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Mechanisms regulating dysfunctional T cell responses in large vessel vasculitis

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Mechanisms regulating dysfunctional T cell responses in large vessel vasculitis

By

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B.S., Spelman College, 2003

Advisor: Cornelia M. Weyand, MD,PhD

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Abstract

Mechanisms regulating dysfunctional T cell responses in large vessel vasculitis
By Kisha A. D. Piggott

Giant cell arteritis (GCA), the most common vasculitis in the Western world, is an inflammatory vasculopathy that preferentially targets the aorta and its extracranial branches. Immune-mediated tissue injury of arteries supplying the head, neck and upper extremities leads to severe and life-threatening complications such as blindness, stroke, and aortic aneurysm. Vasculitic lesions are transmural granulomatous infiltrates composed of CD4 T cells and highly activated macrophages. Immune-mediated damage of the vessel wall results in intimal hyperplasia, luminal occlusion and tissue ischemia. The immune response in the vessel wall is initiated by a tissue-resident population of vascular dendritic cells (vDC). CD4 T cells are key regulators of this response and differentiate into vasculitic T cells after being instructed by vascular DC. Pathogenic T-cell responses are guided and shaped by the unique environment of the blood vessel wall, but the distinctive pathways driving DC-T cell interactions are yet to be fully elucidated. An exploration of unique accessory receptor-ligand pairs assigned a critical role to the Notch-Notch ligand pathway in the initiation and sustenance of large vessel vasculitis. Notch signaling components are elevated in peripheral blood of GCA patients and in tissue biopsies of affected temporal arteries. Notch signaling inhibition resulted in repressed cellular responses, decreasing CD4 T cell activation markers and T-cell proliferation. Both IL-17-producing and IFN-γ-producing T cells are expanded in GCA patients, suggesting that disease-relevant T cells originate from two distinct functional lineages. The in vivo relevance of Notch-dependent T cell responses in vascular inflammation was tested in a humanized mouse model in which human arteries are engrafted into immuno-deficient mice and human T cells are adoptively transferred. Blocking of the Notch pathway through the γ-secretase inhibitor *N*-[*N*-(3,5-difluorophenacetyl)-l-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT) led to a significant decrease in T-cell recruitment and formation of vascular wall infiltrates. Disruption of Notch signaling inhibited in situ T-cell activation, differentially downregulating IFN-γ and IL-17 production. T cells secreting the pro-inflammatory cytokine IL-17 appeared more dependent on Notch signaling than those releasing IFN-γ. In essence, disrupting Notch activation has profound immunosuppressive effects, indicating that Notch pathway modulation may provide novel therapeutic opportunities for large vessel vasculitis.

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Foreword

In this thesis, unless otherwise mentioned below, all of the experiments were performed and reagents were prepared by the author. Experimental and procedural contributions made by others include:

- Proliferation assays and flow cytometry in Chapter 2 conducted in part by Stefan Groschel.
- Western blot in Chapter 2 performed by Karnail Singh.

Chapter 1

Introduction

Giant Cell Arteritis and the Human artery-SCID mouse Model of Vasculitis

Giant cell arteritis (GCA), also known as temporal arteritis, is a granulomatous vasculitis that primarily affects medium and large-sized vessels (1). This inflammatory vasculopathy preferentially targets extracranial branches of the aorta with manifestations in the head, neck and upper extremities leading to severe and life-threatening complications such as blindness, stroke, aortic arch syndrome and aortic aneurysm (2). Several risk factors have been correlated with increased susceptibility of disease and include age, gender and genetic predisposition. Thus, GCA more frequently afflicts females, persons older than 50 years of age and the strongest genetic risk is conferred by the HLA-DR4 haplotype (1).

Studies have revealed that GCA is mediated by a maladaptive immune response which is characterized by in situ activation of CD4 T cells for which the vasa vasorum of the adventitial layer appears to provide a physiologic port of entry. Local activation of T cells results in IFN-γ production (3), a cytokine that regulates the differentiation of the tissue-infiltrating macrophages that contribute to the tissue injury via production of pro-inflammatory cytokines, growth factors, reactive oxygen intermediates (ROIs) and matrix metalloproteinases (MMPs) (4). The resulting cell recruitment, neoangiogenesis and concentric intimal hyperplasia give rise to luminal occlusion and, consequently, to tissue ischemia that accounts for the clinical symptoms that plague GCA patients (Fig 1.1). The clinical spectrum of GCA reflects the selective tissue tropism and includes symptoms such as impaired vision, jaw pain and headache (5). The vasculitis is almost often associated with intense systemic inflammation manifesting with fever, malaise, anorexia and depression. Diagnosis is established by temporal artery biopsy, which typically

shows multinucleated giant cells, thinning of elastic membranes and an obscured vascular lumen.

Much of the progress made thus far in elucidating the pathogenic mechanisms driving early and late events in GCA derives from studies utilizing a human artery-SCID mouse chimera model. The model was developed in order to overcome limitations presented by animal models of vasculitis in which recapitulation of the pan-arteritic lesions of GCA were restricted by small vessel size and vessel wall architecture of the animals. This novel model was established to mimic vascular inflammation via implantation of normal or GCA-affected human arteries into immunodeficient mice. In this model engraftment of the human arteries over a 7 day period is supported by the supply of blood to the graft via the vasa vasorum (6). The integration of the murine and human vascular networks allows for the introduction and delivery of small molecules into the chimera that can be used in the study of the mechanisms of disease induction and maintenance, as well as in the investigation of potential treatment options for the induced vasculitis.

