate that other notch ligands like jagged-1 and jagged-2, which are associated with Th2 responses may synergize to upregulate VEGF production.

1.5.3 VEGF in the immune system

The role of VEGF as a mediator of immune responses has not been extensively studied. The novel function of VEGF as a promoter of Th2 responses has shed new light for the cytokine's involvement in asthma (194). The limited capabilities of DCs, macrophages and

neutrophils to secrete VEGF has been one of the reasons this cytokine's function in mediating immune responses has not been studied.

In the field of tumor immunology, most of the studies have focused on the immunosuppressive role of VEGF on DC development and maturation. The presence of several angiogenesis factors during tumor development also suggests other molecules might be involved in mediating this suppressive effect and not necessarily VEGF alone. One study has reported the ability of alternatively-activated DCs (AADCs) to secrete VEGF and has shown that LPS stimulated myeloid DCs are unable to do so. This study however used prostaglandin E2 (PGE2) and IL-10 to induce an AADC phenotype. PGE2 is known to prime DCs towards a Th2 response and IL-10 is an effective inhibitor of IL-12 production, which makes it conducive for VEGF production (202). Hence, this data does not preclude the ability of myeloid DCs to secrete VEGF. Cockroach allergen triggers the secretion of VEGF from epithelial cells, which makes it an ideal candidate to be tested on myeloid DCs for VEGF production.

CD8 T cells and NK cells lyse tumor cells through their cytolytic activity and IFN- γ production (155). Since, VEGF is now known to induce a Th2 response it is possible that it suppresses IFN- γ production and promotes tumor formation.

In conclusion, dissecting the role of VEGF in mediating different immune responses will open the doors to powerful therapeutic interventions in the field of asthma and cancer.

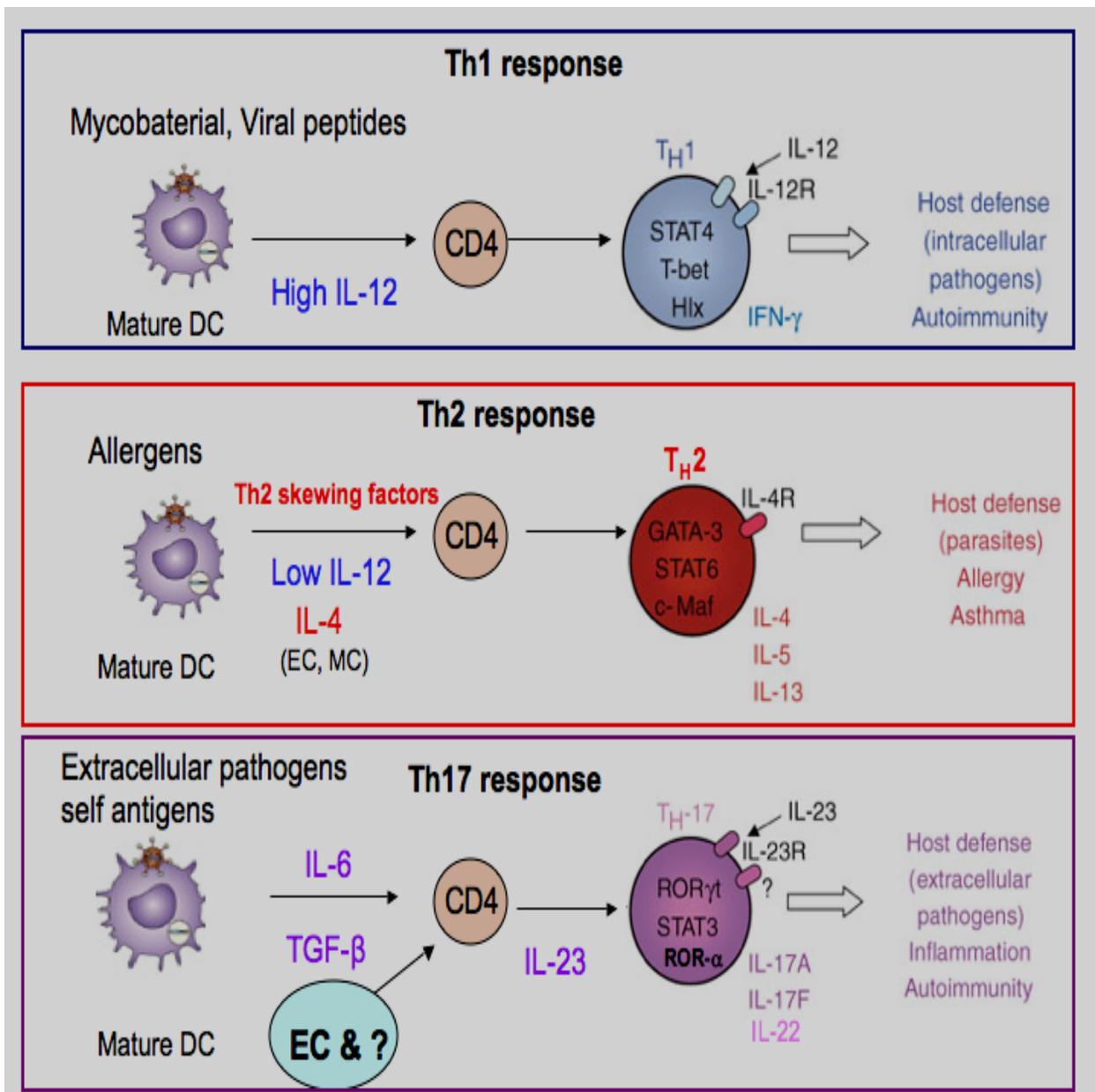


Figure 1: Model depicting CD4 T cell priming in the lung.

Following antigen encounter in the lung, DCs mature and migrate to the lymph nodes to prime naïve CD4 T cells or CD8 T cells. This model depicts the cytokines and transcription factors involved in the priming of CD4 T cell lineages. EC-Epithelial cells and MC-Mast cells are the source of the Th2 skewing cytokine, IL-4. Epithelial cells along with other cell types like regulatory T cells contribute to TGF- β production, which can promote a Th17 response.

1.6 Specific aims

The primary focus of our laboratory has been towards understanding regulation of Th2 responses and how this regulation is altered during allergic inflammation or asthma, which is a result of an exaggerated Th2 response. Recent studies have defined an important role for DCs as the initiators of the inflammatory response seen in asthma and the depletion of DCs during allergen challenge results in diminished airway inflammation (69, 104). These studies collectively show the importance of DCs in regulating different stages of inflammation in the progression to asthma.

Recently, we used a model of antigen (OVA) coupled with CT administered intranasally to induce airway inflammation. This model is efficient in reproducing the hallmarks of asthma and also enables investigation of priming events in lung-draining lymph nodes. Our findings showed that the mucosal adjuvant promoted the migration of myeloid DCs into the lung-draining lymph nodes, which are inflammatory in nature (73). We also wanted to dissect the different genes CT specifically regulates in myeloid DCs that promotes Th2 priming. For this purpose, we undertook a microarray approach to identify novel genes regulated in CT-stimulated DCs. Our microarray data revealed significant upregulation of c-kit, IL-6 and VEGF gene transcripts. The studies in this dissertation focuses on two important pathways in DCs that regulate T cell responses; the first is the involvement of c-kit-PI3 kinase-IL-6 signaling axis and the second that of vascular endothelial growth factor.

1.6.1 Specific Aim 1:

c-kit expression and signaling via PI3 kinase regulates IL-6 production and T cell responses.

c-kit is an important marker of mast cells and signaling via c-kit regulates the production of IL-6 in these cells. However, the upregulation of c-kit on DCs following CT stimulation implicated a role for this molecule in regulating IL-6 production in DCs. IL-6 is also an important cytokine that affects CD4 T responses. No study so far has dissected the mechanism of c-kit signaling in DCs and the role it plays in regulating T cell responses. In chapter 2, we describe a novel mechanism by which upregulation of c-kit and its membrane-bound ligand, SCF

is triggered by CT or allergens (House dust mite and OVA) regulate IL-6 production via the PI3-kinase pathway. We establish the specificity of this pathway in promoting Th2 and Th17 responses by using CpG oligodeoxynucleotide (ODN), which does not promote c-kit or SCF upregulation, but induces a potent Th1 response. The impairment in IL-6 production affected both Th2 and Th17 responses but promoted Th1 response. Overall, this signaling pathway affects CD4 T cell responses.

1.6.2 Specific Aim 2:

A critical role for DC-derived VEGF in priming Th2 responses.

VEGF is an important angiogenic factor that promotes lung development and has been implicated in asthma. A recent study, using transgenic mice overexpressing VEGF under the influence of the lung promoter showed the importance of VEGF in skewing Th2 responses, which resulted in exacerbated inflammation in these mice. This novel role for VEGF as a Th2 skewing cytokine coupled with the upregulation of VEGF gene transcript seen in CT-stimulated DCs warranted the study of VEGF regulation in DCs. This study described in chapter 3 outlines a mechanism for VEGF production by CT-stimulated DCs in promoting Th2 responses. VEGF production is positively regulated by the activation of iNOS, NF- κ B and PI-3 kinase and was inhibited by Th1 skewing cytokine, IL-12. The inhibition mediated by IL-12 explains the inability of LPS-stimulated DCs to induce VEGF production, since these DCs secrete high levels of IL-12. This study details the positive and inhibitory pathways that regulate VEGF production in DCs.

2.0 Activation of c-kit in dendritic cells regulates T helper cell differentiation and allergic asthma

This chapter is modified from the manuscript accepted in Nature Medicine. This chapter includes additional data and description. The use of the data from the published article adheres to copyright guidelines stated by Nature Medicine.

2.1 Abstract

Dendritic cells (DCs) are integral to differentiation of T helper cells into the three subsets, Th1, Th2 and Th17. Interleukin-6 (IL-6) plays a central role in regulating these three arms of the immune response by limiting a Th1 response and promoting Th2 and Th17 responses. In this study, we investigated pathways in DCs that promote IL-6 production. We show that the allergen house dust mite (HDM) or the mucosal adjuvant cholera toxin (CT) promotes cell surface expression of c-kit and its ligand, stem cell factor (SCF), on DCs. This dual regulation of c-kit and SCF results in sustained signaling downstream of c-kit promoting IL-6 secretion. Intranasal administration of antigen into c-kit mutant mice or neutralization of IL-6 blunted the Th2 and Th17 response in lung-draining lymph nodes of mice. DCs lacking functional c-kit or those unable to express membrane-bound SCF secreted lower levels of IL-6 in response to HDM or CT. DCs expressing non-functional c-kit were unable to induce a robust Th2 or Th17 response and elicited diminished allergic airway inflammation when adoptively transferred into mice. Expression of the Notch ligand, Jagged-2, which has been associated with Th2 differentiation, was blunted in DCs from c-kit mutant mice. The Th1-inducing adjuvant CpG oligodeoxynucleotide (ODN) did not promote either c-kit or Jagged-2 expression. DCs generated from mice expressing a catalytically inactive form of the p110 δ (p110D910A) subunit of PI3 kinase secreted lower levels of IL-6 upon stimulation with CT. Collectively, these results highlight the importance of the c-kit-PI3 kinase-IL-6 signaling axis in DCs in regulating T cell responses.

2.2 Introduction

Dendritic cells (DCs) are efficient antigen presenting cells and prime naïve CD4⁺ T cells towards a Th1, Th2 or the recently discovered Th17 response (1, 56, 93). Significant progress has been made in the last decade in our understanding of the molecular basis of T helper responses. We and others have identified GATA-3 as the master regulator of Th2 differentiation (39-41, 91, 203). The transcription factors T-bet and ROR- γ t is now recognized as critical regulators of Th1 and Th17 differentiation respectively (8, 65). Studies have shown that the nature of the antigen, antigen concentration, pattern recognition molecules and cytokine milieu are some of the key regulators that determine the outcome of an immune response (1).

Cytokines play a crucial role in regulating T helper cell differentiation (204-206). Previous studies have shown the Th2-skewing ability of IL-6 (172, 207). More recently, IL-6 has been shown to be the quintessential cytokine regulating Th17 development in both mice and humans (54, 55, 57). Interestingly in certain immunological responses such as those elicited by allergens in asthma, both Th2 and Th17 responses are evident (94, 208-211). In these situations, IL-6 would be a central regulator in not only inhibiting the Th1 response (207), but also promoting Th2 and Th17 responses (54, 55, 57, 67, 172). However, mechanisms that regulate IL-6 production in DCs have not been elucidated. Also, there have been very few studies (161) that have addressed the role of c-kit expressed by DCs in modulating immune responses.

Our goal was to determine molecular mechanisms by which a complex allergen such as house dust mite (HDM) or the mucosal adjuvant cholera toxin (CT) promotes IL-6 production in DCs, which in turn, would induce Th2 and Th17 responses. Using microarray analysis to identify novel genes regulated by CT in bone marrow-derived dendritic cells (BMDCs), we identified significant upregulation of IL-6 and c-kit expression in CT-treated DCs as compared to that in control DCs. Since cytokine expression is triggered by activation of cell surface receptors, we hypothesized that c-kit expression and activation in DCs regulates the production of IL-6 and thereby the induction of a Th2 or Th17 response subsequent to DC-T cell interaction. To elucidate the role of c-kit expressed by DCs in regulating IL-6 production, we used c-kit mutant mice (WBB6F1 kit^{W/W-v}; kit^{W/W-v}). These mice have a deletion in the transmembrane region of c-kit and also a missense mutation in the kinase domain (131, 212). The two mutations cause an

overall decrease in signaling by c-kit. Our *in vitro* data utilizing CT- and HDM-stimulated BMDCs or lung DCs and *in vivo* data using two models of allergic airways disease in mice coupled with an adoptive transfer experiment with wild-type (WT) and c-kit-defective DCs show for the first time that a functional c-kit in DCs is critical for the induction of a Th2 and Th17 response. Our findings also show that CpG oligodeoxynucleotide (ODN), known to be an effective Th1 inducing agent (213) and which also inhibits allergic responses in the lung in both mice and humans (214-217), did not upregulate c-kit expression. Furthermore, DCs generated from mutant mice unable to express membrane-bound SCF did not show increased IL-6 production in response to HDM or CT. The inability of CpG ODN to upregulate or signal through c-kit further strengthens the importance of the molecule in promoting IL-6 secretion from DCs thereby stimulating Th2 and Th17 development. Interestingly, we also found that the ligand of c-kit, stem cell factor (SCF), also showed similar expression characteristics on the cell surface as c-kit in response to CT, HDM and CpG ODN. Interestingly, c-kit expression was also found to influence expression of the Notch ligand, Jagged-2, that has been associated with Th2 responses (34). DCs from c-kit mutant mice showed impaired Akt phosphorylation upon stimulation with CT and DCs from p110 α mutant mice with defective PI3 kinase signaling, which constitutes a major signaling pathway downstream of c-kit(123), showed impaired IL-6 production suggesting that the c-kit/PI3 kinase signaling axis positively regulates the production of IL-6. Thus, we have identified a novel signaling axis in DCs that promotes the induction of Th2 and Th17 responses, while significantly inhibiting the Th1 arm of the immune response.

2.3 Materials and methods

2.3.1 Mice

Balbc/ByJ, C57BL/6, IL-6^{-/-} (Stock number 002254), Kitl^{Sl/Sl-d} (Stock number 100401), c-kit mutant WBB6F1^{W/W-v} (kit^{W/W-v}; Stock number 100410) and the wild type control (kit^{+/+}) were all purchased from The Jackson Laboratory. DO11.10 transgenic mice were originally provided by Dr. Kenneth Murphy at Washington University, St. Louis and were bred at the animal facility at the University of Pittsburgh. All animals were housed under pathogen-free conditions and were used between 6 and 12 weeks of age. Within experiments, the animals were

age and sex matched. All studies with animals were approved by the Animal Care and Use Committee at the University of Pittsburgh.

2.3.2 Cell culture

The media used for cell culture was RPMI 1640 (Gibco, Cat# 22400-089) supplemented with 10% heat inactivated fetal bovine serum (Gemini), penicillin/streptomycin, 1mM Na-pyruvate (Gibco) and 50 μ M 2-mercaptoethanol (Sigma). All reagents used had <0.6 EU/ml of endotoxin. For DC cultures, the femur and tibia of mice were removed and the bone marrow flushed using a 27gauge needle. The cells were washed and cultured with 10ng/ml recombinant murine GM-CSF (Peprotech) at 1×10^6 /ml. On the third day, the cells were refed with fresh medium and cytokine. On day 6, the loosely adherent cells were harvested. Prior to harvest, the flask was gently shaken at 7 hertz per second for 5 min on a MicroMix shaker (DPC instruments). The cells were then subjected to purification using magnetically labeled anti mouse CD11c⁺ beads (Miltenyi Biotech). The labeled cells were passed through the column twice and CD11c expression was >98%. The contaminating population of CD3⁺ and CD19⁺ was negligible. These cells were considered immature DCs as ascertained by low expression of CD40, CD86, and MHC class II (data not shown). The immature DCs were plated at 1×10^6 /ml in 12 well plates in medium containing 10ng/ml GM-CSF with or without Cholera toxin (List Biologicals Labs) or HDM (Greer Labs) for 24 h. CT at 1 μ g/ml and HDM at 10 μ g/ml, both dissolved in PBS, were used for stimulating the DCs unless specified otherwise. Both OVA and HDM preparations in PBS were stripped of LPS using EndoTrap column (Profos AG, Germany). The residual LPS in the solutions was estimated by the Limulus Amebocyte Lysate assay (Cambrex). The level of LPS in the cell cultures containing OVA or HDM was less than 0.1ng/ml. CT as purchased contained undetectable endotoxin. The lung and spleen DCs were isolated as described previously(218). The lung DCs protocol was slightly modified. A high volume lavage was performed to isolate alveolar macrophages. The lung cells after CD11c separation was sorted on FACS ARIA based on the auto fluorescence. The low autofluorescent cells considered to be dendritic cells were sorted from the high autofluorescent cells, the macrophages and used in the experiments. For the inhibition of cyclic AMP, the BMDCs were pre-incubated with the cyclic AMP antagonist Rp-cAMPS, triethylammonium salt (Calbiochem) for 1 h before stimulation of

cells. The cells were harvested and used in various experiments. The CD4⁺ T cells were obtained from splenocytes as previously described (73).

2.3.3 Antibodies

Antibodies for either flow cytometry or blocking experiments were purchased from BD Pharmingen unless otherwise stated. Anti-CD11c (APC; clone HL3), anti-CD40 (PE; clone 3/23), anti-CD86 (PE; clone GL1), anti-CD117 (c-kit) (PE; clone 2B8), anti-MHC class II (Southern Biotech; NIMR-4) were used along with the appropriate isotype controls for flow cytometry. Anti GATA-3, anti T-bet, anti-Jagged-2, anti-Delta-4, anti CREB-1 (Santa Cruz Biotechnology), anti-c-kit, anti-pAkt (Thr-308), and anti-Akt (Cell Signaling Technology) were used for western blot analysis. Anti-SCF from Chemicon was used for immunofluorescence studies and for detection of SCF by flow cytometry, indirect staining was carried out using anti-SCF from Abcam together with PE-conjugated goat-anti-rat IgG (Serotec). Anti IL-6 (clone MP5-20F3) and the isotype control were purchased from BD Pharmingen.

2.3.4 Intranasal antigen instillation

Intranasal administration of OVA/CT or OVA/CpG and *ex vivo* stimulation of single cell suspension of cells from lung-draining lymph nodes with OVA were performed as described previously(73, 218). The experiments with HDM (Greer labs) were performed as described (109) with minor modifications. Mice were anesthetized with isoflurane before intranasal administration of any antigen. In the experiments involving immunization of mice, 25µl of the instilled solution contained 10ng of residual LPS. Intranasal administration of 25µg of HDM was carried out for 7 consecutive days and the animals were rested for 3 days. Single cell suspensions of the lung-draining lymph nodes were stimulated *ex vivo* with HDM (10 µg/ml). Nuclear extracts were prepared from OVA- or HDM-stimulated cells after 5 days. The CpG ODN 1826 (5'-TCCATGACGTTTCCTGACGTT-3'), which is known to be optimal for stimulation of murine cells, and the control ODN 1911 (5'-TCCAGGACTTTCCTCAGGTT-3') were used at a concentration of 1 µM to stimulate DCs. The control ODN did not influence cytokine production or expression of any molecules in DCs in initial experiments as previously

reported by us and was not used in all experiments(218). The LPS level in the ODN preparations was low (<0.1 ng/mg DNA). The oligonucleotides were purchased from Oligos Etc.