GCA is a T-cell mediated disease involving dysfunctional T-memory immune responses that are guided and shaped by the unique environement of the blood vessel wall. In adaptive immunity T cell responses are ultimately driven by three key factors: 1) the strength of the Ag:TCR signal, 2) antigen:T cell receptor accessory signals derived from the antigen-presenting cell, and 3) the microenvironment. Existing and unfolding data provides supporting evidence that the evolution of disease in GCA is characterized by a sequence of events that incorporates all three components of a typical adaptive response.

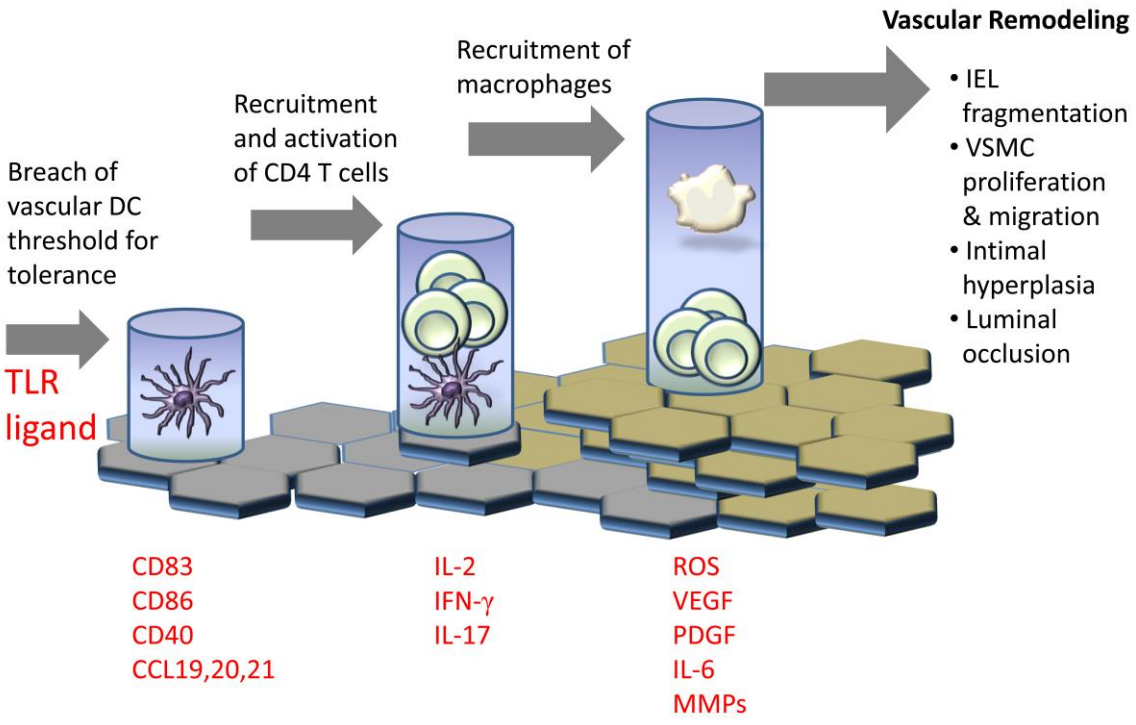

Figure 1.1. Innate and adaptive immune responses lead to vascular damage in GCA. Tissue injury of the vascular wall in GCA is the cumulative effect of a cascade of immune events that shows a mounting stepwise progression eventually leading to remodeling of the wall, intimal hyperplasia and luminal stenosis.

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I. Cellular Immune Response

Unique adaptive immune responses in large vessel vasculitis

Phenotypic studies have shown that Th1 cells are the dominant T-cell type in the vasculitic lesions of GCA (7). Comparison of T-cell populations accumulated in the right and left temporal arteries of patients with GCA has demonstrated identical T-cell receptors (8), a finding that strongly supports the paradigm that antigen is the driving factor (9). The identity of this antigen remains elusive. Immunophenotypic analysis has provided evidence supporting these earlier studies via a comparison of the T-cell receptor (TCR) usage in peripheral blood lymphocytes with that of the lesion infiltrates in GCA patients at the time of diagnosis (10). Disparities in the TCR V gene expansions in peripheral blood lymphocytes and tissue lymphocytes point to the possibility of a selective T-cell recruitment process or local expansion of T cells *in situ*. Non-randomness in the selection of T cells strongly supports a role for antigen.

The peripheral T-cell pool is assembled from a number of different subsets, including naive and memory T cells. Memory T cells dominate the vasculitic infiltrates in GCA. However, multiple functional subpopulations contribute to the memory T-cell compartment. A recent study has analyzed whether vasculitic T cells originate from a particular subset or are representative of all memory T cells (11). Experimental data support the concept that chemokine receptor 6 (CCR6)-expressing T cells are particularly effective at invading the wall layers and survive in the wall structure (Figure 1.2). CCR6 is expressed on several T-cell subsets and in particular has been shown to be an important

functional marker for Th17 cells, a novel CD4 T-cell subset that has been implicated in the pathogenesis of autoimmune disease (12).

Indeed, CCR6-deficient T-cell subsets have been shown to diminish susceptibility to autoimmune diseases such as rheumatoid arthritis and multiple sclerosis (13), partially due to the demonstrated role of CCR6 in directing the targeted migration of these subsets into inflammatory sites. To investigate the role of $CCR6^+$ T cells in GCA, the authors made use of an experimental model in which normal human arteries are engrafted into SCID mice. Infusion of human T cells into such chimeras results in vessel wall inflammation if the arterial DC are appropriately conditioned. In this study, two distinct conditioning regimes were compared. Wall-embedded DC were either triggered with ligands for toll-like receptor (TLR) 4 (LPS) or with ligands for TLR5 (flagellin). Pretreatment with TLR4 ligands resulted in a typical transmural vasculitis with T cells clustering in the adventitia and infiltrating deep into the media (11).

Conversely, pretreatment with a TLR5 agonist led to a perivasculitic pattern of inflammation in which T cells accumulated in the adventitia but failed to invade into the media. To answer the question of whether different T-cell populations could contribute to vasculitis, markers of T-cell recruitment and activation were compared. T-cell activation as quantified by IFN-γ was similarly induced in arteries with transmural and perivascular infiltrates. However, T cells migrating deep into the vessel wall were markedly enriched for $CCR6^+$ T cells. Subsequent experiments showed preferential recruitment of $CCR6^+$ T cells into the media in LPS-treated human artery-SCID chimeras, an invasive property that was abrogated following depletion of the $CCR6^+$ cells or upon blockade with an

antibody specific for the CCR6 receptor. This skewing of the T-cell responsive phenotype was shown to be driven by a differential and enhanced production of CCL20, the unique ligand for the CCR6 receptor (14), by TLR4-triggered DC, a response which was not mirrored by flagellin-stimulated DC.

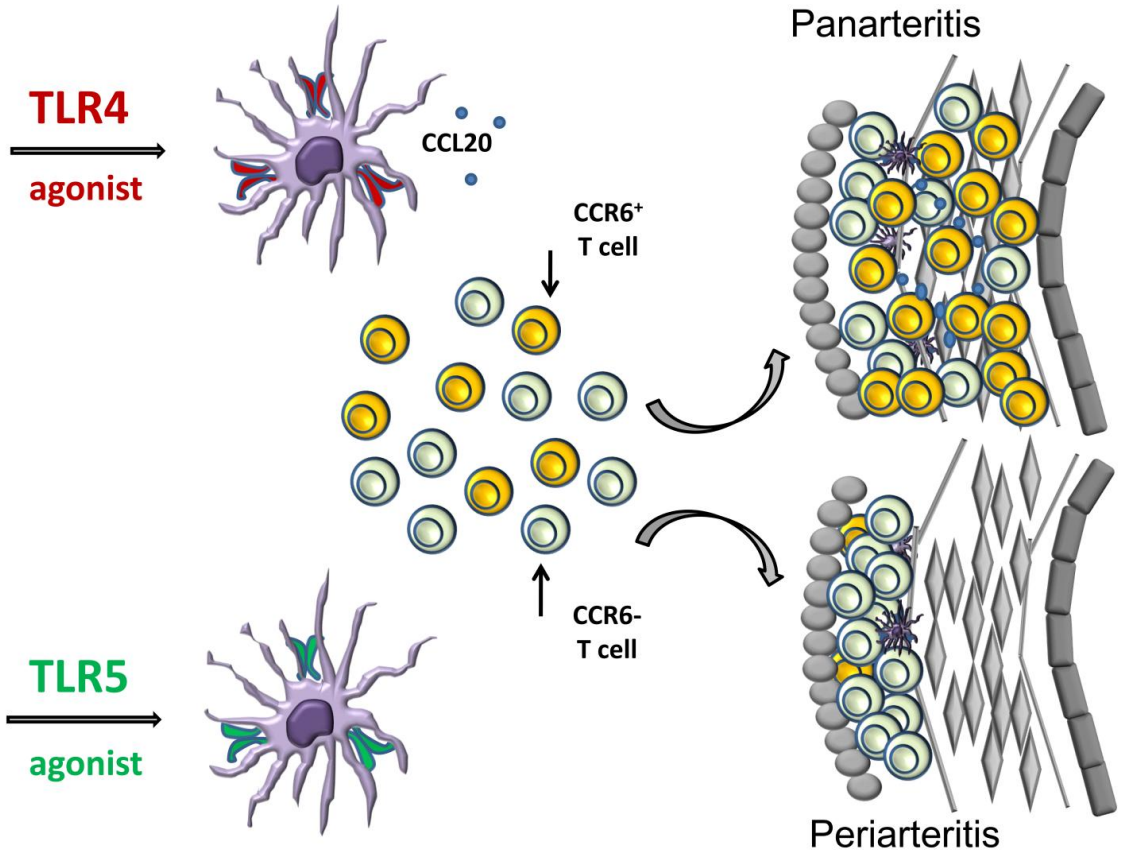

Figure 1.2. Vascular DC shape the arrangement and composition of T cell responses in the arterial wall. Triggering of wall-embedded DC by different TLR ligands results in distinct architectures of vessel wall inflammation. TLR4 ligands induce the release of CCL20, the preferred recruitment of $CCR6^+$ T cells and the establishment of wall-penetrating inflammation. TLR5 agonists facilitate T cell recruitment with T cells clustering in the adventitia, assembling a perivascular infiltrate.

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Antigen presentation in the vessel wall

While the tissue-damaging inflammation in GCA is ultimately maintained by adaptive immunity (Fig 1.1) innate immune responses are instrumental in bringing immune cells to the artery (15). Hierarchical studies have positioned the vascular DC at the helm of the immunological apparatus, navigating the inflammatory response in diseased vessels (16, 17). In normal arteries, the gatekeeper function of the vascular DC facilitates a commensal relationship with the vessel via the mitigation of potentially destructive immune responses. Under physiological conditions, the vessel wall is an immunoprivileged site, with local DC actively guarding this privilege. In experimental systems, and likely also in the patient, this privilege can be breached by innate stimuli that override the tolerogenic mechanisms of the DC (6). This breakdown in self-tolerance leads to the propagation of a chronic inflammatory response distinguished by CD4 T-cell influx, formation of granulomatous infiltrates, neovascularization, intimal hyperplasia and luminal occlusion (1).