2.3.5 Adoptive transfer of DCs into mice

CD11c⁺ BMDCs from kit^{+/+} and kit^{W/W^v} were isolated and incubated overnight with OVA (100µg/ml) and CT (1µg/ml). The cells were harvested, washed and 1x10⁶ cells were adoptively transferred via the intratracheal route into C57BL/6 recipients. After transfer, the mice were rested for 10 days and then challenged by exposure to aerosolized 1 % OVA for 7 consecutive days using an ultrasonic nebulizer (Omron Healthcare). 24 h after the last aerosol, the mice were sacrificed and the lungs were examined for inflammation. The cell count in the BAL fluid and lung histology were performed as previously described (73, 88, 91)and PAS staining was performed according to manufacturer's instructions (Richard Allan Scientific)(88). For tracking the cells *in vivo*, the cells were labeled with CFSE dye after stimulation with OVA+CT. 10 X10⁶ BMDCs were labeled with CFSE dye (1 µM, Invitrogen) for 15 min at 37°C. The labeling was stopped by washing the cells twice with PBS containing 2%FBS. The cells were re-counted and suspended at a concentration of 1 X10⁶ BMDCs in 50 µl of PBS.

2.3.6 Flow cytometry

The staining procedures were essentially as described previously(73). The live cells were gated using propidium iodide staining and at least 10,000 events within the live gate were collected using a FACSCalibur flow cytometer. (BD) Staining for SCF was performed by first adding the unconjugated primary antibody (1µg) and incubating on ice for 1h. The tubes were washed and the secondary antibody (PE conjugated goat-anti-rat IgG) was added at the same concentration and incubated for 0.5h. The data were analyzed using FlowJo software (Tree Star corporation).

2.3.7 Cell extracts and immunoblotting

Nuclear extracts and total cell lysates were prepared as stated previously(33, 72). Total cell lysates were prepared from DCs at 24 h post- stimulation using 1X cell lysis buffer (Cell signaling).

2.3.8 Microarray analysis

The isolation of RNA and microarray analysis was performed essentially as described previously(33). RNA was isolated from BMDCs incubated with or without cholera toxin (1 $\mu\text{g/ml}$) for 24h. Total RNA quality was assessed prior to cRNA target preparation and labeling. cRNA was synthesized from the total RNA samples by using the CodeLink Expression Assay Reagent Kit (GE Healthcare). The labeled cRNA was fragmented at and hybridized to CodeLink UniSet Mouse I Bioarrays (GE Healthcare) that identifies 10012 unique murine genes. The hybridized bioarrays were scanned using the GenePix 4000B Array Scanner (Axon Instruments, Union City, CA). The Normalized Intensities of all slides were then imported into Spotfire to create one file, which included annotations supplied by CodeLink. Only the Discovery genes and genes with Good or Low quality flags were selected with the Spotfire query devices. These selected genes were then exported into Excel. Then the data was formatted into the acceptable form for the DOS based data analysis program, Scoregene. Scoregene globally normalized the data by calculating the geometric mean across the controls and experimentals and then log transformed the data to base 2. T-test p-values were also calculated by Scoregene. Genes with T test p-values < 0.05 and a fold change of 1.5 (SLR signal log ratio of 0.5849625) were determined to be statistically significant and selected for hierarchical clustering in Spotfire. The clustering method used was UPGMA (Unweighted Pair-Group Method Average) with a Euclidean distance similarity measurement. Genes were considered to be significantly upregulated or down regulated if the fold change was greater than +1.5 or less than -1.5. RNA was isolated in three independent experiments from control and CT-treated BMDCs and bone marrows were pooled from at least 4 mice in each experiment. Gene Expression Omnibus microarray accession code, GSE10815 can be used for the analysis of the microarray data.

2.3.9 Immunofluorescence

1×10^5 cells were cytopspun on slides and fixed with 95% ethanol and 5% glacial acetic acid. The slides were blocked with 2% goat serum diluted in PBS and incubated overnight at 4°C with the primary anti-SCF antibody. The slides were washed with PBS and incubated with the secondary antibody tagged with Cy3 for visualization. The slides were mounted with mounting medium Vectashield containing DAPI stain (Vector labs) to identify the nuclei. The specificity of staining was ensured using control slides in which the incubation with the primary antibody was skipped.

2.3.10 ELISA

ELISAs for IL-12p70, IL-6, IL-4, IL-13 and IFN- γ were performed with kits from R&D Systems per manufacturer's instructions. ELISA for soluble SCF was performed using a kit from Peprotech. Unless otherwise indicated, all statistical analyses were carried out using mean values of triplicate wells and results shown are representative of two to four independent experiments. The OVA-specific IgE levels were measured as previously described (73). The concentration of IgE was derived from standard curves generated using pooled sera from animals immunized with OVA/alum and repeatedly challenged with aerosolized OVA and the standard was assigned an arbitrary unit of 10,000 U/ml.

2.3.11 ELISPOT

IL-13, IL-17 and IFN- γ ELISPOT assays were performed using kits from eBioscience as per the manufacturer's specifications. Briefly, ELISPOT plates (Millipore 96-well MultiScreen HTS) were pre-coated with the primary antibody at 4°C overnight. Lymph nodes cells were plated at a concentration of 1×10^5 or 1×10^4 per well and stimulated with PMA (25ng/ml) and ionomycin (500 ng/ml) for 24 h. Biotin-conjugated secondary antibody was used to detect the secreted cytokine. The plates were developed with Avidin-HRP and peroxidase substrate (Vectastatin). The spot forming units (sfu) were quantified using an automated ELISPOT plate reader (ImmunoSpot; Cellular Technology).

2.3.12 Statistical analysis

Student's unpaired two-tailed t-test was used for all statistical analyses. Differences between groups were considered significant if $P < 0.05$. All statistical analyses (except that performed for microarray data) were performed using GraphPad Prism software.

2.4 Results

2.4.1 IL-6 secretion from CT or HDM-stimulated DCs prime Th2 and Th17 responses

The mucosal adjuvant property of CT has been associated with a Th2 response. However the ability of the toxin to induce secretion of high levels of IL-6 strongly suggests an influence on the Th17 arm of the immune response as well. Allergens such as HDM, that cause asthma in humans (219) as well as in experimental models in animals(109) have been also associated with IL-6 production(25) and recent studies have implicated an important role for IL-17 in allergen induced inflammation(94, 95, 211). To dissect the importance of the cytokines secreted by DCs in influencing the T cell response, we analyzed cytokine levels in culture supernatants of BMDCs stimulated with CT or HDM. As shown in **Fig. 2a**, a higher level of IL-6 was detected in the culture supernatants of BMDCs stimulated with CT or HDM compared to that in the supernatants of control cultures. The level of secretion of the Th1 skewing cytokine IL-12p70 was not significantly different between HDM- and CT-treated DCs and control DCs. A similar cytokine profile of high IL-6 and low IL-12 production by lung DCs has been shown to be important for the induction of Th2 response (207) and an important role for IL-6 in triggering a Th17 response is now well recognized (54, 55, 57, 220). We, therefore, examined the importance of this cytokine balance in promoting a Th2 and Th17 response while inhibiting a Th1 response. Towards this end, anti-IL-6 antibody was added to the co-cultures of CT-stimulated BMDCs and DO11.10 CD4⁺ T cells. Neutralization of IL-6 resulted in a cytokine profile that showed a switch in the Th response with a dominant Th1 response with elevated levels of IFN- γ coupled with inhibition of IL-13 and IL-17 production (**Fig 2b**).

Intranasal immunization of IL-6^{-/-} mice with OVA/CT or HDM resulted in significant attenuation of both Th2 and Th17 responses (**Fig. 2c**). These results show that whether T cell responses are triggered by BMDCs or lung DCs, IL-6 plays an important role in regulating T cell responses.

We next investigated the role of IL-6 in the shaping of the immune outcome *in vivo* in response to a natural allergen such as HDM. Mice were immunized intranasally with the allergen and the lung-draining lymph nodes were harvested. This protocol of immunization has been shown to prime the T cells from the lung-draining lymph towards a Th2 response (109). Cells harvested from the lymph nodes were restimulated with HDM in the presence or absence of neutralizing anti-IL-6 antibody. Immunization with the allergen increased the Th2 and Th17 response while neutralization of IL-6 crippled the dominant Th2+Th17 immune response (**Fig 2c**).

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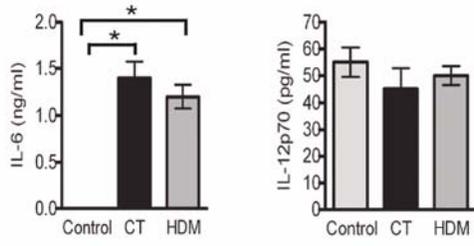
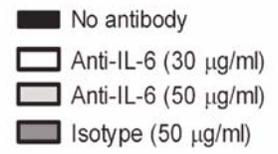
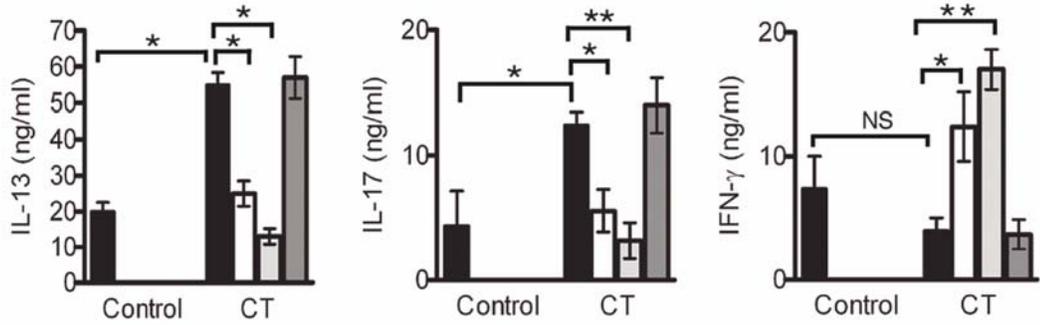
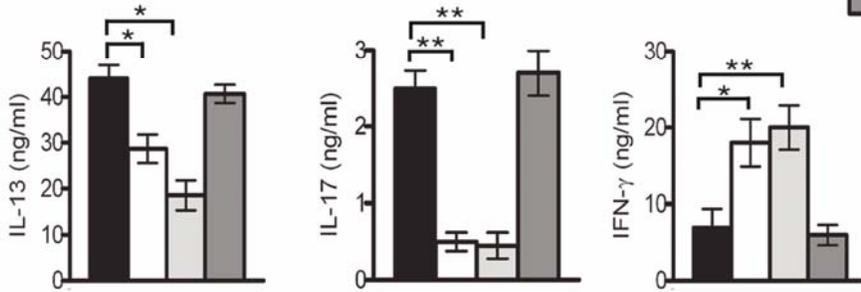
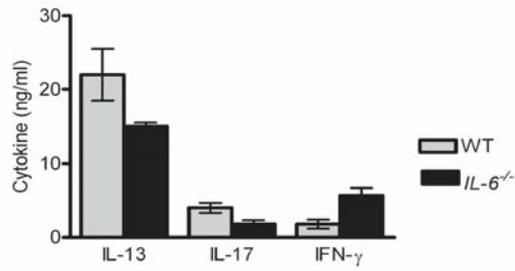
a**b****c****d**

Figure 2: IL-6 secretion from CT or HDM-stimulated DCs prime Th2 and Th17 responses.

(a) BMDCs were incubated with or without CT for 24 h. The cell supernatants were analyzed for IL-6 and IL-12p70 levels by ELISA. Cytokine levels shown are mean \pm s.e.m. of triplicates and are representative of three independent experiments *, $P < 0.001$. (b) Anti-IL-6 antibody inhibits Th2 and Th17 response but promotes Th1 response. BMDCs were incubated with or without CT for 24 h and then co-cultured with DO11.10 T cells in the presence of OVA peptide. As shown, some wells containing BMDCs+CT also contained anti-IL-6 antibody or an isotype control. The cytokines present in the culture supernatants were assayed for IL-13, IL-17 and IFN- γ . The values represent means of triplicates \pm s.d. and are representative of two independent experiments. *, $P < 0.05$ and ** $P < 0.001$. (c) Lymph node cells derived from WT mice show diminished Th2 and Th17 response but increased Th1 response upon treatment with anti-IL-6 antibody. Lymph node cells from WT mice following HDM instillation were cultured with anti-IL-6 antibody or an isotype control and IL-13 and IFN- γ levels in the culture supernatant were measured. Results shown represent means of triplicates \pm s.e.m. and are representative of three independent experiments. *, $P < 0.05$ **, $P < 0.001$ (d) IL-6^{+/+} or IL-6^{-/-} mice were immunized as described in Methods. The lung-draining lymph nodes were harvested and the cells were re-stimulated with HDM for 3 days. The cytokines from the cell culture supernatants were assayed by ELISA.

2.4.2 High levels of IL-12 inhibit Th2 and Th17 responses

The addition of IL-12 in two different concentrations to control or CT-stimulated BMDCs switched the response of T cells in co-culture to a Th1 response by promoting T-bet and suppressing GATA-3 expression. The analysis of cytokines from the cell culture supernatants also showed increase in IFN- γ and decrease in IL-13 and IL-17 production (Fig. 3). These results from Fig 2 and 3 showed that altering the cytokine balance by neutralizing IL-6 or adding IL-12 diverted the dominant Th2 and Th17 -skewing property of CT to a more Th1 response.

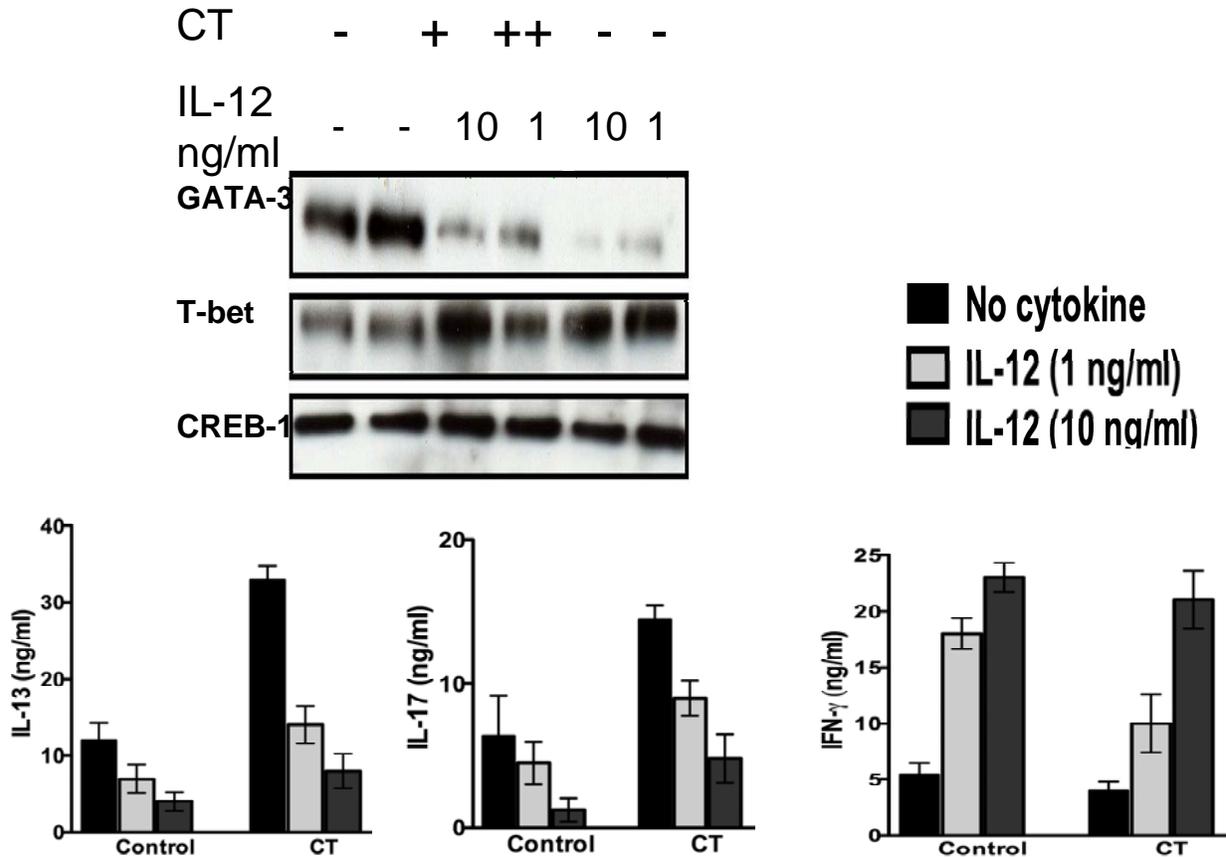


Figure 3: High levels of IL-12 inhibit Th2 and Th17 responses.

BMDCs were co-cultured with naive DO11.10 T cells in the presence or absence of IL-12. The transcription factors GATA-3 and T-bet were assayed from nuclear extracts by immunoblotting. The culture supernatants were assayed for levels of different cytokines as shown in the lower panel.

2.4.3 CT- and HDM-stimulated DCs upregulate c-kit expression

A prior study has shown that CT does not upregulate IL-12p70 production in DCs and also downregulates the expression of IL-12 β 1 and IL-12 β 2 receptors on DCs(23). A low IL-12 level is necessary but is not sufficient to induce a Th2 response(1). As shown in **Fig. 2b, 2c and 2d**, high levels of IL-6 contributed to the Th2- and Th17-skewing ability of CT-stimulated BMDCs. To further dissect and identify molecular mechanisms underlying IL-6 upregulation by CT, we undertook a microarray approach to identify genes differentially regulated in BMDCs upon CT treatment. A similar approach was used by us previously to identify Ym1 as a statin-induced gene involved in Th2 differentiation (33). In three independent experiments, RNA was

isolated from BMDCs after incubation in medium with or without CT and the RNA was hybridized to Codelink Uniset Mouse Expression Bioarray that identifies 10012 unique murine genes. For data analysis, Scoregene software was used for comparisons between the processed arrays and for calculating fold change in transcript levels of genes using various statistical methods. The genes whose expression was consistently increased or decreased were selected and were further filtered based on Student's t-test. The microarray data analysis (details to be published elsewhere) revealed the c-kit gene to be significantly upregulated along with IL-6 in BMDCs upon CT treatment (**Fig. 4a**). The significant upregulation of c-kit suggested that signaling through this receptor could regulate the expression of IL-6. To confirm our microarray data, we investigated the expression of c-kit protein after stimulation with CT for 24 h. Immunoblotting of total cell lysates revealed greater c-kit expression in CT-stimulated BMDCs compared to that in control DCs (**Fig. 4b**). As determined by flow cytometry, the frequency of cells expressing c-kit on the cell surface was increased by CT treatment (**Fig. 4c**) and this increase was dose-dependent (data not shown). We also investigated whether c-kit was induced by specific agents such as CT or whether an allergen such as HDM could also upregulate c-kit expression in DCs. As shown in **Fig. 4c**, exposure of BMDCs to HDM increased the frequency of c-kit expressing-cells, which was comparable to that observed with CT treatment. Next we wanted to dissect the mechanism involved in the upregulation of c-kit. CT is known to activate adenylate cyclase via G-protein coupled receptor resulting in an increase in the level of intracellular cyclic AMP (cAMP) (221). Stimulation of human monocytes with CT has been also shown to suppress IL-12 levels via a cAMP-dependent pathway(24). We, therefore, examined the role of cAMP in c-kit upregulation by CT using the specific cAMP antagonist Rp-cAMPS that blocks cAMP-dependent downstream pathways such as protein kinase A (222). As shown in **Fig. 4c**, the addition of the cAMP antagonist caused a significant decrease in c-kit-expressing cells in CT-stimulated DCs. This decrease was dose-dependent as the higher dose (10 μ M) of the antagonist shifted the expression of c-kit to that seen in the control DCs. Addition of the cAMP antagonist also inhibited HDM-induced c-kit expression (**Fig. 4c**). Analysis of IL-6 levels in the culture supernatant showed dose-dependent decrease in cytokine release in the presence of the antagonist whether CT or HDM was used to stimulate the DCs (**Fig. 4c**). To determine whether c-kit expression was modulated by IL-6 secreted from the stimulated cells, BMDCs from IL-6^{+/+} and IL-6^{-/-} mice were incubated with or without CT and the expression of c-kit was determined

by flow cytometry. The data showed that c-kit expression on DCs derived from IL-6^{-/-} mice was comparable to that on cells derived from IL-6^{+/+} mice suggesting that IL-6 is a downstream target of c-kit (Fig. 4d).

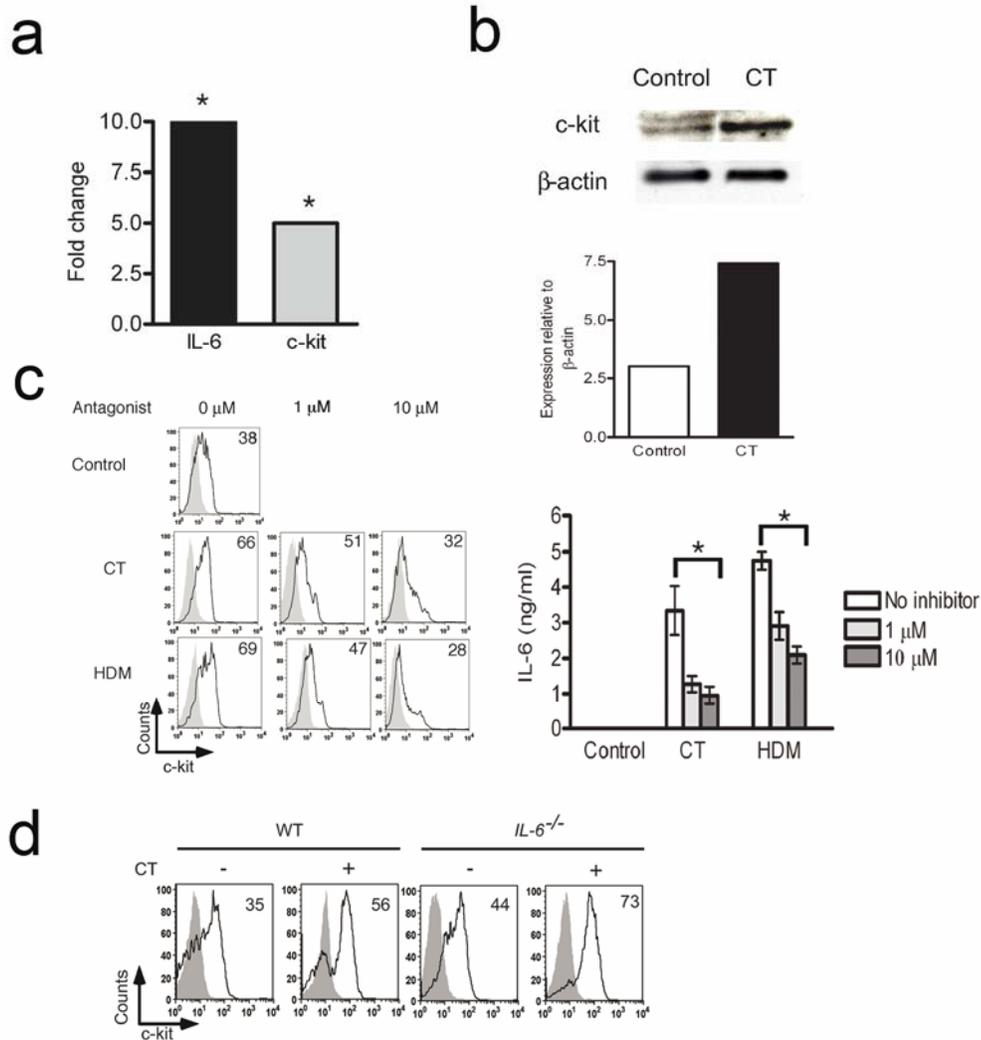


Figure 4: CT- and HDM-stimulated DCs upregulate c-kit expression.

(a) Increased IL-6 and c-kit mRNA expression in CT-stimulated BMDCs as revealed by microarray analysis. The fold change in c-kit and IL-6 mRNA expression in CT-stimulated BMDCs with respect to control DCs incubated in medium is shown. *, P<0.005. (b) c-kit expression in CT-stimulated BMDCs as revealed by immunoblotting. Total cell lysates prepared from CT-stimulated BMDCs were probed for c-kit and the same blot was stripped and re-probed for the loading control β-actin. The intensity of c-kit bands was quantified relative to the loading control β-actin. Data are representative of two independent

experiments. (c) CT and the allergen HDM upregulate c-kit expression on BMDCs in a cAMP-dependent fashion. BMDCs were pre-treated with the cAMP antagonist Rp-cAMPS at indicated concentrations for 1 h and then stimulated with CT (1 $\mu\text{g/ml}$) or HDM (10 $\mu\text{g/ml}$) for 24 h. The cells were harvested and analyzed for c-kit expression by flow cytometry. The culture supernatants were analyzed for IL-6 production by ELISA and the data shown are mean values of triplicates \pm s.d. This experiment was performed twice with similar results. *, $P < 0.05$. (d) BMDCs from IL-6^{+/+} (WT mice) and IL-6^{-/-} mice were incubated with or without CT for 24 h and the expression of c-kit was analyzed by flow cytometry. The shaded grey represents the isotype control and the black overlay represents staining with the anti-c-kit antibody. The numbers in the histograms represent the percentage of CD11c⁺c-kit⁺ cells. This experiment was repeated twice with similar results.

2.4.4 c-kit positive BMDCs from CT-stimulation induce a Th2 response

The data shown in Fig. 3 indicated that CT-stimulated BMDCs yielded 2 distinct populations of cells based on the expression of c-kit. Also, inhibition of expression of c-kit directly affected the production of IL-6. This suggested that expression of c-kit could determine the nature of the induced T cell response. To further understand the functional significance of c-kit upregulation on DCs, we sorted c-kit positive and negative cells after stimulation with CT. The purity of the sorted populations was greater than 93% (**Fig. 5a**). The sorted populations were then co-cultured with T cells from naïve DO11.10 mice in the presence of OVA peptide. The supernatants from the co-culture were assayed for IL-13, IL-17 and IFN- γ production by ELISA. The CT-stimulated BMDCs that were c-kit positive skewed the T cells towards production of high levels of IL-13 and IL-17 and low levels of IFN- γ . This cytokine profile was different in culture supernatants that included the c-kit negative population showing higher IFN- γ and lower IL-17 and IL-13 levels (**Fig. 5a**). In contrast to generation of a higher frequency of c-kit positive cells in response to CT, BMDCs stimulated with CpG ODN, a Th1-skewing adjuvant, resulted in the majority of the cells being c-kit negative and this population of sorted DCs strongly promoted a Th1 response (**Fig. 5b**). These results strengthened our notion that the upregulation of c-kit on DCs elicits a strong Th2 and Th17 response while suppressing a Th1 response.

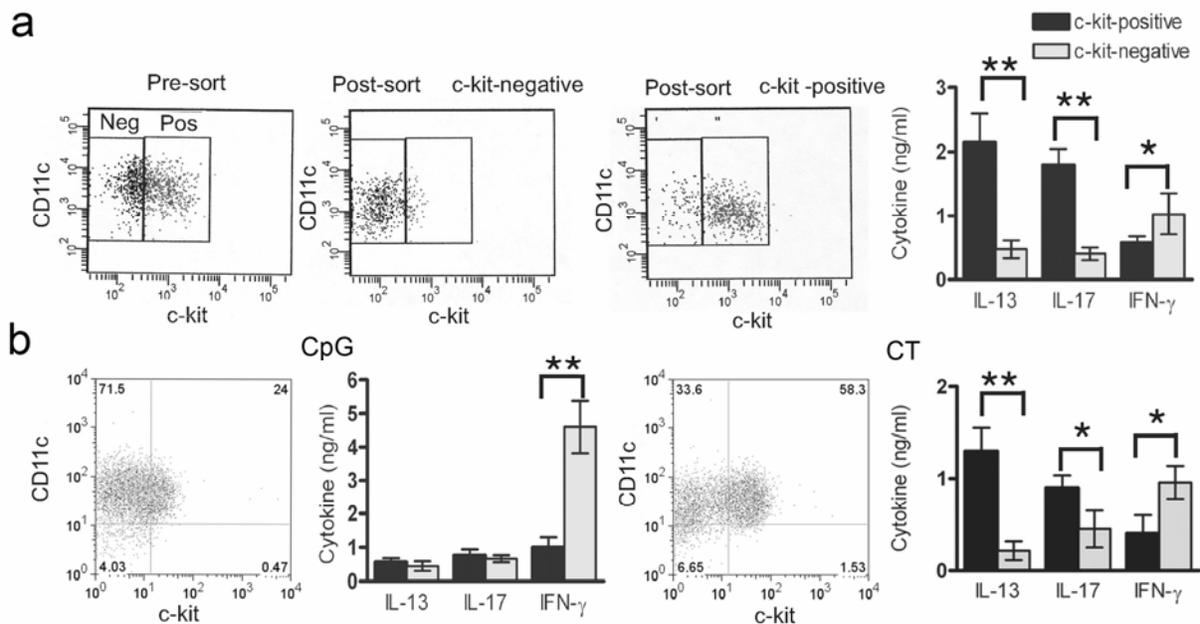


Figure 5: c-kit positive BMDCs from CT-stimulation induce a Th2 response.

(a) BMDCs were stimulated with CT (1 μ g/ml) and sorted based on the expression of ckit. The purity of the c-kit⁺ and c-kit⁻ populations after sorting was >93%. The supernatants from the co-cultures of either c-kit⁺ or c-kit⁻ cells with CD4⁺ T cells were assayed for IL-13, IL-17 and IFN- γ by ELISA. (b) BMDCs were stimulated with either CpG ODN (1 μ M) or CT (1 μ g/ml) and sorted based on c-kit expression as in panel a resulting in similar purity of c-kit⁺ and c-kit⁻ populations. The sorted cells from each treatment were co-cultured with CD4 T cells and the culture supernatants were assayed for cytokine levels by ELISA. Data shown are means of triplicates \pm s.d. and are representative of two independent experiments *, P<0.05, **, P<0.001.

2.4.5 CT- or HDM-mediated c-kit signaling regulates IL-6 production and the expression of Notch ligands

The ability of c-kit negative cells to trigger a strong Th1 response (Fig. 5) suggested that the lack of c-kit upregulation could be a potential mechanism to subvert a Th2 response. To test this hypothesis, we isolated lung DCs and stimulated them with Th2- or Th1-skewing agents - HDM or CT were used to induce a Th2 response whereas CpG ODN was used to induce a Th1 response. The lung DCs were stimulated for 24h and the expression of different molecules

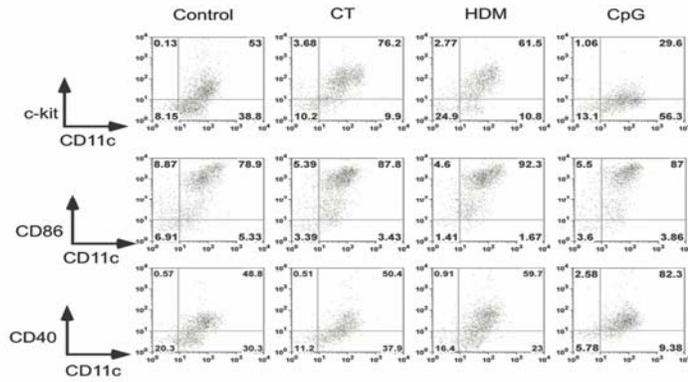
associated with DC maturation and T helper differentiation was examined. A significant increase in the percentage of c-kit-expressing DCs was noted upon treatment with CT or HDM, but not CpG ODN. In contrast, CpG ODN, but not HDM or CT, promoted expression of CD40, which is associated with increased IL-12 production in DCs and Th1 response (15)(**Fig. 6a**).

Since CT and HDM upregulated c-kit expression in BMDCs, and the expression of this molecule was critical for IL-6 production, we next investigated whether c-kit signaling was an important component of IL-6 production in BMDCs and, in turn, in the regulation of T cell responses. BMDCs were cultured from WT ($kit^{+/+}$) or kit-mutant ($kit^{W/W-v}$) mice. There was no difference in BMDCs between $kit^{+/+}$ and $kit^{W/W-v}$ mice with respect to purity, yield and expression of MHC class II and the co-stimulatory molecules CD40 and CD86 (**Fig. 6b**). The DCs were incubated with or without CT or HDM and the cytokine profile was analyzed by ELISA. DCs from $kit^{W/W-v}$ mice secreted significantly lower levels of IL-6 upon CT or HDM stimulation indicating that c-kit signaling in BMDCs is important for IL-6 production in response to these agents. The level of IL-12p70 in the culture supernatants showed no significant difference between the mouse groups, however CpG ODN upregulated the production of this cytokine (**Fig. 6c**). We also wanted to investigate the ability of tissue-specific DCs to secrete IL-6 in response to CT and HDM and the role of c-kit signaling in regulating the levels of IL-6 produced. Lung DCs and spleen DCs were isolated from $kit^{+/+}$ and $kit^{W/W-v}$ mice and stimulated by CT or HDM. Both spleen and lung DCs from the $kit^{+/+}$ secreted more IL-6 in response to the stimuli when compared to control DCs, which was significantly impaired when cells were isolated from $kit^{W/W-v}$ mice (**Fig. 6c**). CpG ODN did not trigger secretion of IL-6 from the lung DCs and the levels were not altered when cells were derived from $kit^{W/W-v}$ mice, which confirmed that CpG ODN-induced signaling was independent of c-kit. These data clearly show that mutations that impair c-kit signaling affect IL-6 secretion from BMDCs as well as spleen and lung DCs in response to Th2/Th17-skewing agents.

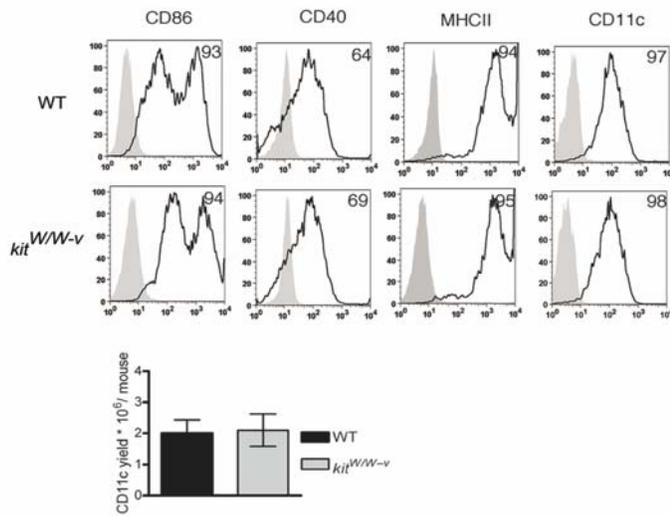
Antigen presenting cells have been shown to upregulate Notch ligands in response to specific Th1- and Th2-inducing stimuli. Jagged-2 and Delta-4 ligands on antigen presenting cells have been associated with promoting Th2 and Th1 responses respectively (34). We, therefore, investigated whether the expression pattern of these ligands was altered in DCs from $kit^{+/+}$ and $kit^{W/W-v}$ mice. As shown in Fig. 4, panel c, at the basal level, BMDCs from $kit^{W/W-v}$ mice showed markedly reduced levels of Jagged-2 compared to that observed in DCs generated from WT mice

suggesting a developmental link between c-kit and Jagged-2. Also, CT stimulated Jagged-2 expression in BMDCs from $\text{kit}^{+/+}$ mice (**Fig. 6d**). The expression of Delta-4 on the other hand was not compromised by c-kit defect and was slightly elevated upon CpG ODN treatment (**Fig. 6d**).

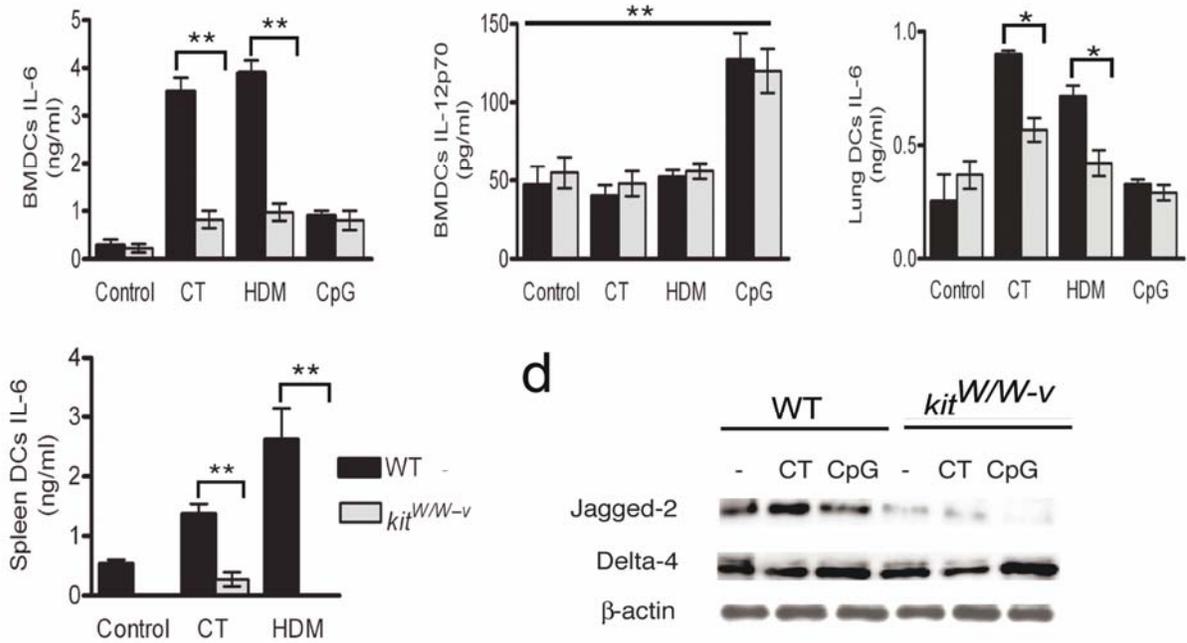
a



b



c



d

Figure 6: CT- or HDM-induced c-kit signaling regulates IL-6 production and the expression of Notch ligands.

(a) Lung DCs were stimulated with different stimuli and phenotyped for the markers shown. Numbers denote percentage of cells in that quadrant. (b) Cytokine production by BMDCs, lung DCs and spleen DCs was assayed by ELISA. Data shown are means of triplicates \pm s.d. and are representative of two independent experiments. * $P < 0.05$, **, $P < 0.01$. (c) BMDCs generated from $\text{kit}^{\text{W/W-v}}$ and WT mice after 6 days of culture and purified by CD11c positive selection were analyzed for the expression of the indicated cell surface molecules. The shaded grey areas represent staining with isotype control and the numbers denote the percentage of cells expressing the respective molecules. Lower panel: The number of CD11c positive cells recovered after 6 days of culture. (d) Total cell lysates of variously treated BMDCs containing equal amounts of protein were sequentially analyzed by immunoblotting technique for expression of the Notch ligands Jagged-2 and Delta-4. The blot was stripped and reprobbed for β -actin levels to ensure similar protein loading.