The localization of DC in peripheral tissues is widespread but not ubiquitous, and they are strategically located in certain tissues that include skin, the gut and the respiratory mucosa, all of which have immediate and frontline access to pathogens in the external environment (18). Their presence in artery walls, therefore, is novel. Early evidence that arteries may contain professional antigen-presenting cell (DC) came from histological studies identifying an $S100^{+}$ cell population in human aortic and carotid arteries (19). Initially, these DC were associated with atherosclerotic disease, and it was not clear whether they were indigenous residents or responded to early tissue injury. Architectural analysis of the healthy temporal artery identified an indigenous resting

population of DC localized at the media-adventitia border in the vessel wall. Further characterization studies showed that in temporal arteries of GCA patients this resting, immature population of DC is replaced by an activated, mature one that begins to infiltrate the media (6). Triggering of TLR4 with its cognate ligand, lipopolysaccharide (LPS) (20), is a well known and potent maturation stimulus for DC and *in vivo* studies utilizing a human-severe combined immunodeficiency (SCID) mouse chimera have shown that TLR4 stimulation is sufficient to trigger a chain of events leading to recapitulation of the key features of vasculopathy observed in GCA. *In situ* triggering of TLR4, which is found on the resident population of DC in the vessel wall, induces DC differentiation, with the upregulation of MHC, CD83 and CD86, which in turn renders them capable of stimulating the T cells that mediate disease.

Besides DC, monocytes and macrophages are known to have antigen-presenting and immunostimulatory functions. These cells are typically excluded from the healthy vessel wall, but are present in the inflammatory lesions of GCA (21). It stands to reason then that these innate immune cells may also play key roles in driving T-cell inflammatory responses. The capacity of these cells to mediate development of T-cell effector functions has been addressed utilizing bioengineered human arteries and was compared to that of DC by selective reconstitution of bioarteries with each of these three DC populations (17).

Remarkably, TLR4 stimulation established a hierarchical response pattern in which the DC emerged as the master stimulator, proving to be the most effective T-cell recruiter and showing the greatest upregulation of CD83, CD86 and CD40L. Conversely,

while the macrophages were able to elicit T-cell recruitment, they failed to provide the necessary signals to promote T-cell activation. Monocytes neither recruited nor stimulated T cells. These studies conducted within the three-dimensional space of the bioengineered artery support earlier studies describing the absolute requirement for DC in the mediation of disease via the sensing of microbial patterns and reaffirmed the role of the DC as the major DC in vessel wall inflammation. It is currently unknown why only DC are capable of serving as DC. This may be a direct reflection of the conditions encountered in the microspace of the adventitial, the medial and the intimal layers.

Toll-like receptors: bridging the gap between innate and adaptive immunity in the artery

The classic paradigm for DC maturation describes a population that resides in peripheral tissues as immature cells that have a characteristically high capacity for antigen capture via macropinocytosis, receptor-mediated endocytosis and phagocytosis (22). These cells express low levels of histocompatibility complex (MHC) and costimulatory molecules, and antigen-induced maturation drives the phenotypic and functional changes that endow the DC with the proficiency to bridge innate and adaptive immune responses (23, 24). These events describe a typical antigenic response that is instigated by pathogen sensing and culminates in the initiation of an orchestrated inflammatory response (25). One of the most well studied mechanisms for modulation of dendritic cell function is via the toll-like receptor (TLR) expressed by these cells (26). This family of pattern recognition receptors comprises at least 10 known receptors

characterized by extracellular leucine-rich repeats and an intracellular Toll/ IL-1 receptor homology (TIR) domain (27). Signaling via the MyD88 pathway ultimately leads to upregulation of NFκB transcription and a consequent increase in the production of inflammatory cytokines and costimulatory molecules (28).

Notably the indigenous DC integrated into the vessel wall are typically quiescent, which may be part of their tolerogenic capacity (16). Losing the immature state is now considered an enabling step in the early stages of vessel wall inflammation, and once vasculitic infiltrates are established, DC continue to express a highly activated phenotype, releasing chemokines and cytokines (6). In studies where wall-residing DC are depleted, the disease process is disrupted. Antibody mediated depletion of the indigenous DC population successfully abrogated the disease process (6). It seems that the integrity of the vessel and its protection from inflammation is ultimately regulated by the innate immune system. Utilizing TLR, vascular DC serve as sentinels and monitor events occurring in the walls of macroarteries. The family of TLR confers dual functionality on DC by providing the thermostat that facilitates sensing of their surroundings and either allowing for the consequent maintenance of equilibrium under tolerizing conditions or initiating an inflammatory cascade.

Among the major distinguishing features of the inflammatory vasculopathies is their distinct predilection for the colonization of unique vascular territories. GCA is no different in this regard and selectively targets extracranial branches of the aorta with manifestations in the head, neck and upper extremities (7). These observations led to the recent investigation and characterization of six human vascular beds in order to gain

insight into the tissue tropisms of these inflammatory diseases (21). While TLR are frequently expressed on DC, they are also found on monocytes, macrophages, and B cells.