2.4.6 c-kit signaling in DCs is critical for priming of Th2 and Th17 responses

The results presented in Figures 4b and 4c show that DCs from $\text{kit}^{\text{W/W-v}}$ mice with reduced ability for IL-6 production and Jagged-2 expression were less potent in priming T cells towards a Th2 or Th17 lineage when compared to those generated or isolated from $\text{kit}^{+/+}$ mice. To confirm that impaired c-kit signaling has physiological relevance in T cell responses, we used an in vivo model of airway inflammation. In this model, OVA in combination with CT was administered via the intranasal route for 3 consecutive days (73) to $\text{kit}^{+/+}$ and $\text{kit}^{\text{W/W-v}}$ mice. After 4 days of rest, the animals were sacrificed and lung-draining lymph nodes were harvested. We first assessed the difference in priming using ELISPOT technique. The lymph node cells from the $\text{kit}^{\text{W/W-v}}$ mice showed reduced IL-13 and IL-17 spot forming units (sfu) and a slight increase in IFN- γ sfu (Fig. 7a). We then examined whether the defective priming resulted in reduced Th2 and Th17 responses during a recall response upon stimulation with antigen. For this purpose, the lymph node cells were cultured in the presence of OVA for 5 days. Nuclear extracts were prepared after 5 days and the expression of Th2 specific transcription factor GATA-3(39, 40) and the Th1 regulator T-bet(8) was analyzed by immunoblotting. Our results show that c-kit signaling is critical for GATA-3 expression as lower GATA-3 expression was

detected in nuclear extracts derived from $kit^{W/W-v}$ mice compared to that in extracts from $kit^{+/+}$ mice (Fig. 7b). The expression of T-bet did not decrease in extract prepared from mutant mice and instead a slight increase was noted. Correspondingly, when assayed for cytokines, a decreased level of IL-13 was noted in cultures containing lymph node cells from $kit^{W/W-v}$ mice as compared to those containing cells from WT mice and the converse was noted for IFN- γ levels (Fig. 7b). The increase in IFN- γ levels in the $kit^{W/W-v}$ mice suggested a decrease in IL-17 production due to known crossregulation by IFN- γ . Indeed measurement of IL-17 levels by ELISA revealed a decrease in the production of this cytokine from the culture supernatant of lymph nodes derived from $kit^{W/W-v}$ mice. As expected, the level of IL-6 in cultures established from lymph nodes of $kit^{W/W-v}$ mice was also lower as compared to that in cultures generated from the $kit^{+/+}$ mice (Fig. 7c).

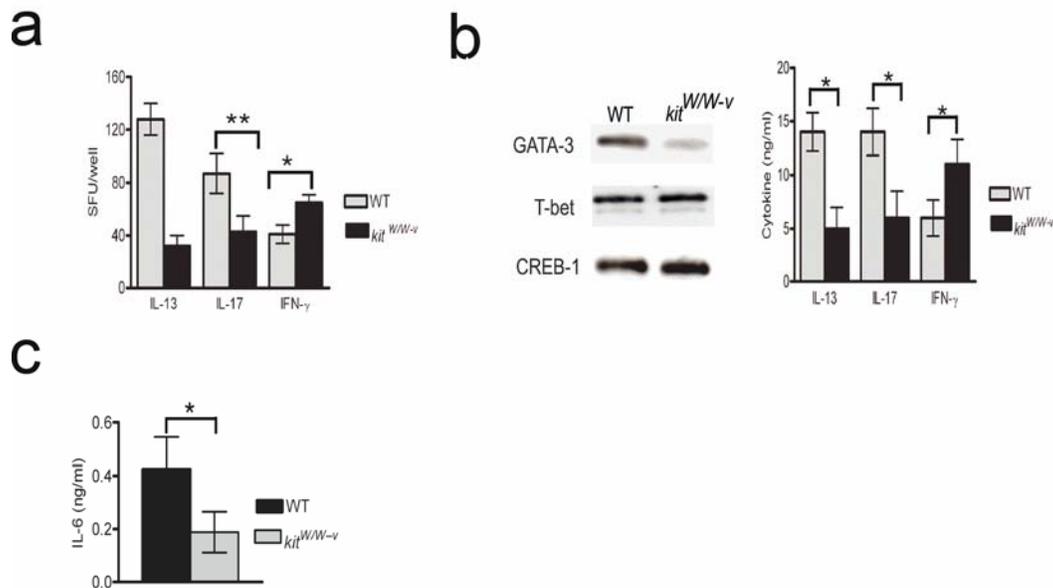


Figure 7: c-kit signaling in DCs is critical for priming of Th2 and Th17 responses.

(a) Cells from lung-draining lymph nodes of mice after OVA/CT immunization were stimulated with PMA (25 ng/ml) and ionomycin (500 ng/ml) for 24 h to assess T cell priming by ELISPOT methods. 1×10^5 lymph node cells from the individual mice were plated in each well and the spot forming units (sfu) for each cytokine shown were identified using ELISPOT techniques. *, $P < 0.05$, **, $P < 0.005$. (b) Lung-draining lymph nodes were harvested after intranasal administration of OVA/CT into c-kit mutant ($kit^{W/W-v}$) and WT ($kit^{+/+}$) mice. The harvested lymph nodes cells were cultured for 5 days in the presence of OVA. Nuclear extracts prepared from the cultures were analyzed sequentially by immunoblotting for

GATA-3 and T-bet expression. The blot was last stripped and probed for CREB-1 as a marker for protein loading. This experiment was performed three times. The supernatants from the co-cultures were analyzed for the cytokines IL-13, IL-17 and IFN- γ by ELISA and data shown are means of triplicates \pm s.e.m. *, P<0.05. Data shown are representative of three independent experiments.

2.4.7 Increased IFN- γ levels in c-kit mutant mice following *Klebsiella pneumoniae*

We also addressed the role of c-kit in a different model associated with a Th17 response, and used the *Klebsiella pneumoniae* model of lung infection (223). WT and kit^{W/W^v} mice were infected with the bacteria and the mice were sacrificed on day 4 following the infection. Analysis of cfu in the mice showed that the c-kit mutants had significantly lower cfu in the spleen, liver and lungs. Interestingly, the c-kit mutants also had a higher IFN- γ response in the lung-draining lymph nodes as assayed by ELISPOT when compared to that in WT mice (**Fig. 8**). This was similar to increased IFN- γ secretion upon neutralization of IL-6 (**Fig. 2**) and in response to OVA/CT in c-kit mutant mice as shown in **Fig. 7b**. Thus, in the *Klebsiella* infection model too, compromised c-kit signaling in the c-kit mutant mice impaired the Th17 response, but increased the IFN- γ response and the mice showed less bacterial burden in different organs. This implicates a role for IFN- γ in reducing bacterial.

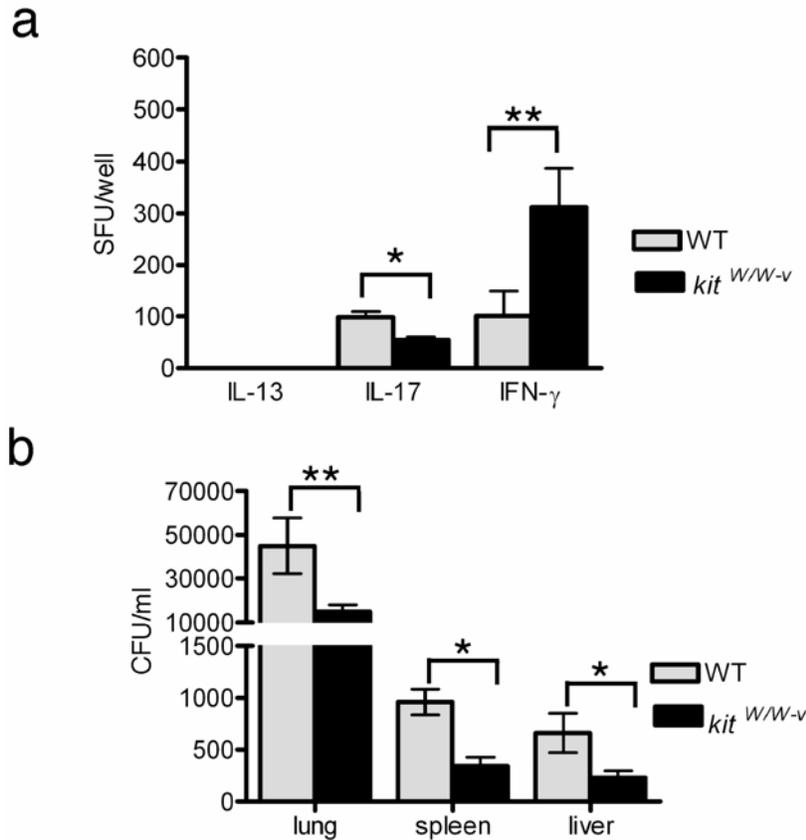


Figure 8: Increased IFN- γ levels in c-kit mutant mice following *Klebsiella pneumoniae* infection.

(a) 150 cfu of the pathogen was administered intratracheally into *kit*^{W/W-v} and *kit*^{+/+} mice. The mice were sacrificed 4 days following the pathogen administration and the lung-draining lymph node cells were isolated. The lymph node cells were plated at a concentration of 1×10^5 cells per well and the cells were re-stimulated with PMA (25 ng/ml) and ionomycin (500 ng/ml) for 24h. The spot forming units (sfu) corresponding to the indicated cytokines per well are shown. The results represent the means from triplicate wells \pm s.d. *, $P < 0.05$. **, $P < 0.005$. (b) CFU was assayed 4 days post-infection from the different organs in *kit*^{W/W-v} and *kit*^{+/+} mice.

2.4.8 c-kit signaling in DCs does not regulate Th1 cell priming

Next, we examined the effect of immunizing *kit*^{+/+} and *kit*^{W/W-v} mice with the Th1-inducing agent CpG ODN. OVA coupled with CpG ODN was intranasally administered to *kit*^{+/+} or *kit*^{W/W-v} mice. After three consecutive administrations of this mixture followed by 5 days of rest, the T cell response was examined. The combination of OVA and CpG ODN elicited a

potent Th1 response evident from the high number of IFN- γ sfu and reduced number of IL-13 sfu. It is important to note that the T cell responses elicited from *kit*^{+/+} mice and *kit*^{W/W-v} mice were not different (**Fig. 9a**). The recall response to antigen was assayed by stimulating the lymph node cells with OVA for 3 days. Nuclear extracts prepared from the cells displayed high levels of T-bet and was also associated with high levels of IFN- γ secretion. In comparison, the Th2 response was subdued as evident from the expression of GATA-3 and the production of IL-13. Overall the T cell response was not different in the *kit*^{+/+} or *kit*^{W/W-v} mice in response to CpG ODN unlike that noted with CT or HDM (**Fig. 9b**). Taken together, the lack of effects of CpG ODN on c-kit expression and the absence of effects on priming or recall responses in response to this stimulus in c-kit mutant mice showed that c-kit signaling is not utilized in the elicitation of a Th1 response.

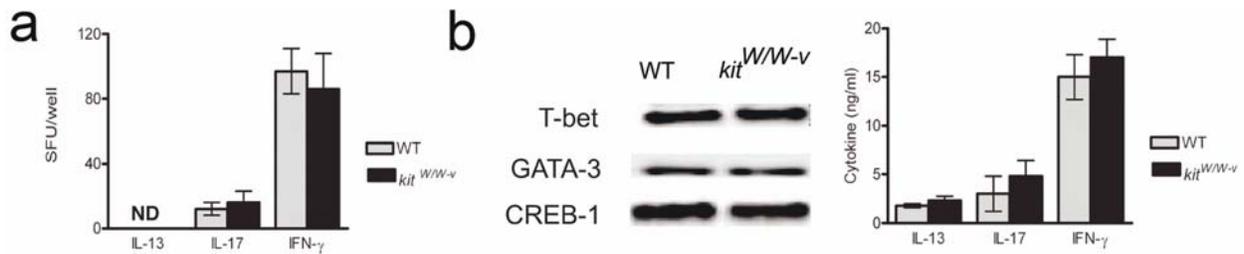


Figure 9: c-kit signaling in DCs does not regulate Th1 cell priming.

(a) Cells from lung-draining lymph nodes following OVA/CpG immunization were stimulated for 24 h with PMA+ionomycin and the sfu were assayed by ELISPOT. ND: not detected and TNTC: too numerous to count. (b) The recall response was assayed by stimulating the lymph node cells with OVA for three days. The transcription factor profile was assayed by immunoblotting technique and the cytokine levels in the culture supernatants were assayed by ELISA.

2.4.9 DCs with defective c-kit signaling elicit poor allergic airway inflammation.

Given that the *kit*^{W/W-v} mice showed reduced Th2 and Th17 priming ability, which was also achieved by neutralizing IL-6 in DC-T cell co-cultures (**Fig. 2**), an important role for both of these molecules in DCs in mounting antigen induced inflammation seemed likely. However, *kit*^{W/W-v} mice also have a defect in mast cells and IL-6 secreted by mast cells could potentially skew T cells towards a Th2 response. To address the importance of c-kit signaling specifically

in DCs in the induction of a Th2 response *in vivo*, we adoptively transferred either $\text{kit}^{+/+}$ (WT) DCs or $\text{kit}^{\text{W/W-v}}$ DCs into WT recipients with a complete repertoire of all cell types including mast cells and asked whether a specific defect in c-kit signaling in DCs would hinder the development of an optimal Th response and allergic airway inflammation. The cartoon in **Fig. 10a** depicts our experimental strategy. **Fig. 10b** shows that BMDCs from $\text{kit}^{\text{W/W-v}}$ mice produced less IL-6 compared to those from $\text{kit}^{+/+}$ mice after incubation with OVA/CT. In one part of the experiment, we made sure that both WT and mutant DCs migrated similarly to lung draining lymph nodes subsequent to intratracheal transfer. CFSE-labeled DCs from the two groups of mice were transferred intratracheally and 24 h following transfer, cells from the lung-draining lymph nodes were analyzed for CFSE expression. As shown in **Fig. 10c**, lymph node migration of BMDCs derived from the mutant mice was comparable to that of cells from WT mice. In other mice, 10 days after transfer, antigen challenge was initiated by exposing animals to 1% aerosolized OVA for 7 consecutive days and the animals were then sacrificed to assess inflammation in the lung. As shown in **Fig. 10d**, the two types of DCs elicited different responses when adoptively transferred into mice. Histological examination of lung sections indicated that recipients of BMDCs from $\text{kit}^{\text{W/W-v}}$ mice mounted less inflammation when compared to those that had received BMDCs from $\text{kit}^{+/+}$ mice. Likewise, mucus production, which is a hallmark feature of human asthma, and is also seen in mice (79, 88, 224) was also less in the recipients of BMDCs from $\text{kit}^{\text{W/W-v}}$ mice (**Fig. 10d**). Differential analysis of cells recovered from the bronchoalveolar lavage (BAL) fluid showed a higher number of cells infiltrating the airways of recipients that received BMDCs from $\text{kit}^{+/+}$ mice and, as expected, the majority of the infiltrated cells were eosinophils (**Fig. 10e**). The antigen-specific IgE level was also lower in mice that received BMDCs from $\text{kit}^{\text{W/W-v}}$ mice compared to that in mice that received cells from $\text{kit}^{+/+}$ mice (**Fig. 10f**). Likewise, the level of both IL-13 and IL-17 in the lung tissue of mice that received DCs with c-kit mutation was ~50% of that in mice that received WT cells but there was no difference in the production of IFN- γ between the two groups of recipients (**Fig. 10g**). The results of this experiment showed the importance of functional c-kit in DCs in influencing the magnitude of a Th2 and Th17 response *in vivo*.

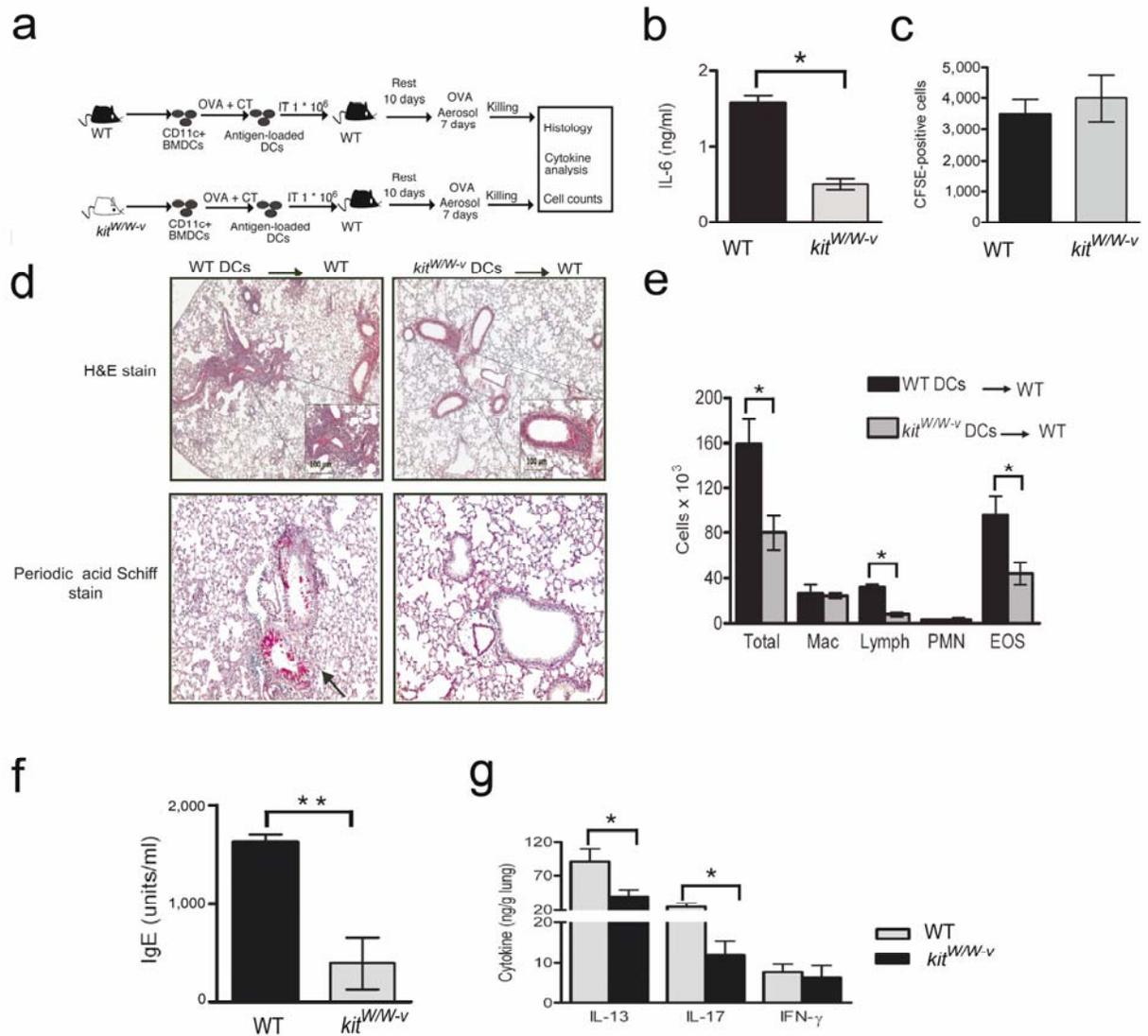


Figure 10: DCs with defective c-kit signaling elicit poor allergic airway inflammation.

(a) Cartoon depicting experimental scheme. (b) Supernatants from BMDCs of *kit^{+/+}* and *kit^{W/W-v}* mice stimulated with OVA/CT overnight were assayed for IL-6 production by ELISA. Data shown are means of triplicates \pm s.e.m. *, $P < 0.05$. Data are representative two independent experiments. (c) The presence of CFSE-labeled CD11c⁺ BMDCs in lung-draining lymph nodes subsequent to intratracheal transfer was assessed 24 h following transfer. This experiment was performed twice and data shown are mean \pm s.d. (d) Histological examination of lung sections of recipient mice stained with hematoxylin and eosin (for assessment of inflammation) or PAS (to assess mucus production). Magnification 100X with the inset at higher magnification (200X). (e) Total and differential counts of cells recovered in the BAL fluid are shown. *, $P < 0.05$. The statistical analysis was performed over 4-5 mice in each group. (f) Antigen-

specific IgE levels were measured by ELISA. The results represent the means of triplicates \pm s.d. *, $P<0.01$. (g) The 17 and IFN- γ in lung homogenates were measured by ELISA and shown are mean values of triplicates \pm s.d. *, $P<0.05$. Shown is a representative experiment of two.