The results of these profile studies, however, indicated that the functional TLR in the vascular tissues are essentially restricted to the $CD11c^{+}$ wall-residing cells. All arteries examined abundantly expressed transcripts for most of the nine TLR tested. All arteries contained sequences specific for TLR 2 and 4, and overall TLR-binding bacterial products were favored. The most surprising finding was that the combination of TLR assigned a unique fingerprint to each of the six different vascular regions. The DC also showed differential localization, as an adventitial network was detected in all six arteries, while a second intimal DC network was identified only in the two largest arteries, the carotid and the aorta. The distinct TLR profiles not only conferred unique immunologic identities to each of the vascular beds, but also correlated with their functional heterogeneity as demonstrated by differential responses to stimulation with cognate ligands for TLR 4 and 5 in arteries displaying disparate profiles for these two receptors.

II. Accessory signaling

The evolution of the immune system allows for broad and comprehensive recognition of infiltrating pathogens providing ample protection against most potentially harmful elements. This defense system is further refined by the implementation of mechanisms that facilitate the self-non-self discrimination, a process that is intrinsically

imperfect with implications for pathology that include allergies and autoimmune diseases such as GCA. In the battle of discrimination of self from non-self the outcome is ultimately driven by the co-signals provided to the T cell. The two-signal hypothesis of lymphocyte activation provides a platform for better understanding the potential for the divergent outcomes of T cell activation vs. tolerance (29). This model describes an antigen-specific response whose ultimate success is driven not only by the recognition of a specific antigen (signal 1), but equally importantly also by the context within which antigen presentation occurs (signal 2). The delivery of signal 1 occurs via the interaction of the T cell receptor with a cognate major histocompatibility/peptide complex, while signal 2 is provided by cell surface co-signaling molecules expressed by antigen presenting cells (DC). Emerging studies in the past decade have highlighted signal 2 as the primary component that shapes the response mounted by the T cell. The "two-signal model", however, belies the complexity of the immune response generated, as multiple signals, both positive and negative, may be received by the responding T cell. Indeed, it is the coincident delivery and the integration of signals generated by co-stimulatory and co-inhibitory molecules that function to enhance or attenuate immune responses (30, 31). Thus, the balance of stimulatory and inhibitory signals is paramount to optimizing the generation of protective immune responses, while ensuring the maintenance of self-tolerance.

The expression of accessory molecules by the T cell facilitates the generation of a breadth of responses generated by the processing of the DC derived signals received via the T cell receptor, and from co-signaling molecules such as those of the B7-CD28 family. Ongoing studies continue to expand this pool of co-signaling molecules and the

recent implication of the Notch pathway, long appreciated for its role in the regulation of T cell fate and hematopoesis, in the regulation of peripheral immune responses poises it as a candidate for inclusion in this category.

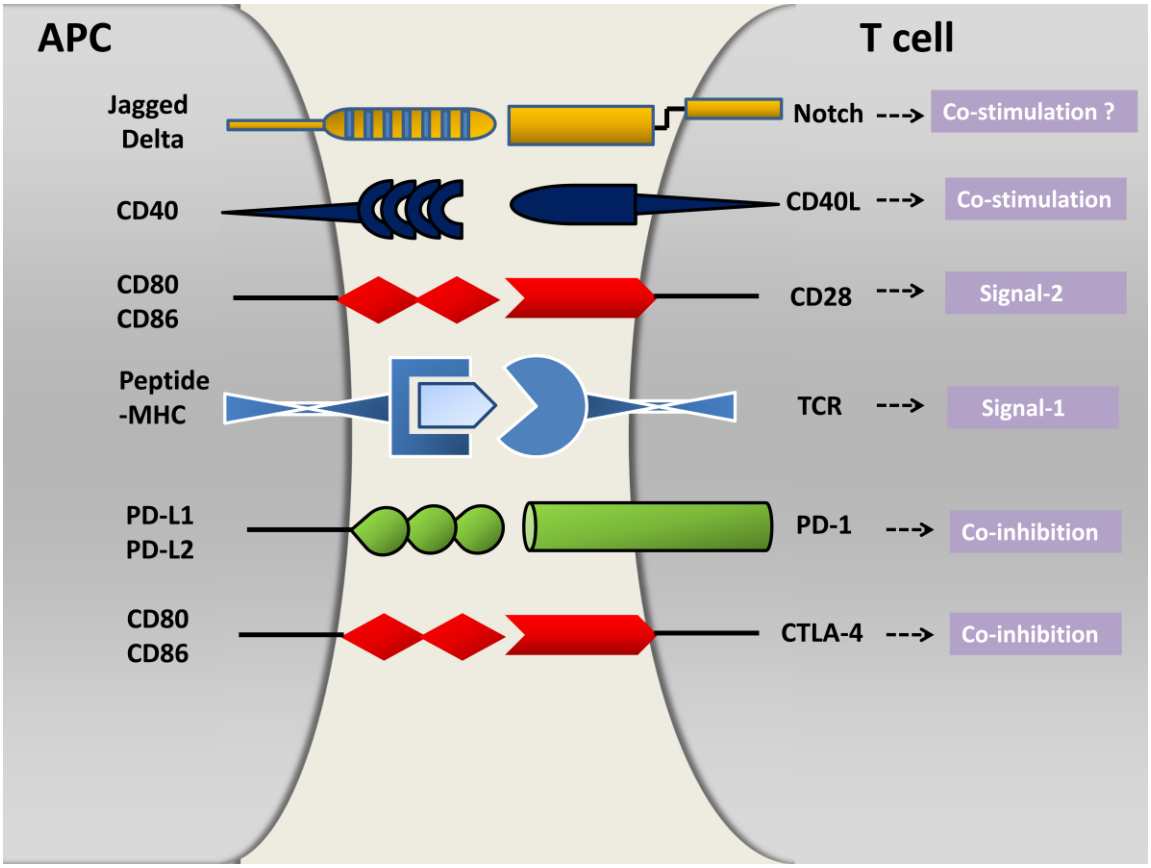

Figure 1.3. Co-signaling overview. Optimal T cell activation requires at least 2 signals. Signal 1 is mediated by the TCR complex which recognizes antigenic peptides presented by the MHC complex. Signal 2 is initiated by CD80/86 binding to CD28 which delivers a co-stimulatory signal. Additional co-signaling molecules can modulate the outcome of an immune response which ultimately depends on a balance of co-stimulatory and co-inhibitory signals.

B7-CD28 Signaling Pathway

One of the most well characterized T cell co-stimulatory pathways is the B7-CD28 superfamily, which includes the classical B7-1/B7-2-CD28/CTLA4 and the more recently delineated PD-1 –PD-L1/PD-L2 pathways (32). The B7-1/B7-2-CD28/CTLA4 pathway has been the most extensively studied co-stimulatory pathway. B7-1(CD80) and B7-2(CD86) have dual specificity for the constitutively expressed CD28 and the activation induced CTLA-4 receptors (33, 34). Signals transmitted via the stimulatory CD28 receptor synergize with the TCR signal to enhance T-cell activation largely through its modulating effect on the T cell threshold required for activation (35). The B7-1 and B7-2 ligands thus play significant roles in the initiation and maintenance of effective T cell responses through their engagement of CD28. The key contribution of these ligands to T cell stimulation does not, however, preclude their ability to generate inhibitory T cell responses. The ligation of the inhibitory receptor CLTA-4 by B7-1 and B7-2 provides a negative signal which actively suppresses TCR and CD28 mediated signals (36-38). The greater affinity of CTLA-4 for the B7 ligands underscores the importance of implementing self-restricting mechanisms on ongoing stimulatory T cell responses (37, 38). This superfamily of co-stimulatory molecules also includes the more recently delineated PD-1 –PD-L1/PD-L2 pathway (39). T cell expression of the receptor "programmed cell death 1" (PD-1) is induced by TCR signaling. This cell surface monomer delivers an inhibitory signal following simultaneous engagement by its cognate ligands PD-L1/PD-L2 coupled with TCR ligation (40). The cytoplasmic domain of this inhibitory receptor contains two tyrosine-based signaling motifs, an immunoreceptor tyrosine based inhibition motif (ITIM) and an immunoreceptor tyrosine based switch

motif ITSM. Phosphorylation at the tyrosine within the ITSM results in the recruitment of phosphatases such as SHP-2, which in turn dephosphorylate signaling molecules activated by TCR signaling (41). The combined effects of dephosphorylation and reduced cytokine mRNA synthesis results in modulation of the threshold for T cell activation and an overall inhibition of the T cell response. The inducible ligands are expressed on antigen presenting cells that include the dendritic cell, and are also upregulated on T cells upon TCR stimulation (39). The kinetics of ligand expression provides strong support for the model which describes PD-1 as an inhibitor in the execution of function during the effector phase of T cells.

Notch Signaling Pathway

Notch signaling is a highly conserved pathway and in mammals 4 Notch receptors (Notch1-4) and five Notch ligands (Jagged1 and 2, Delta1, 3 and 4) have been identified thus far (42). Notch encodes a single pass transmembrane receptor that is cleaved within the Golgi by a furin-like convertase, subsequent to which it is transported to the cell surface where it is expressed as a heterodimer. The heterodimeric complex is comprised of several conserved extracellular and intracellular domains (43). Extracellular Notch contains epidermal growth factor-like repeats that are responsible for ligand binding (44). There are 36 such EGF-like repeats in drosophila Notch, Notch1 and Notch 2; 34 in Notch 3 and 29 in Notch 4. More proximally, the receptor contains 3 cysteine rich Notch/LIN12 repeats that impede ligand-independent signaling. Intracellular Notch is the

signal transducing component of the receptor, a role that is facilitated by several functional domains. These include 2 protein-protein interaction domains: the RAM domain, and six ankyrin repeats, both of which interact with downstream effector proteins. The cytoplasmic portion also contains two nuclear localization sequences which direct the cleaved intracellular domain to the nucleus for downstream signaling. Notch 1 and 2 contain a transactivation domain that is absent in Notch 3 and 4, while all 4 receptors express a C-terminal PEST (proline, gluatamine, serine, threonine) sequence that is responsible for regulation of the stability of the receptor (45). Initial studies of Notch signaling first described the Notch receptor as a full length cell surface molecule. Extensive biochemical analyses however, have since demonstrated that Notch synthesis in the endoplasmic reticulum is followed by a furin-convertase-type cleavage at the S1 site in the trans Golgi network (46). The processing of the single polypeptide precursor yields an N-terminal fragment that constitutes the extracellular domain, and a C-terminal transmembrane fragement, which are subsequently reassembled at the plasma membrane into the mature heterodimeric receptor, held together by a calcium coordinated bond (47). The Notch cascade is initiated by regulated intramembranous proteolysis (RIP), a mechanism described in several physiological processes including amyloid beta peptide generation (48), in which transmembrane proteins are liberated from the cell membrane by proteolytic cleavage within the membrane (49). Ligand binding induces cleavage at the extracellular site S2, between alanine 1710 and valine 1711. The ligand-induced, extracellular domain shedding event is mediated by the ADAM protease TACE, resulting in the Notch Extracellular Truncation (NEXT) product. The generation of this proteolytic intermediate facilitates the subsequent intramembranous cleavage at the S3 site between

glycine 1743 and valine 1744, that leads to the cytoplasmic release of the intracellular domain (NICD) (50). The second cleavage of this dual address protein is catalyzed by the γ-secretase activity of a multiprotein complex consisting of presenelin and nicastrin (51). The latter cleavage event liberates the Notch intracellular domain (NICD) to translocate to the nucleus where it binds to the transcription factor CSL(CBF-1/RBP-Jκ in mammals, suppressor of Hairless in *Drosophila*, and Lag-1 in C. elegans) (52). Constitutively, CSL binds components of a corepressor complex (CoR) and thereby inhibits transcription. Upon heterodimerization with CSL, NICD displaces corepressors , recruits coactivators (CoA) and consequently initiates transcription resulting in the transcription of target genes that include Hes1, Hes5 and pre-Tα (53). Undoubtedly, there are other downstream target genes essential to each biological event regulated by this pathway that have yet to be elucidated.