2.4.10 Decreased expression of soluble SCF in CT stimulated BMDCs

We next investigated how c-kit was stimulated in DCs to induce IL-6 production. Prior studies have shown that c-kit is internalized upon binding its ligand indicating an important role for the ligand in regulating cell surface expression of c-kit(225, 226). Analysis of SCF levels in culture supernatants of control versus CT-stimulated BMDCs after 24 h showed lower levels in the latter (**Fig. 11a**). We then tested whether soluble SCF results in internalization of c-kit in DCs. In these experiments, soluble SCF was added to BMDCs at two different concentrations during stimulation with or without CT and the expression of c-kit was analyzed by flow cytometry. As shown in **Fig. 11, panel b**, the expression of c-kit was reduced upon treatment with soluble SCF showing that soluble SCF results in internalization of c-kit.

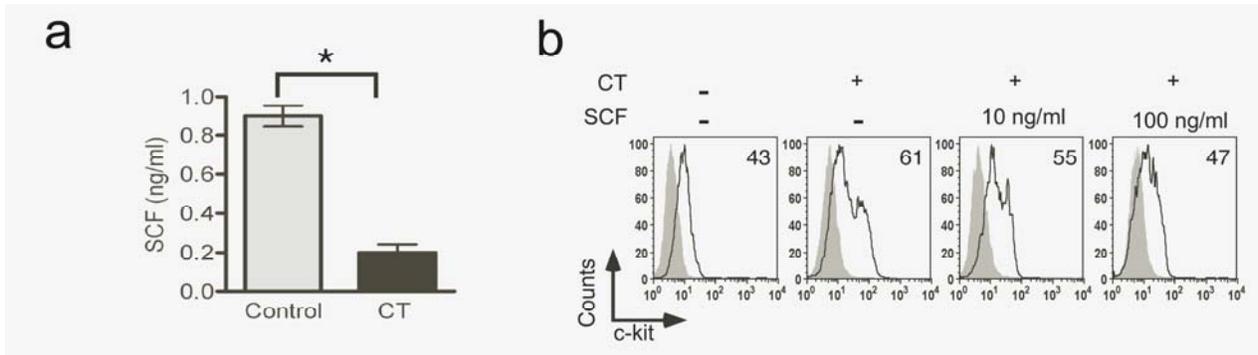


Figure 11: Decreased expression of soluble SCF in CT-stimulated BMDCs.

(a) Supernatants collected 24 h after incubation of BMDCs with or without CT were assayed for soluble SCF by ELISA. The results shown are representative of four independent experiments. The error bars indicate means of triplicates \pm s.e.m. *, $P<0.001$. Data are representative of four independent experiments. (b) Soluble SCF reduces c-kit expression in CT-stimulated BMDCs. BMDCs were incubated with or without CT for 24 h in the presence or absence of soluble SCF as indicated. The cells were stained for c-kit expression by flow cytometry.

2.4.11 Increased expression of membrane-bound SCF in CT or HDM-stimulated BMDCs

Since the c-kit ligand is also expressed in a membrane-bound form due to alternative splicing(148), we analyzed the expression of membrane-bound SCF by immunofluorescence and flow cytometry techniques. Cytospins of BMDCs cultured with or without CT for 24 h were stained for membrane-bound SCF. The results of the immunostaining showed that CT-treatment of BMDCs promoted expression of membrane bound-SCF rather than the soluble factor that was produced by control cells (**Fig. 12a**). When expression of membrane-bound SCF was analyzed by flow cytometry, a greater frequency of SCF-expressing BMDCs was detected when cells were stimulated with CT or HDM, which was not seen with CpG ODN treatment (**Fig. 12b**). To further elucidate the functional significance of the differential expression of the kit ligand upon stimulation with CT or HDM, we used DCs from kit^{sl/sl-d} mice. In these mice, the expression of the membrane-bound form of SCF is compromised, while that of the soluble form of is retained. The mice have a null mutation in one allele of the kit ligand (145, 227) while the other allele is mutated to produce only the soluble form of the ligand(148, 149, 228). Treatment with CT or HDM significantly upregulated the production of IL-6 from the WT DCs, but this robust cytokine expression was lost in DCs generated from kitl^{sl/sl-d} mice (**Fig. 12c**). BMDC from the mutant mice were also used to ensure the specificity of staining for the membrane-bound isoform of the ligand (**Fig. 12c**). The differential expression of soluble versus membrane-bound SCF in CT- or HDM-treated DCs could be key to the function of c-kit in Th2 differentiation, since the membrane-bound ligand is expressed only upon treatments with CT or HDM and is also required for the production of IL-6.

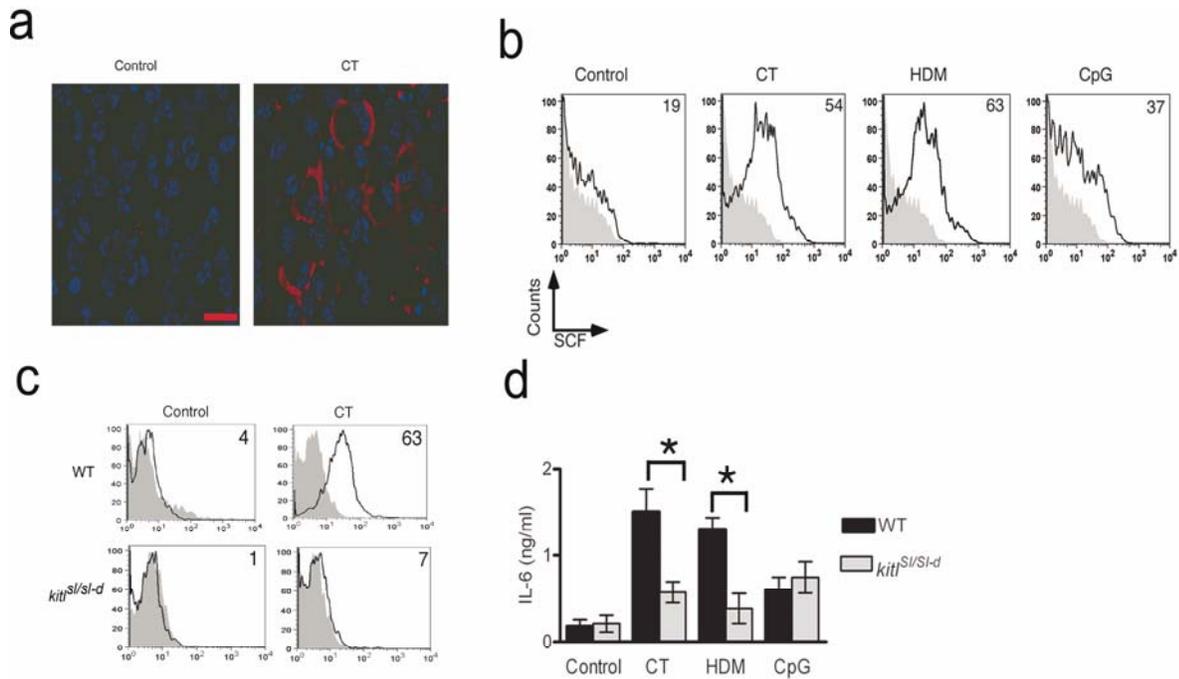


Figure 12: Increased expression of the membrane-bound SCF in CT or HDM-stimulated DCs.

(a) BMDCs were incubated with or without CT for 24 h. Cytospins prepared from the cultures were immunostained with anti-SCF antibody and the nuclei were identified with DAPI. (b) The BMDCs from the different stimulations were assessed for expression of membrane-bound SCF. The numbers represent the percentage of cells expressing SCF on the cell surface. The shaded grey represents staining with the isotype control. (c) BMDCs generated from WT and SCF mutant mice were stimulated for 24h as indicated and IL-6 production was assayed by ELISA. Data shown are means of triplicates \pm s.e.m. and are representative of two independent experiments. *, $P < 0.01$.

2.4.12 c-kit signaling via PI3 kinase pathway regulates IL-6 production

BMDCs from *kit^{W/W-v}* mice upon CT stimulation showed decreased IL-6 production indicating that the downstream signaling by c-kit was critical for IL-6 production (**Fig. 11a**). Ligand-bound c-kit can activate multiple signaling pathways in cells(123). Among these pathways, PI3K has been shown to be important for signaling by the c-kit receptor in different cell types and mutations in c-kit prevent the binding of PI3 kinase to the kinase domain of c-kit (135, 229). The sustained expression of c-kit after 24 h coupled with the high level of expression of membrane-bound SCF suggested persistent signaling through c-kit via PI3 kinase in the DCs.

We, therefore, examined whether Akt phosphorylation was evident even at 24 h post CT treatment of BMDCs and whether it was impaired in the $kit^{W/W-v}$ mice. As shown in **Fig. 13b**, phosphoAkt was detectable in cell extracts prepared from BMDCs stimulated with CT for 24 h, which was drastically inhibited when BMDCs were derived from $kit^{W/W-v}$ mice. Since CT caused Akt activation, which is dependent on PI3 kinase(230, 231), we investigated whether CT-stimulated IL-6 production was compromised in PI3 kinase 110 δ mutant mice ($p110\delta^{D910A/D910A}$). These mice lack the 110delta subunit of PI3 kinase and have impaired B and T cell signaling and mounting of allergic airways disease(232, 233). BMDCs derived from $p110\delta^{+/+}$ and $p110\delta^{D910A/D910A}$ mice were stimulated with CT. CT-stimulated BMDCs from $p110\delta^{D910A/D910A}$ mice secreted less IL-6 as compared to cells from $p110\delta^{+/+}$ mice showing the involvement of the PI3 kinase pathway in IL-6 production (**Fig. 13b**). Thus, c-kit expressed by BMDCs in conjunction with membrane-bound SCF causes prolonged activation of the PI3 kinase/Akt pathway in DCs that promotes a higher IL-6 and lower IL-12 expression profile in DCs, which in turn, promotes T cell differentiation towards the Th2 and Th17 lineage.

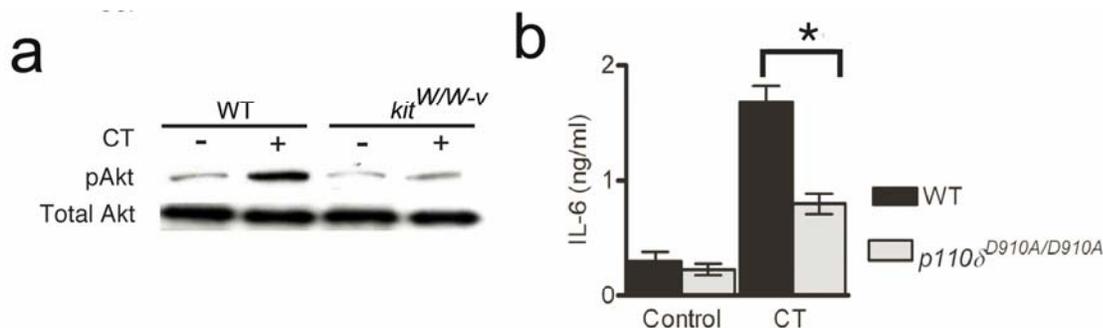


Figure 13: c-kit signaling via PI3 kinase pathway regulates IL-6 production.

(a) Total cell lysates prepared from BMDCs of $kit^{W/W-v}$ mice and $kit^{+/+}$ mice incubated with or without CT for 24h were immunoblotted for pAkt. The same blot was stripped and re-probed for Akt. (b) BMDCs from $p110\delta^{D910A/D910A}$ and $p110\delta^{+/+}$ mice were incubated with or without CT for 24 h. IL-6 levels in the culture supernatants were assayed and data shown are means of triplicates \pm s.e.m. The experiments were repeated twice with similar results. *, $P < 0.05$.

2.5 Discussion

The discovery of DCs has enhanced our knowledge of the divergent pathways of Th1, Th2 and the recently discovered Th17 responses. Specific cytokines are known to bias differentiation towards these 3 lineages. High levels of IL-12 promote Th1 differentiation, and conversely a low level of IL-12 is important for Th2 and Th17 differentiation, both of which are downmodulated by IL-12 and IFN- γ (1). While IL-4 promotes Th2 differentiation, it is not secreted by DCs. Once T cells are induced to differentiate to Th2 cells, IL-4 secreted by the T cells consolidates the Th2 phenotype. IL-6, on the other hand, secreted by DCs has been associated with both Th2 and more recently, Th17 differentiation. IL-6 was shown to increase the expression of the Th2 specific transcription factors GATA-3 and c-maf (49). Studies have also shown that IL-6 can inhibit the production of IL-12p70, which is critical for induction of a Th2 response (176). Recent studies have shown that IL-6 is also a dominant regulator of IL-17 responses. The development of human and murine Th17 cells is critically dependent on IL-6. While IL-17 is important for defense against pathogens (220, 223), IL-17 has been also associated with asthma (94, 208-211). The early recruitment of neutrophils to the airways in asthma is orchestrated by IL-17 (208, 210) and IL-17 has been shown to negatively regulate established disease via effects on DCs (95). Although IL-6 is clearly an important regulator of Th differentiation, there is limited knowledge of molecular mechanisms that underlie IL-6 production in DCs. In this study, we show that sustained activation of the c-kit/PI3 kinase signaling axis via upregulation of both c-kit and membrane-bound SCF on DCs is important for IL-6 production. Increased IL-6 production was found to be important for induction of Th2 and Th17 responses both *in vitro* in BMDC-T cell co-culture systems as well as *in vivo* in lung-draining lymph nodes in response to a combination of the model antigen OVA and the Th2-skewing adjuvant CT or a complex allergen such as HDM. Defective c-kit signaling, associated with reduced IL-6 production, compromised the ability of DCs to mount a robust Th2 and Th17 response and allergic airway inflammation in mice.

Impairment of c-kit expression translated into dampening of Th2/Th17 responses with amplification of Th1 response due to upregulation of IFN- γ expression. This shows an important role for IL-6 in providing the cytokine balance that determines a Th1 versus a Th2 or Th17 response in lung-draining lymph nodes in response to an allergen such as HDM. Our results also

demonstrate the specificity of c-kit and its ligand SCF for IL-6 since the Th1-inducing adjuvant CpG ODN failed to upregulate expression of c-kit. IL-12 production in DCs by microbial stimuli has been tightly linked to CD40 ligation (15) and IL-12 promotes Th1 differentiation. While IL-6 production by DCs has been associated with Th2 and Th17 differentiation, no cell surface event in DCs has been identified heretofore that promotes IL-6 and limits IL-12 production. Interestingly, in mice lacking RABGEF1 (Rab guanine nucleotide exchange factor 1), which is a negative regulator of c-kit, the levels of IL-6 were found to be elevated (234). We also show that the population of c-kit positive BMDCs from CT stimulation induced a strong Th2 and Th17 response, while a higher Th1 response was seen with the population of c-kit negative BMDCs. Based on our findings, it is tempting to speculate that via activation of the c-kit-PI3 kinase axis in DCs, IL-6 serves the function of an important regulator that limits a Th1 response in contrast to IL-12 that promotes it.

Exposure to the allergen HDM or to a combination of OVA and CT resulted in decreased Th2 and Th17 priming in kit^{W/W-v} mice. We and others have shown the importance of elevation of cAMP in T cells in the induction of a Th2 response (235, 236). In DCs, an increase in intracellular cAMP level has been associated with decreased IL-12 production, which is conducive to Th2 priming (24). Increased IL-6 levels in a wide range of cell types has been associated with elevated cAMP levels and cAMP also promotes c-kit expression (237-239). The suppression of c-kit expression seen in DCs upon addition of the cAMP antagonist correlated with a decrease in IL-6 production. These results, in combination with the data from the two models of inflammation, strongly suggest that the c-kit signaling pathway in DCs can be utilized by allergens to regulate cytokine production in a cAMP-dependent fashion.

Not only did this study unravel a link between c-kit and IL-6 production in DCs, but the expression of a Notch ligand, Jagged-2, specifically aligned with Th2 responses (34), was also found to be dependent on functional c-kit. The Notch signaling pathway is an evolutionarily conserved pathway influencing cell fate during development (240). Important regulatory roles for Notch have been recently identified in the immune system. For example, Notch has been implicated in T cell polarization, which has been associated with upregulation of specific Notch ligands in response to Th2 or Th1 stimuli on APCs (34). We have recently demonstrated cross-talk between Notch- and TGF- β induced pathways in antigen-induced tolerance in the airways (241). Here, we show that DCs expressing functionally inactive c-kit express low levels of

Jagged-2 compared to DCs from WT mice identifying a potentially important role for c-kit during development in the expression of a specific Notch ligand. It will be interesting to investigate in further detail the developmental relationships between c-kit and Notch ligand expression in different cell types. The relationship between Jagged-2 and c-kit is also in line with previous observations showing that the Notch-Jagged 2 axis promotes IL-6 secretion in different cell types (242, 243). While Notch activation in CD4⁺ T cells is important for Th2 differentiation (34, 244, 245), bidirectional Notch-Notch ligand signaling has been documented in Th:DC conjugates with evidence of nuclear translocation of Hes1 and STAT3 in both cell types (246). Thus, the Notch pathway may be important in promoting IL-6 gene expression in DCs with c-kit playing a key role in regulating Jagged-2 expression.

SCF has been shown to regulate the expression and signaling of c-kit. SCF is expressed in membrane-bound and soluble forms and the two forms have distinct biological properties(148). The binding of soluble SCF to c-kit causes the internalization of c-kit and the ligand-receptor complex is targeted for ubiquitination(226). Membrane-bound SCF, on the other hand, has been shown to prevent c-kit receptor internalization and instead causes persistent signaling via c-kit(247). As suggested by our data, the differential ligand expression seen in CT or HDM-stimulated BMDCs could be an important mechanism by which prolonged activation of c-kit and its downstream targets is achieved. It should be noted that soluble SCF can transiently cause Akt phosphorylation, and phosphorylated Akt can no longer be detected after 15 min of addition of SCF (234). In our experiments, even at 24 h after treatment with CT, phosphorylated Akt could be detected in the DCs. Furthermore, our data show that impaired c-kit signaling in DCs derived from *kit*^{W/W^{-v} mice results in markedly attenuated Akt phosphorylation and IL-6 production in DCs and lower Th2 and Th17 responses in lung-draining lymph nodes subsequent to allergen administration. The data from the *kit*^{sl/sl^{-d} mice also reiterates the fact that absence of membrane-bound SCF significantly reduces signaling via the receptor resulting in decreased IL-6 production. Collectively, our data suggest that the dual upregulation of c-kit receptor and membrane-bound form of SCF contributes to increased IL-6 production from allergen-stimulated DCs.}}

CT-induced intestinal secretory responses are dependent on c-kit signaling since *kit*^{W/W^{-v} mice were found to be unresponsive to CT(248). In a model of infection with *Giardia*, the administration of anti-c-kit antibody resulted in mice succumbing to infection from the parasite.}

The authors elegantly showed that IL-6 was a critical player in clearing the infection since IL-6^{-/-} mice succumbed to the infection despite having normal mast cell numbers(249). While c-kit has been best studied in the context of mast cells, in some studies, administration of SCF *in vivo* was shown to have mast cell-independent effects(250). This suggests that the effects of c-kit mutation may not be restricted to mast cells alone in other models as well. Zitvogel and colleagues have recently demonstrated that the c-kit receptor on DCs has the ability to modulate the function of NK cells by increasing the cytolytic and IFN- γ -secreting ability of NK cells, thereby defining the importance of this receptor in one aspect of DC function(161). Another important aspect of this study to note is that the authors have clearly demonstrated that DCs are the pharmacological target of Gleevec administration *in vivo*, indicating mast cell-independent effects of Gleevec. While mast cells are known to fine tune T cell responses due to their ability to secrete a variety of cytokines, being professional antigen-presenting cells, DCs play a quintessential role in the orchestration of the adaptive immune response. The experiment involving adoptive transfer of DCs demonstrated that crippling c-kit signaling in DCs with intact mast cell c-kit signaling in recipients blunts the Th2 response, which prevents induction of full-blown allergic airway inflammation. Thus, although mast cells can modulate T cell responses, they are unable to supplant DCs in eliciting a strong Th2 response. Based on these results, it seems likely that defective DC functions may be responsible for the reported lower susceptibility of kit^{W/W-v} mice to allergic airways disease (251, 252).

The importance of c-kit signaling via PI3 kinase has also been explored in our study. Interestingly, p110 δ ^{D910A/D910A} mice have been shown to be resistant to allergic responses indicating a positive role for PI3 kinase in mediating Th2 responses(232). A prior study has shown a negative role for PI3 kinase signaling in IL-12p70 production from DCs(253). Our study for the first time shows a positive role for this pathway in IL-6 production from DCs and our data suggest that sustained phosphorylation of Akt in DCs is required for the expression of specific cytokines such as IL-6 which can influence the outcome of an immune response. Prolonged c-kit expression coupled with expression of membrane-bound SCF ensures sustained Akt activation as is evident from our study and from previous studies in which soluble SCF was shown to impede signaling via c-kit (226) while membrane-bound SCF prolonged c-kit signaling (247).

The cytokine balance in the microenvironment is a key regulator of the net immune response. Our study highlights the importance of this balance in regulating T cell responses by demonstrating a central role for c-kit expressed by DCs in the fine-tuning of the IL-6/IL-12p70 expression profile. Our findings also have important ramifications in DC-based vaccines in cancer therapy where inhibition of c-kit may promote the efficacy of the vaccine by augmenting a Th1 response.

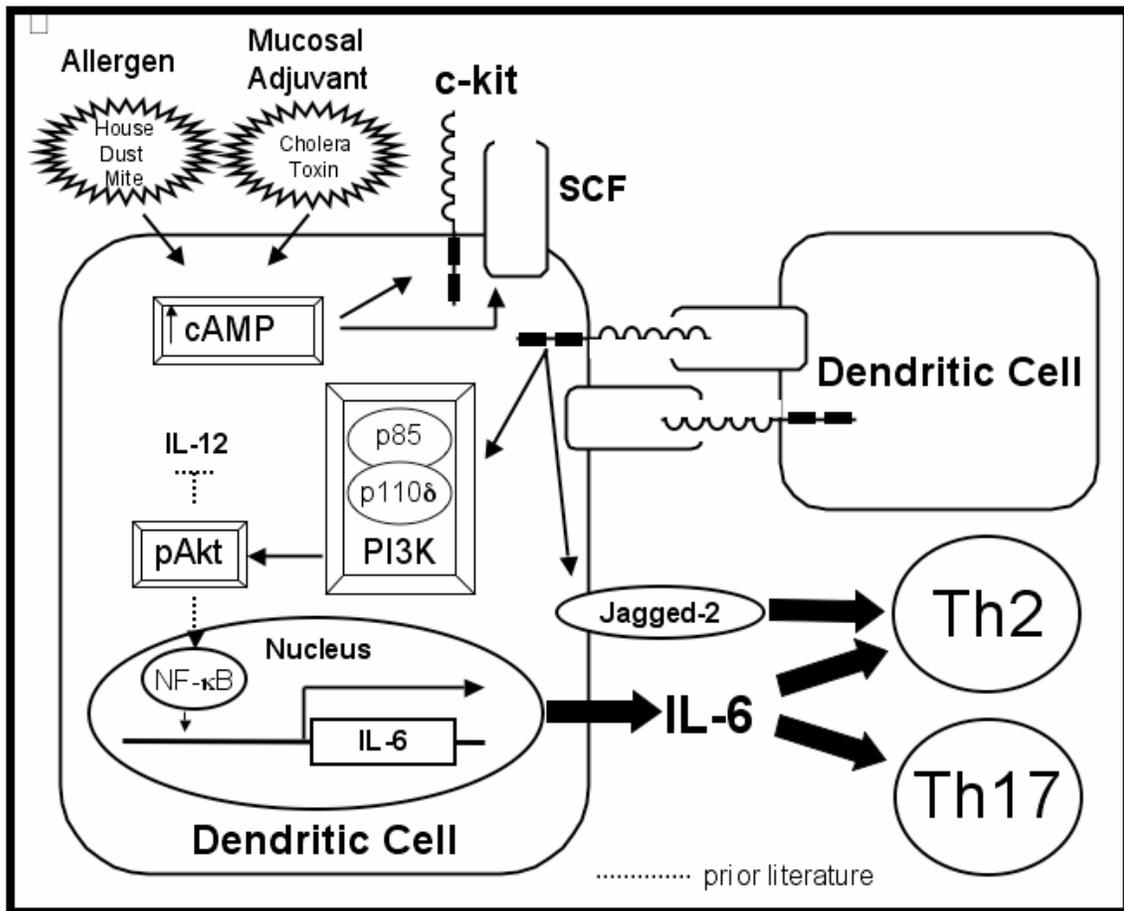


Figure 14: Cartoon depicting the c-kit-PI3 kinase-IL-6 pathway

The importance of the dual upregulation of c-kit and membrane-bound SCF in prolonging PI3 signaling and promoting IL-6 production is depicted here. The upregulation of Th2 signature notch ligand, Jagged-2 is also seen. The IL-6 produced by DCs promotes Th2 and Th17 responses but inhibits a Th1 response.

3.0 A critical role for vascular endothelial growth factor (VEGF) secreted by DCs in priming Th2 responses

3.1 Abstract

Vascular endothelial growth factor (VEGF) is an important angiogenic factor that has multiple effects in lung development and disease, including asthma. VEGF has recently been implicated as a mediator of T helper 2 (Th2) immune response in the context of asthma pathogenesis. We investigated the regulation of VEGF expression in dendritic cells (DCs), and the downstream functions of DC-derived VEGF on T cell activity. iNOS and NF- κ B enhance production of VEGF in DCs whereas IL-12 potently inhibits VEGF synthesis. VEGF secreted by DCs promotes the differentiation of Th2 cells, and alters established T cell responses. In summary our study details the complexity of Th2 immune responses by highlighting a critical but previously unknown role for DC-derived VEGF.

3.2 Introduction

Asthma is a debilitating inflammatory disease of the small airways caused by an exaggerated CD4 T helper 2 (Th2) immune response and characterized by the overexpression of the pro-inflammatory cytokines IL-5, IL-13, and IL-4 (70). Animal models involving administration of allergen or antigen coupled with adjuvant have been successful in recapitulating the hallmarks of asthma. Several researchers have shown that OVA coupled with mucosal adjuvant cholera toxin when administered intranasally into mice trigger a robust, eosinophil-rich inflammation in the lungs (73). *In vitro* CT-stimulated DCs prime naïve T cells towards a Th2 response but the mechanism of CT-induced Th2 response is not well elucidated. We undertook a microarray approach to dissect the mechanism underlying promotion of Th2 responses by CT-primed DCs, and found significant upregulation of expression of vascular endothelial growth factor (VEGF). Prior studies have identified increased levels of VEGF in asthma pathogenesis and documented its role as a vascular permeability factor in asthmatics (254-256). Interestingly, a novel but poorly understood immunologic role for VEGF in Th2

polarization has recently been discovered, which suggests that DC-derived VEGF could be critical in priming Th2 responses (34).

The differentiation of naïve T cells into effector Th2 cells is mediated by dendritic cells (DCs). Often overlooked in the past, DCs are increasingly gaining recognition for their critical role in bridging the innate and adaptive immune system. Several studies have shown that specific cytokines secreted by DCs are essential for T cell polarization and dictate the outcome of the immune response (25). However, given the previously unknown immunologic function of VEGF no study has comprehensively analyzed the secretion of VEGF from DC. A prior study has demonstrated the exclusive ability of DCs alternatively activated in the presence of PGE2 to secrete VEGF and also found that DCs matured with LPS are unable to do so (202). Given the role of PGE2 as a well known Th2 skewing agent, we were interested in further investigating whether classically activating DCs with Th2 agents would induce the production of VEGF.

In the present study, we examined the capacity of DCs to secrete VEGF in response to different stimuli. Concurring with our microarray data, CT induced secretion of VEGF from bone marrow and tissue DCs. Our experiments also confirm that LPS, which is a known inducer of several cytokines in DCs failed to trigger the production of VEGF.

The addition of VEGF to DC:T cell co-cultures in the presence of antigen promoted a Th2 phenotype whereas adding an inhibitor specific to the VEGF receptor abrogated the Th2 priming ability *in vitro*. These results highlight the role of VEGF as a potent Th2 skewing cytokine.

We also found that iNOS and NF- κ B regulate the expression of VEGF in DCs. The inhibition of these molecules using iNOS knockout mice or specific pharmacological inhibitors decreased the production of VEGF thus establishing their important role in the signaling axis of VEGF. We have also identified that IL-12 acts as a negative regulator of VEGF - high levels of IL-12 secreted by LPS-stimulated DC limits the production of VEGF. This study provides a unique perspective on the importance on the role of DC-derived VEGF in modulating T cell responses and identifies molecular events that regulate the production of the cytokine.

3.3 Material and Methods

3.3.1 Mice and Reagents

C57BL/6, iNOS $-/-$ and BALB/c were purchased from Jackson laboratory. The iNOS specific inhibitors used in the study L-NIL and L-NAME were purchased from Sigma. NF- κ B blocking (SN50) and mock (SN50M) cell-permeable inhibitors were purchased from Calbiochem. Cholera toxin and LPS from *E.coli* were obtained from List biological labs and Sigma respectively. The iNOS antibody was purchased from BD biosciences and the STAT-1 and p65 antibodies were from Santa Cruz Biotechnology. The beta-actin antibody was purchased from Novus Biologicals. The ELISA kits for VEGF and IL-12p70 were from R&D systems and Peprtech. The recombinant cytokine VEGF and IL-12p70 were obtained from Peprtech.

3.3.2 DC preparation

BMDCs were obtained as described previously. Briefly femur and tibia were dissected from mice and the bone marrow progenitor cells were grown in RPMI medium (Gibco) supplemented with 10% heat inactivated fetal bovine serum and 50 μ beta-mercaptoethanol. The medium also contained the antibiotics penicillin and gentamycin. The bone marrow cells were plated at 1 million/ml. GM-CSF (Peprtech) was added to the culture at 10ng/ml concentration. The culture was replenished with fresh medium and cytokine on the third day. On day 6 the loosely adherent cells were harvested and were purified using CD11c magnetic bead (Miltenyi). The cells were considered immature DCs from the low levels of MHCII, CD40 and CD86. The cells were then stimulated with CT (1 μ g/ml), LPS (10 μ g/ml) and HDM (10 μ g/ml). Lung and spleen DCs were obtained exactly as described previously.

3.3.3 Intranasal Immunization protocol

The immunization protocol to induce tolerance with OVA instillation and inflammation with OVA/CT was performed exactly as described in chapter 2.

3.3.4 Immunoblotting of nuclear and cellular extracts

The nuclear and cellular extracts were prepared as described previously. 10µg of the protein estimated by Biorad kit was immunoblotted on 4-15% gradient SDS-PAGE gel and transferred on a PVDF membrane. All the antibodies were used in 1:1000 dilutions in 5% non-fat dry milk dissolved in Tris-buffered saline with 0.01% Tween 20. Beta-actin was diluted at 1:10000. The membranes were incubated overnight in the antibodies and stained with anti-rabbit or anti mouse (1:10000) conjugated to horseradish peroxidase. The chemiluminescence was developed by addition of specific substrates from the Pierce

3.3.5 ELISA

The ELISA for VEGF and IL-12p70 were performed according to Manufacturer's instruction.

3.4 Results

3.4.1 DCs secrete VEGF upon specific Th2 stimuli

We used a microarray approach to identify novel Th2 skewing factors associated with CT-stimulated DCs. The gene analysis revealed 258 genes to be differentially regulated in DCs upon treatment with the toxin. One of the genes significantly upregulated was VEGF. Although, prior research has established that VEGF is secreted by epithelial cells as well as CD4 Th2 cells during airway inflammation (34), our objective was to determine whether DCs secrete VEGF upon stimulation with CT and if this property was exclusive for the toxin or was shared by other antigens such as LPS. Interestingly, BMDCs when stimulated with CT secreted high levels of VEGF as compared to LPS stimulated-DCs (**Fig 15a**). We also investigated the capacity of tissue specific DCs to induce the production of VEGF and found that lung DCs induced higher amounts of VEGF compared to the control treatment, after stimulation with CT for 48 h. However, LPS-stimulation of lung DCs did not trigger secretion of VEGF. This suggests that the

Th2 response triggered by low concentrations of LPS in the lung is not mediated by VEGF. It is interesting to note that DCs from the spleen secreted lower levels of VEGF when compared to lung DCs when stimulated with the same antigens (**Fig 15a**). This corroborates our finding that the immune response in the lung is skewed towards a Th2 response when compared to the spleen. Several studies including ours have shown that DCs from different tissues are programmed to induce different cytokine profiles in response to the same stimuli(33, 207). In support of this preferential Th2 skewing in the lung, we found lower levels of IL-12p70 secretion in lung DCs than in spleen DCs. (**Fig 15b**). The inability of LPS to induce secretion of VEGF was surprising since it triggers the production of a spectrum of cytokines in DCs. Subsequently in our experiments we used CT as the agent to induce the secretion of VEGF and LPS as a negative control.

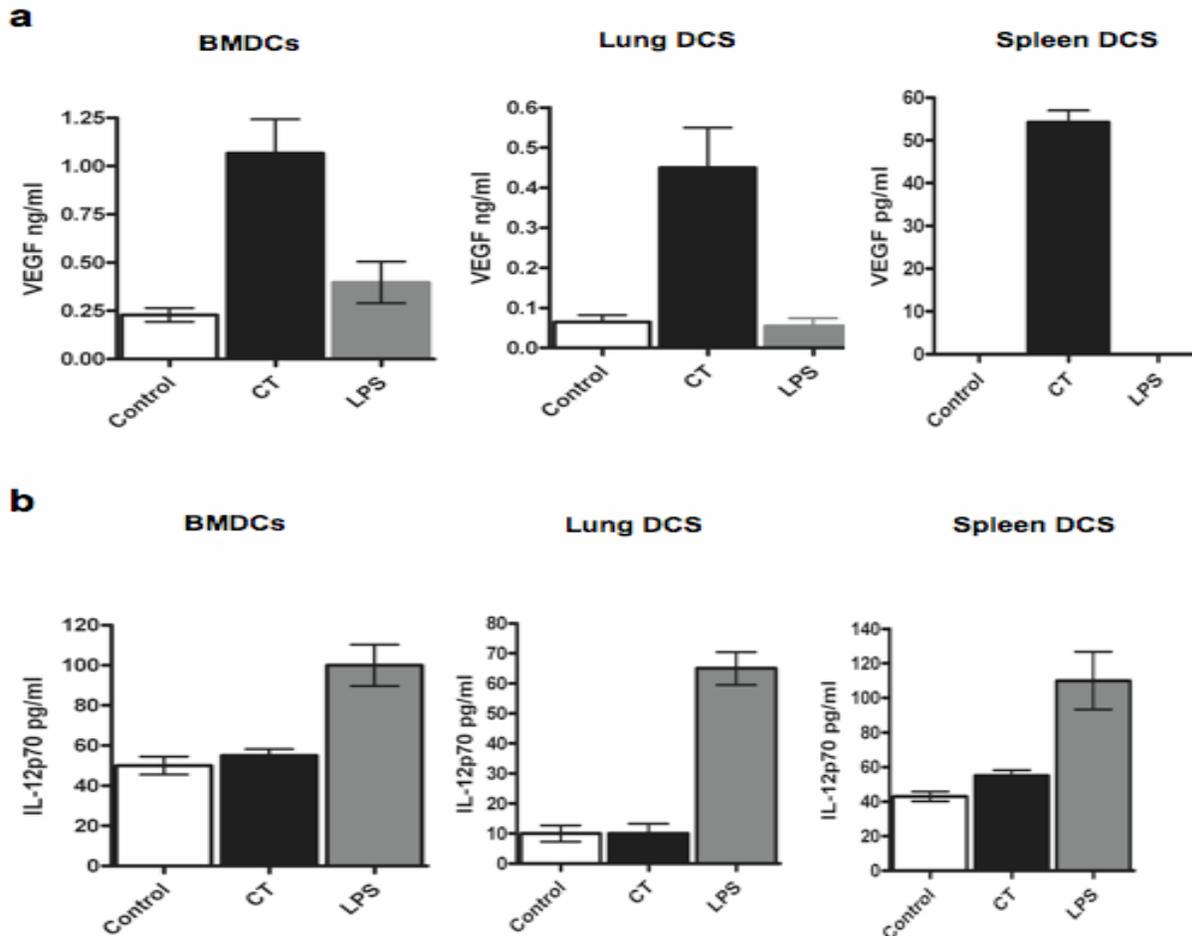


Figure 15: CT-stimulated DCs secrete VEGF.

BMDCs and tissue-derived Dcs from the spleen and lungs were stimulated with CT or LPS.

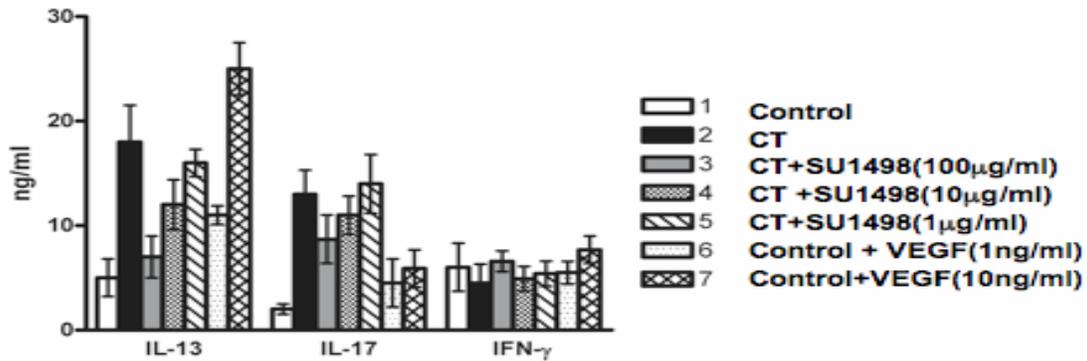
The levels of VEGF (**a**) and IL-12p70 (**b**) was assayed by ELISA from the culture supernatants. The experiment was performed four times with BMDC supernatants and two times with tissue DCs. The error bars are means with SEM between triplicate wells.

3.4.2 VEGF production *in vitro* and *in vivo* is critical for Th2 priming and airway inflammation

Fig 15 shows that antigen presenting cells have the capacity to secrete VEGF in response to specific Th2 stimuli. To investigate the Th2 skewing capacity of VEGF in a DC:T cell co-culture, DCs stimulated with CT were co-cultured with T cells from naïve DO11.10 mice in the presence of OVA peptide and added VEGF of 10 ng/ml and 100 ng/ml concentrations to the control cultures. Upon assaying the production of cytokines associated with Th1, Th2 and the recently discovered Th17 by ELISA, we found that CT-stimulated DCs induces a mixed T cell response with increased production of IL-13 and IL-17 without changing IFN- γ levels. The addition of VEGF to control cultures impressively increased the expression of IL-13 to levels secreted by CT-stimulated DCs (**Fig 16a**). Thus, we found that VEGF promotes a Th2 response but does not inhibit the Th1 response. This effect contrasts with other Th2 skewing cytokines like IL-4 and IL-6, which are known to concomitantly promote the Th2 arm of the immune response and inhibit the Th1 response (172, 207). The addition of the VEGF-receptor inhibitor, SU1498 to the DC:T cell co-culture significantly abrogated the Th2 response, evidenced by the reduced secretion of IL-13 (**Fig 16a**).