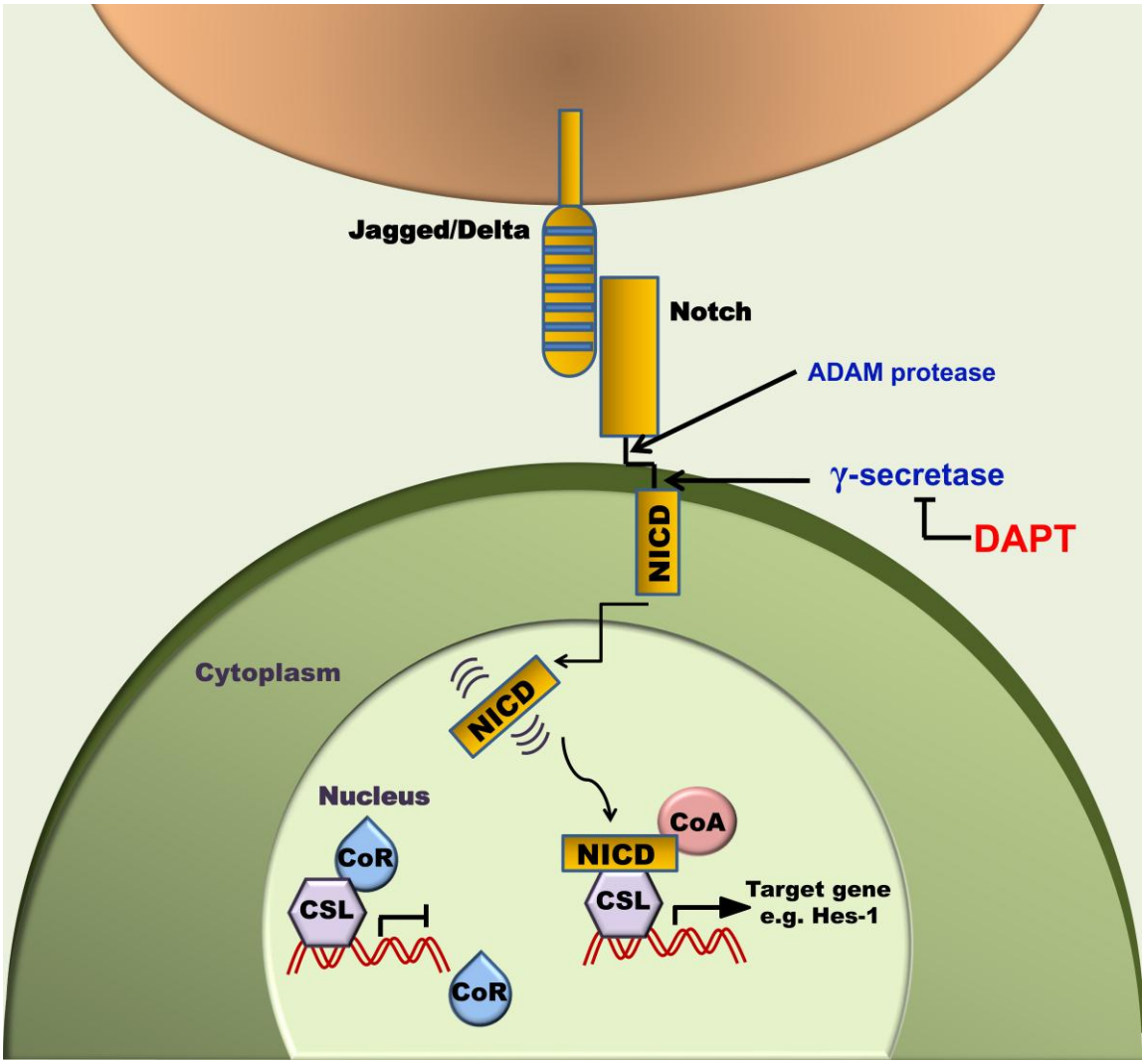

Fig 1.4. Notch signaling pathway. Notch receptor is expressed at the cell surface as a heterodimer. Notch signaling is initiated by Notch ligand binding which leads to two proteloytic cleavage processes. The first cleavage occurs external to the transmembrane domain and is mediated by the ADAM metalloprotease tumor-necrosis factor α-converting enzyme (TACE). The second intra-membranous cleavage is catalyzed by the γ-secretase. The liberated cytoplasmic domain (NICD) translocates to the nucleus where it competes with corepressors (CoR) for binding to CSL, recruits coactivators (CoA) and forms a transcription-activating complex.