DCs in the lung-draining lymph nodes polarize T cell responses by secreting cytokines and via other mechanisms. Since the presence of VEGF primes a Th2 response, it is critical to determine its *in vivo* role in airway inflammation. Mice were immunized with three consecutive instillations of OVA alone, OVA coupled with CT or OVA coupled with CT along with the VEGF-receptor inhibitor, SU1498. After five days of rest, the mice were subjected to OVA aerosol for 7 consecutive days (**Fig 16b**). The inflammation in the lung was assessed by H&E staining and also by IgE titers in the serum.

a



b

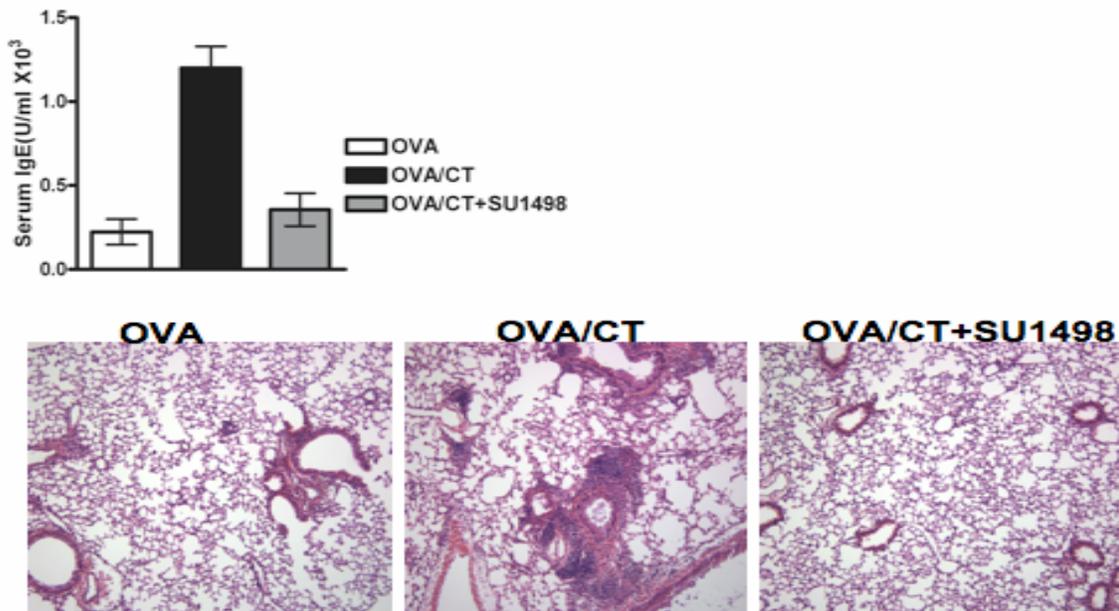


Figure 16: VEGF production *in vitro* and *in vivo* is critical for Th2 priming and airway inflammation.

(a) DCs were incubated for 24 h with CT and then co-cultured with naïve CD4⁺ T cells from DO11.10 mice in the presence of ova peptide. VEGF receptor inhibitor or VEGF were added as shown. Supernatant from the cultures were assayed for different cytokine production. The error bars represent mean between wells. This experiment was performed twice. (b) Mice were immunized with OVA, OVA/CT or OVA/CT+SU1498. The mice were rested for 5 days and then subjected to OVA aerosol for seven consecutive days. At the end of this time point, the mice were sacrificed and analyzed for inflammation in the lungs by H&E staining. The serum IgE titers was assayed by ELISA. This experiment was performed twice.

3.4.3 VEGF secretion in DCs is regulated by iNOS

Studies relating to liver regeneration have shown a strong association between iNOS activation and VEGF production (257). In asthmatics, an increase in exhaled nitric oxide has been documented and the inhibition of iNOS has shown to reduce airway inflammation (196, 258). Our next objective was to explore the mechanism of regulation of VEGF production in DCs and whether iNOS plays a role in mediating its production. The expression of iNOS was assayed from total cell lysates of CT or LPS-treated DCs and found elevated levels of iNOS (**Fig 17a**). The activity of iNOS was determined by measuring the levels of nitric oxide from the culture supernatants and CT or LPS-stimulated DCs induced higher production of nitric oxide compared to the control samples, as measured by Greiss assay (**Fig 17a**). Although, the expression of iNOS in CT-stimulated DCs seemed slightly elevated when compared to LPS treatment, the levels of NO produced was contrastingly different. NO produced by LPS was significantly higher at 24 h and it is possible that the high levels of NO reduces the iNOS expression. To determine whether the production of VEGF was dependent on iNOS, bone marrow derived DCs from wild type mice and iNOS knockout mice were stimulated in the presence of CT or LPS. DCs from the WT mice showed increased secretion of VEGF upon stimulation with CT, but this upregulation was lowered in DCs from iNOS knockout mice (**Fig 17b**). This suggests an important role for iNOS in regulating VEGF expression. Although LPS-stimulated DCs did not significantly upregulate the production of VEGF, the inhibition of nitric oxide further decreased the production of the cytokine (**Fig 17b**). The pharmacological inhibition of iNOS using inhibitors L-NIL and L-NAME also resulted in decreased secretion of VEGF from the DCs stimulated with CT or LPS (**Fig 17c**). Collectively, the results from these experiments provide compelling evidence that that the upregulation and activation of iNOS is a critical factor in the production of VEGF. We then evaluated whether the decreased VEGF production from DC stimulated with CT from the iNOS knockout mice resulted in compromised Th2 skewing capacity of naïve T cells. DCs from WT or iNOS knockout mice were stimulated with or without CT and co-cultured with CD4 T cells in the presence of soluble anti-CD3. The CT-stimulated DCs from the WT and iNOS knockout mice showed no difference in IL-13

production when co-cultured with CD4 T cells from the WT mice. However, CT- stimulated DCs from iNOS knockout mice when co-cultured with CD4 T cells from iNOS knockout mice were severely compromised in the Th2 arm of the immune response. The response was marked by decrease in IL-13 production whereas the Th1 and Th17 responses remained unaffected. This defect was not necessarily due to impairment in the CD4 T cells from iNOS knockout mice since CT-treated DCs from WT mice when co-cultured with CD4 T cells from iNOS deficient mice showed upregulation of Th2 immune response (**Fig 17d**). This experiments further strengthens the role of VEGF in skewing Th2 responses.

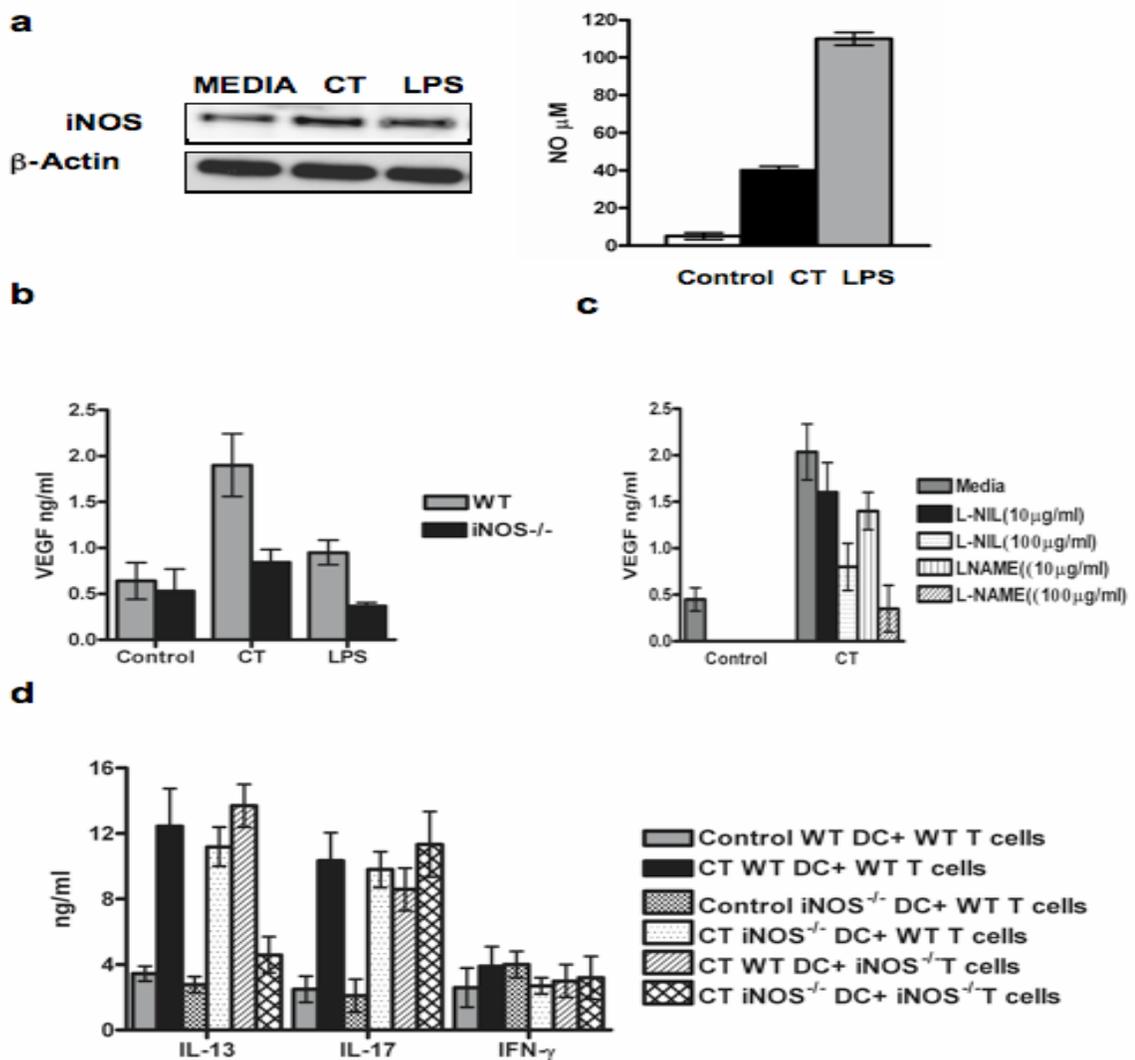


Figure 17: Nitric Oxide positively regulates VEGF production.

(a) DCs were incubated for 24 h with CT or LPS and total cell lysates were analyzed for iNOS expression by western blotting. Nitric Oxide levels in the culture supernatants of DCs was measured by

Greiss assay. **(b)** DCs from WT and iNOS^{-/-} mice were incubated for 24 h with CT or LPS. The level of VEGF production in the culture supernatants of DCs was measured by ELISA. **(c)** DC were pre-treated with specific iNOS inhibitors for 1h and then stimulated with CT. The levels of VEGF produced after 24 h was measured by ELISA **(d)** DCs from WT and iNOS^{-/-} were incubated with or without CT for 24 h and then co-cultured with naïve CD4 T cells from WT and iNOS^{-/-} mice. The levels of cytokines in the culture supernatants was measured by ELISA. All of the experiments have been performed twice and the error bars represent the mean + SEM between triplicate wells

3.4.4 Inhibition of NF-κB results in decreased nitric oxide and VEGF production

Since iNOS deficiency weakened the production of VEGF from CT-stimulated DCs, we wanted to evaluate the consequence of blocking important activators of iNOS in regulating the production of VEGF. The expression of iNOS is tightly regulated by several transcription factors. NF-κB is a known regulator of several cytokines and has been shown to bind to the iNOS promoter and regulate the production of nitric oxide (259, 260). We wanted to determine whether the inhibition of NF-κB could affect the production of VEGF via an iNOS/nitric oxide dependent pathway. We first verified the extent of NF-κB activation in DCs stimulated with CT or LPS. We probed nuclear extracts from the stimulated DCs for expression of p65 (RelA) and found increased nuclear translocation of the subunit thus indicating increased activation of the transcription factor (**Fig 18a**). We inhibited the activation of the transcription factor using specific pharmacological inhibitors and found that the addition of NF-κB specific inhibitor SN50 to BMDCs stimulated with CT or LPS decreased nitric oxide production from the DCs (**Fig 18b**). The mock peptide SN50M used as control did not affect the levels of nitric oxide produced. We also analyzed the supernatants for the production of VEGF and observed that the inhibition of NF-κB also decreased the production of VEGF from LPS or CT-stimulated DC (**Fig 18c**). This decreased production of VEGF indicates an important role for the transcription factor in regulating the production of the cytokine via a nitric oxide dependent pathway.

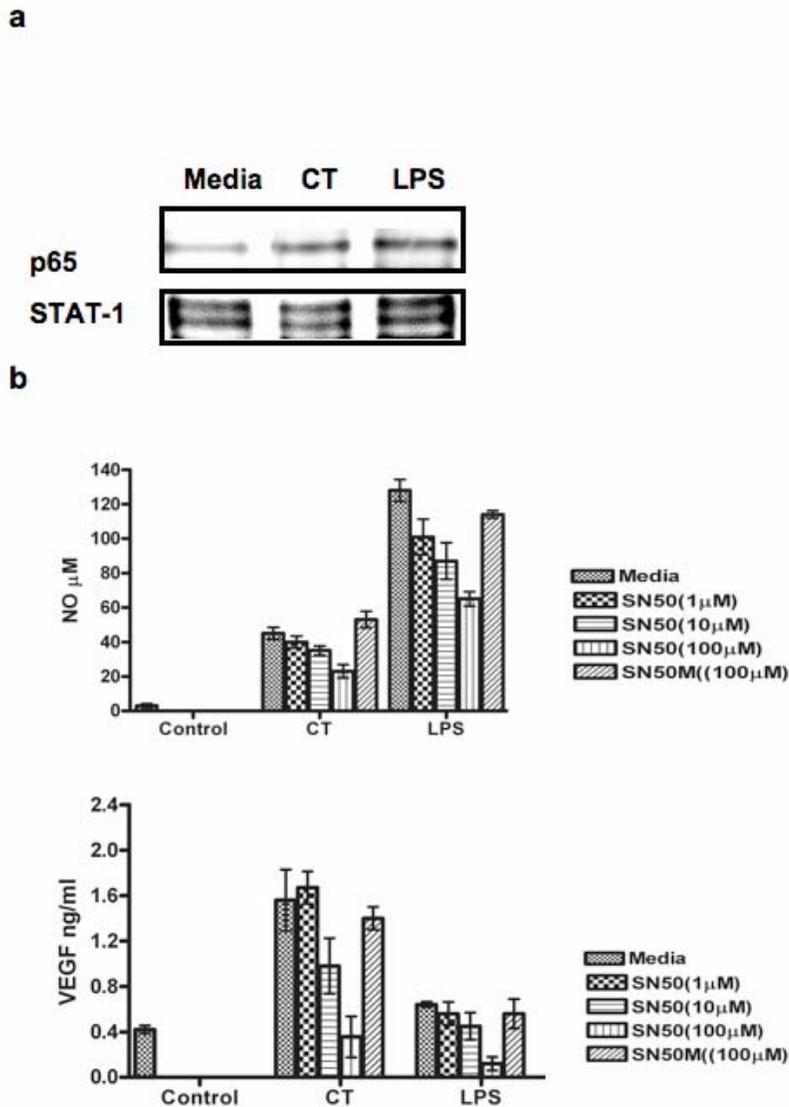


Figure 18: NF- κ B positively regulates Nitric Oxide and VEGF production.

(a) Nuclear translocation of p65 subunit of NF- κ B assayed by western blotting from BMDC nuclear extracts. The blot was stripped and re-probed for STAT-1 for loading control. (b) Levels of VEGF produced in the supernatant was measured by ELISA. The same supernatants were also used to estimate NO levels by Greiss assay. The NF- κ B inhibitor (SN50) and the mock peptide (SN50M) were added to BMDCs for 1 h prior to stimulation with CT. or LPS

3.4.5 IL-12 acts as a negative regulator of VEGF

LPS-stimulated DCs secrete substantially higher levels of nitric oxide when compared to the CT treatment but are unable to trigger the production of VEGF (**Fig 18**). This suggested that the secretion of VEGF in LPS-stimulated DCs is limited by a potent inhibitor. Several recent studies in the field of cancer biology have shown the therapeutic effect of administering IL-12 in various tumor models. Deficiency in IL-12 results in the promotion of pro-angiogenic factors, specifically VEGF, which results in increased tumor growth (197, 198). These studies collectively establish a role for IL-12 in downregulating the production of VEGF. As shown here (**Fig 15b**) and in other studies, LPS-stimulated DCs secrete significantly higher levels of IL-12p70 compared to CT-stimulated DCs. To test whether IL-12p70 inhibits the production of VEGF in DCs, we treated CT-stimulated DCs with IL-12 and assayed the culture supernatants for VEGF production by ELISA 24 h after the stimulation. We found that CT-stimulated DCs upregulated the production of VEGF as compared to control DCs but the addition of IL-12 decreased the production of VEGF, correlating with the dose of IL-12 added to the cultures (**Fig 19a**). We also found that stimulation of DCs with the combination of CT and LPS also downregulated the production of VEGF suggesting that the high levels of IL-12p70 secreted by LPS-stimulated DCs is a possible cause for the reduced production of VEGF. We assayed the production of nitric oxide and found that the addition of IL-12p70 decreased the capacity of CT-stimulated DCs to upregulate nitric oxide, thus strongly suggesting the inhibition of VEGF mediated by IL-12p70 was dependent on iNOS activation (**Fig 19b**). As discussed earlier, the VEGF-receptor signals via the PI3 kinase pathway (182) and PI3 kinase is a negative regulator of IL-12 production (200, 253). We also assayed the levels of VEGF produced in BMDCs from WT mice and p110 $\delta^{D910A/D910A}$ mice upon stimulation with CT. The supernatants from the BMDCs of the mutant mice showed decreased levels of VEGF production 24 h post-treatment with CT (**Fig 19c**). This experiment highlights the role of PI 3 kinase in positively regulating VEGF production in DCs, whereas IL-12, which is negatively regulated by PI 3 kinase, also negatively regulates VEGF production from DCs.

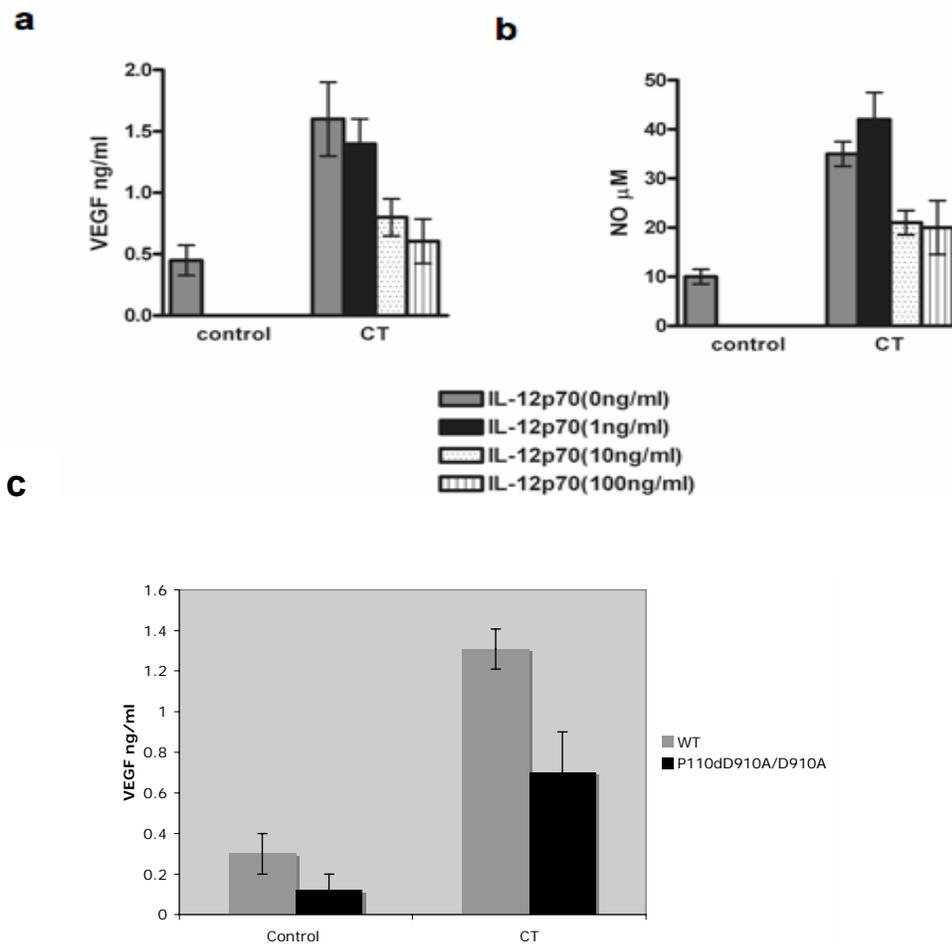


Figure 19: IL-12 antagonizes VEGF production.

(a,b) IL-12p70 was added in different concentrations to BMDCs. The culture supernatants were assayed for levels of VEGF and NO production. (c) BMDCs from WT and p110δ^{D910A/D910A} mice were stimulated with CT. The level of VEGF produced was measured by ELISA. These experiments were performed twice.

3.5 Discussion

The pathway for Th2 differentiation is no longer considered a default pathway that occurs in the absence of a Th1 polarizing influence but regarded as a finely tuned response. Recent studies suggest that a Th2 driving force coupled with a low IL-12 environment are important prerequisites for a Th2 response. Kalinski and colleagues initially proposed the concept of “Signal 3,” the polarizing signal in DCs that causes T cell differentiation (2, 3). Recent advances in the DC cell field have identified several molecules to be Th2 polarizing candidates but the mechanism of activation and regulation of several Th2 skewing agents in the DCs is poorly understood. Our study not only identifies the importance of VEGF derived from DCs in tailoring a Th2 specific response, but specifies the key molecular events involved in regulating the expression of this cytokine.

Cytokines secreted by DCs are potent T cell skewing factors. In the field of asthma and allergy, elevated levels of VEGF have been attributed to extensive re-modeling and infiltration of cells into the lungs. Prior research in murine models of asthma has shown that blocking the action of VEGF in vivo by the administration of the VEGF-receptor inhibitor can significantly reduce inflammation in the lungs. However these studies have not assigned a definitive role for DC-derived VEGF in modulating the inflammatory response, despite the established role of DCs as proinflammatory cells that promote inflammation in asthma (193). The finding of a novel role for VEGF as a Th2 polarizing cytokine warrants the study of this cytokine in DCs. The discovery of the new helper T cell subset Th17 makes the interactions between three CD4 T cell subtypes further more intricate. Cytokines that promotes a Th1 response have been shown to directly affect the Th2 and Th17 arms of the immune response but we have found VEGF to influence only Th2 responses.

Recently, LPS and cholera toxin (CT) have gained importance as bacterial adjuvants used in vaccines. Monophosphoryl lipid A (MPLA), the modified low-toxic derivative of LPS is close to being approved to be used in human trials as a vaccine adjuvant and the cholera toxin B subunit has been successfully tested as a mucosal adjuvant in a novel method of “needle –free” vaccine delivery (261, 262). As these molecules gain prominence as adjuvants, it is critical to understand the different signaling axes they trigger which make them effective candidates in the field of vaccine engineering. A prior study has shown the importance of GM-1 ganglioside in

regulating the mucosal adjuvant property of CT via the NF- κ B pathway. It would be interesting to explore whether the loss of adjuvant capacity mediated by CT in the GM-1 ganglioside deficient mice could be the result of reduced VEGF secretion (263). Our study defines a role for VEGF in priming Th2 responses in vitro and the capacity to alter primed T cell responses.

Nitric oxide is an important second messenger in the cell and prior studies have shown the critical role it plays in both Th1 and Th2 responses. Mycobacterium and viruses that induce a Th1 response are known to activate macrophages via IFN- γ to release nitric oxide, which is essential for protection against the pathogen (264). On the other hand, there is also substantial evidence to support the role of nitric oxide in Th2 responses: iNOS deficient mice are susceptible to parasitic infections and nitric oxide acts as a negative regulator of IL-12 production in DCs and macrophages (265-267). The confounding role of nitric oxide in both Th1 and Th2 adaptive immunity suggests that a wide range of activators and inhibitors tightly regulate the expression of this second messenger. Also, the concentration of nitric oxide secreted could be key in determining the outcome of immune responses. In this study the role of nitric oxide in triggering VEGF production initially appeared to paradoxical since LPS-stimulated DCs were handicapped in inducing the production of VEGF despite high levels of nitric oxide secreted when compared to CT. The role of IL-12 in inhibiting the production of VEGF illustrates the inherent balance between Th1 and Th2 skewing cytokines that is maintained in DCs when exposed to antigens and IL-12 has a dominant role in suppressing Th2 skewing agents in DCs and thereby limiting the response itself. PI 3 kinase is a known negative regulator of IL-12 (200, 253) and in this study we show that it promotes VEGF secretion. This clearly illustrates the role for PI 3 kinase in regulating Th2 skewing.

NF- κ B activation is a central regulator in immunity and is known to have various targets. The transcription factors documented to be important in VEGF regulation are AP-1 and hif-1 α . Recent research has demonstrated that NF- κ B mediated signaling in hypoxic condition is independent of hif-1 α and that the activation of NF- κ B is critical for VEGF production (268-270). We have demonstrated the importance of NF- κ B in Th2 development rather than Th1 (91). In the context of cytokine regulation, NF- κ B is known to have a dominant effect in regulating various cytokines, and this regulation is extremely intricate due to cross talk between cytokines. In our study we see increased activation of the transcription factor upon treatment with CT and

LPS, and this implicates an important role for NF- κ B in regulating the production of VEGF via a nitric oxide dependent pathway.

We highlight the important role of VEGF secreted by DCs as a Th2 skewing agent rather than an immunosuppressive cytokine as previous studies have suggested. Our study focuses on the underlying molecular mechanisms of VEGF production mediated by the cooperative interaction between nitric oxide and NF- κ B and also underlines the negative inhibition mediated by IL-12. The findings in this study emphasize novel therapeutic approaches of inhibiting VEGF in asthma, cancer and also other Th2 dominated diseases.

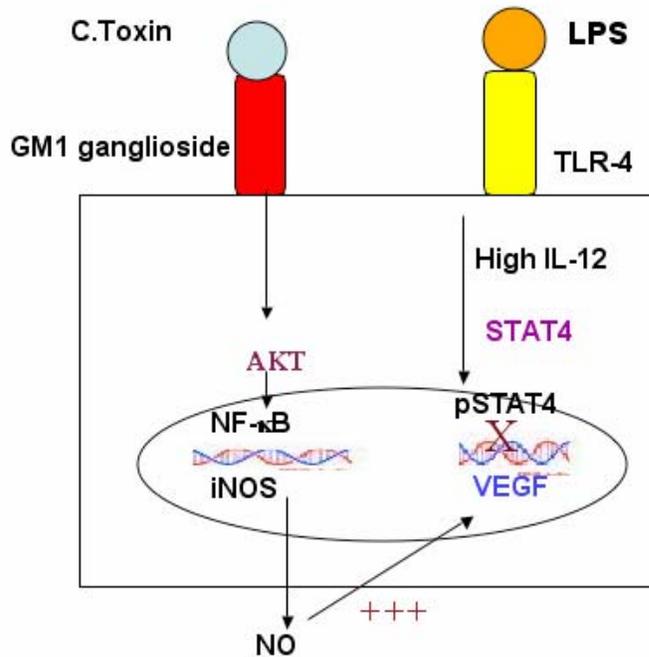


Figure 20: Cartoon depicting regulation of VEGF production.

Cholera toxin induces AKT activation triggering NF- κ B translocation to the nucleus. This results in NO production, which positively regulates VEGF(+++) production. LPS signaling through TLR-4 results in high IL-12 production resulting in activation of STAT-4, possibly regulating VEGF production negatively (X).

4.0 Conclusions and future directions

The seminal discovery of DCs by Steinman and Cohn marked an important milestone in immunology and has spawned a new field of research in innate immunity (271, 272). The papers documenting the discovery of this novel cell type was initially treated with much skepticism by immunologists. Some researchers dismissed the cell type as a mere cell culture artifact, and others were highly suspicious that these sparsely distributed cells among the populous macrophages in the spleen could have any significant biological function. Undeterred by the criticism, Steinman and his graduate student Michel Nussenzweig carried out elegant experiments defining the role for DCs as antigen presenting cells (273, 274). Wesley Van Voorhis, a leading immunologist commented, "Ralph was the lone voice in the desert. It took almost ten years for people to accept that DCs and their immune potency were for real. But history proved him right." History indeed proved Steinman right, for in 2007 he received the *Albert Lasker Award for Basic UnMedical Research* for his path-breaking discovery (275). Today, our understanding of the biology and functions of DCs has exploded and it is understood that every phenomenon mediated by the immune system is contributed, triggered or inhibited in some fashion by DCs. The ability to perform live-cell imaging and capture nanosecond events relating to antigen uptake and presentation by DCs has revolutionized our understanding of these basic DC function (276, 277).

Another significant finding that shaped the field of adaptive immunity was the divergence of CD4 T cells into two distinct lineages, Th1 and Th2 (278). The ability of DCs to induce the proliferation of CD4 T cells in co-culture prompted investigators to explore and identify novel factors in DCs that dictate this lineage -decision making choice in T cells. A surprising revelation from these studies was that the Th2 response is not merely a default pathway that occurred in the absence of a Th1, but is mechanistically intricate and influenced by several molecules. Our group has made significant contribution towards better understanding this CD4 T cell lineage. The discovery of GATA-3 as the molecular switch promoting Th2 responses and T-bet as the Th1-signature transcription molecule has been critical in furthering our understanding of molecular events that regulate development of these pathways (8, 39, 40). Furthermore, the emergence of Th17 as a novel CD4 T cell subset and the identification of its transcriptional

regulators, ROR- γ and ROR- α were critical in resolving prior findings that could not be explained within the realm of Th1 and Th2 subsets (50, 65, 66). A classic example is our improved understanding of EAE achieved by defining a role for Th17 in mediating the pathogenesis. Before the discovery of Th17, EAE was regarded as a Th1 immune disorder associated with high levels of IFN- γ secretion (206). However, the increased severity of the disease seen in IFN- γ and IL-12p35 knockout mice confounded investigators. Also, IL-6 knockout mice were not susceptible to EAE in spite of IL-6 being able to restrict the development of Th1 responses (52). These findings provoked investigators to think about the possibility of a novel CD4 T cell subset rather than dismissing the findings as compensatory mechanisms seen in knockout mice.

An important concept that emerged from these studies was that CD4 T cell pathways co-evolve during immune protection against pathogens, autoimmune disorders and allergic inflammation with one of the pathways becoming dominant eventually. The amelioration of EAE seen in mice treated with the cholesterol-lowering drug and also Th2- promoting agent statin shows the possibility of reversal or suppression of established T cells (279). Again, the molecular events regulating the crosstalk between CD4 T cells can explain these processes. The inhibition of GATA-3 by T-bet is mediated by phosphotyrosine kinase ITK, which physically interacts with T-bet and causes its phosphorylation. The phosphorylated form of T-bet sequesters GATA-3 from binding to the IL-5 promoter. This study confirmed the molecular mechanism behind the process (280). The discovery of ROR- γ and ROR- α as Th17-specific transcription factors has opened the doors to further understanding of molecular mechanisms that regulate CD4 T cell responses. It is interesting to note that molecular regulation is eventually dictated by chromatin remodeling by histone proteins that promote cytokine production.

Our group has conducted extensive research on Th2 regulation and mechanisms that promote allergic inflammation in the lung. The discovery of GATA-3 as a Th-2 regulator and the amelioration of airway inflammation seen in GATA-3 conditional knockout mice have been important in better understanding asthma (88). The spontaneous development of asthma in T-bet knockout mice emphasizes the immunological importance of regulation between T cell subsets (281). Although asthma is a Th2 driven response, the presence of IFN- γ and IL-17 during the peak of inflammation strongly suggests that these cytokines reduce the severity of the disease by constraining the Th2 response. Importantly, the progression of allergic inflammation into full-

blown asthma is not due to absence or reduction of Tregs in the lungs, but rather an impairment in their suppressive activity (282). This suggests that the dynamics and kinetics of CD4 T cell subsets vary greatly and depend on the nature of the immune response. Understanding the kinetics and dynamics of CD4 subsets was fundamental in the interpretation of several epidemiological studies that helped understand the different pathogenic infections and immune disorders.

For example, the increased incidence of asthma seen in westernized countries, partly due to better hygiene conditions, emphasizes the need to understand the regulation between the different subsets of T cells. Strachan proposed the hygiene hypothesis in 1989 upon discovering a strong inverse correlation between incidences of hay fever and the number of siblings in a family. This led him to postulate that early childhood infections triggered by unhygienic conditions present in larger families offered protection against allergic diseases. This hypothesis received support from other epidemiological studies that showed neonatal exposure to endotoxin from dirty mattresses or by growing up on a farm mitigated the chance of acquiring asthma in adulthood. The hygiene hypothesis functioned within the paradigm of Th1/Th2 responses; acquiring Th1 responses during childhood blunted Th2 responses in adulthood. However, studies also showed infections with parasitic worms offered protection against allergic disorder. These studies strained the validity of hygiene hypothesis since Th2 responses triggered by helminthes are expected to exacerbate allergic disorders and so cannot be protective. The missing piece of this puzzle was Tregs. The hygiene hypothesis is not a mere postulate regarding Th1/Th2 balance, but delves into the core of an immune response, which involves the development of Tregs to restrain the inflammatory response during pathogenic infections and autoimmune disorders. The development of Tregs upon exposure to Th1 or Th2 agents can also be protective during allergic reactions (283).

The mucosal surfaces of the lung and the gut are constantly subjected to antigenic provocation and the balance between inflammation and tolerance is a tightrope walk performed by the immune system. This has led Inflammation to be framed as the double-edged sword of immune responses. The importance of DCs in orchestrating inflammatory pathways in various diseases has been recently highlighted by several studies. An important recurring theme emerging from these studies is that alteration of pathways in DCs is sufficient to exacerbate or ameliorate inflammatory diseases. A classic example is the novel finding that DCs play an

important role in inflammatory bowel disease. T-bet, the Th1 transcription factor expressed on T cells, was found to be sufficient for driving experimental colitis (284). Recently, a novel role for T-bet in DCs has also been discovered. The expression of T-bet in DCs promotes IFN- γ production and enhances Th1 response by this positive feed back loop (285, 286). T-bet is also an important regulator of TNF- α production and its loss in DCs results in increased TNF- α production, which leads to experimental colitis (287). Impairment of PI3 kinase similarly results in defective suppressive function in Tregs, which promotes inflammatory bowel disease (IBD) (288). However, careful evaluation of the innate component of P110 δ mutant mice has revealed a defect in DC –mediated regulation of IL-12 production. Absence of the catalytic subunit of PI3 kinase triggers excess IL-12p70 production from DCs promoting the pathogenesis of IBD (JK Uno, K Rao, K Matsuoka, F Li, R Santor and SE Plevy; University of North Carolina at Chapel Hill; Altered innate immunity contributes to the development of colitis in the PI3K p110delta mutant mice; AAI 2008)

In allergic inflammation mediated by an overwhelming Th2 response, DCs have gained a prominent role in orchestrating the immune response. The importance of DCs in asthma has become indisputable and they have become known as fulfilling the “*Koch postulates*” of asthma; removal of DCs completely abrogates allergic inflammation and experiments from adoptive transfer experiments suggest that DCs alone are sufficient to induce all the hallmarks of asthma (104, 289). These findings further emphasize the importance of the two novel pathways discussed in this dissertation: c-kit/SCF and VEGF had previously been thought to have very different roles in asthma and have not been given due credit for regulating the function of DCs.

Our study focuses on a regulatory balance promoted by DCs that influences regulation of different T cell subsets and is critical in modulating allergic inflammation. Upregulation of c-kit and its ligand, SCF, in DCs promotes a signaling axis that specifically regulates priming and effector Th2 and Th17 responses via the PI3 kinase pathway and notch pathway. The defective priming of Th2 and Th17 responses seen in c-kit mutant DCs and inhibition of Th2 priming upon addition of VEGF inhibitor focuses on the initiating events of T cell priming triggered by DCs. The balance of this T cell response mediated by c-kit and VEGF signaling in DCs might have implication in other non-lymphoid organs like the brain and skin, which have a rich population of DCs and an upset in the immune balance triggers autoimmune diseases like multiple sclerosis and psoriasis.

As mentioned, a regulatory role for PI3 kinase in DCs is critical in maintaining gut homeostasis and in preventing unnecessary IL-12 production in response to normal gut flora. Interestingly, our study and other groups show a similar function for the molecule in maintaining the lung homeostasis. Lung DCs secrete higher levels of Th2 cytokines like IL-6 and VEGF as compared to the spleen, and this promotes a Th2-milieu in the lung (207). This Th2 environment is critical for protection against innocuous bacterial and viral antigens that can trigger a strong Th1 response. Mice deficient in PI3 kinase have reduced airway inflammation upon allergen sensitization (232). These studies illustrate the role of PI 3 kinase as a molecular switch in restricting Th1 and promoting Th2 responses. Activation of c-kit is critical in triggering this molecular switch. The ability of PI3 kinase to promote VEGF production further strengthens its importance as the converging molecule for promoting Th2 responses.

Furthermore, our study also illustrates the importance of the notch pathway. c-kit acts as an initiating molecule in specifically promoting the expression of Jagged-2 ligand. It is interesting to note that recent studies have established the importance of delta-4 ligand in negatively regulating the production of VEGF and being important in restricting tumor growth. Furthermore, IL-12 is a known inducer of Delta-4 ligand on DCs and this reiterates the balance operated by Th1 and Th2 cytokines in immune responses (201). This balance also extends to other CD4 subsets. The Th17 response mediated by IL-6 and TGF- β directs us to the crosstalk between regulatory T cells and Th17, since the phenotype and function of Tregs is greatly influenced by these two cytokines. Decreased IL-6 production in c-kit mutant mice compromises the Th17 response to allergens and intracellular pathogens. It is also possible that reduction in IL-6 levels can alter suppressive function of Tregs. We are unaware of any study investigating the alteration of function in Tregs in c-kit mutant mice that may be important in influencing the path of allergic inflammation and host protection in pathogens.

Dr. Elias' team and our group have shown that VEGF- mediated signaling specifically influences only the Th2 arm of the immune (194). Our study identified and characterized the important regulators of VEGF in DCs. An important area for future research remains to understand the molecular and immune mechanisms underlying VEGF receptor signaling in the lung. It is possible that expression of VEGF between allergic inflammation and tolerance as well as the expression of soluble vs. membrane-bound VEGF receptor are both altered. The

antagonistic function of the soluble receptor in regulating VEGF production suggests that a balance in receptor expression could also be key in maintaining lung homeostasis.

Our study has attempted to further understand the labyrinthine nature of the cytokine network that is tightly regulated by receptors and signal transducers. The discovery of Natural killer DCs a year before Dr. Steinman received the Lasker award suggests that we have traversed a full circle in our understanding of DC biology. Natural killer DCs share common features with NK cells and DCs and were labeled as “platypus” due to their hybrid nature. These hybrid cells initially triggered controversy with several experts questioning their existence. However, due to rapid strides taken by DC biology in, Zitvogel and Housseau have been able to convince innate immunologists that the novel ‘platypus’ cell type had come to stay (162, 290, 291).

5.0 Publications

Activation of c-Kit in dendritic cells regulates T helper cell differentiation and allergic asthma

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Distinct responses of lung and spleen dendritic cells to the TLR9 agonist CpG oligodeoxynucleotide.

Li chen, Meenakshi Arora, Manohar Yarlalagadda, Timothy B Oriss, Nandini Krishnamoorthy, Anuradha Ray & Prabir Ray.

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