Therapeutic potential of γ-secretase inhibitors

Gamma-secretase is a protease complex comprised of four integral membrane proteins. The main catalytic subunit, presenilin is a highly conserved multispan protein whose enodproteloytic activity is dependent on the accessory subunits, nicastrin, Aph-1, and Pen-2 (54). This conserved membrane protein complex was first identified as the catalytic driver of amyloid-β-protein generation from the amyloid-β precursor protein (APP), implicating the enzyme in the pathogenesis of Alzheimer's (55). Despite the discovery of APP as one of its first substrates, more in depth studies led to the characterization of γ-secretase as a proteasome that had primarily evolved to mediate the cleavage of type I membrane proteins such as Notch, assigning it a key role in development and differentiation. The known biological functions of γ-secretase have led to its investigation as a potential therapeutic target through paralysis of its activity utilizing γ-secretase inhibitors. One of the first inhibitors tested in vivo was *N*-[*N*-(3,5-Difluorophenacetyl)-l-alanyl]-*S*-phenylglycine *t*-butyl Ester (DAPT), developed by Elan and Eli Lilly, in which oral administration induced a dose-dependent decrease in cortical Aβ production in young APP transgenic mice (56). The efficacy of the inhibitor was later confirmed by a subsequent study in which subcutaneous DAPT administration led to a dose dependent decrease in both CSF and plasma Aβ levels (57). Although existing data support the use of γ-secretase inhibitors in AD therapeutics, the potential adverse effects from the interruption of Notch processing, has lead to the investigation of alternative Notch sparing inhibitors that include the non-steroidal anti-inflammatory drug (NSAID) γ-secretase modulator, R-flurbiprofen, which has already moved into Phase III clinical trials (58). The limitations for the use of γ-secretase inhibitors in AD however, do not

preclude their potential utility as agents of therapeutic intervention in other human diseases mediated by the γ-secretase complex. Indeed, it is precisely this role in Notch processing from which the potential advantages of their use derives, particularly as it pertains to certain cancers. Excessive Notch signaling has been shown to be a key mechanism in tumorigenesis (59). The first mammalian homolog of drosophila Notch was identified in T cell acute lymphoblastic leukemia (T-ALL), in which a gain of function mutation in Notch1 has been detected in more than 50% of patients (60), with a chromosomal translocation that results in a constitutively active form of Notch1 being found in 10 % of cases. Apart from T-ALL (61), aberrant Notch expression has also been implicated in other cancers that include lung, ovarian and breast cancer. Several γ-secretase inhibitors have been evaluated for anti-tumor effects (62-65) and studies using a mouse model of T-ALL showed that DAPT treatment caused cell cycle arrest and apoptosis in Notch dependent tumor cells (66). Other γ-secretase inhibitors have been investigated in cancer models and one inhibitor MK0752 developed by Merck has entered Phase I clinical trials for treatment of breast cancer and relapsed or refractory T-ALL (67). As is the case in AD, the possible accompanying side effects of immunouppression and GI cytotoxicity (68, 69)serve to underscore the importance of achieving a balance between efficacy and toxicity of γ-secretase inhibitors in cancer therapeutics.

Notch and peripheral T cells

Although the role of Notch is well defined in lymphocyte development (70, 71), and despite the fact that the expression pattern suggests a key function in peripheral T cells, little is known regarding its specific effects on peripheral immunity. Differential expression and regulation of Notch receptors and ligands extends into the peripheral immune system (72). Notch 1 and 2 appear to be constitutively expressed in murine naïve CD4 T cells while expression of all 4 receptors increases following antigen stimulation (73). Murine CD4 T cells have also been shown to express the Notch ligands Delta1, Jagged1 and 2, while Delta4, Jagged1 and 2 were found on murine DC. Studies of the endogenous expression patterns of Notch ligands in primary human CD4 T cells have detected Delta1, Jagged1 and 2 but not Delta3 or 4, similar to the ligand expression pattern observed in human DC (74).

In adaptive immunity T cell responses are ultimately driven by three key factors; the strength of the Ag:TCR signal, the microenvironment and DC-derived accessory signals. It is within this latter capacity that recent evidence indicates a contributory effect by the Notch pathway. Experimental data suggest cross-talk between the TCR and the Notch receptor (75). Specifically, Notch is required in TCR-mediated activation of peripheral T cells. Pharmacological Notch inhibition resulted in decreased Notch expression and simultaneously decreased T cell proliferation, CD69 expression, NFkB activity and IL-2 and IFN-γ production. In surface expression studies Notch 1 co-localized with CD4 on activated T cells (76). Pharmacological blockade attenuated CD4 T cell activation, decreasing TNF-α, IFN-γ, IL-4 and IL-10 secretion (77). Beside its function in TCR mediated activation, Notch signaling also regulates the cytokine

dependent survival of activated T cells as interference of Notch in activated T cells triggered ROS dependent, Fas-independent apoptosis (78). Notch^{+} cells showed resistance to apoptotic stressors. Cumulatively, Notch signaling positively regulates T cell activation and survival thereby amplifying adaptive immune response (79). Other studies have implicated Notch signaling in driving differentiation of naïve T cells towards a regulatory or tolerizing phenotype (80-83). Jagged-1 overexpression induced antigen specific regulatory T cells (Tregs) (43) that inhibited proliferative and cytotoxic T cell responses. Similarly, Jagged-1 overexpression by murine DC promoted Treg differentiation. Notch ligation by Delta-1 resulted in unresponsiveness to transplantation antigens, while constitutive expression of the Notch 3 ICD in transgenic mice enhanced Treg development with a correlate protection against experimental autoimmune diabetes (84). This direction of T cell fate by Notch ligands on DC has also been shown to contribute to CD4 T helper cell differentiation (85, 86). Differential expression of Notch ligands was observed in DC following exposure to Th1 vs. Th2-promoting stimuli providing evidence that the two ligand families Delta and Jagged alternately regulate T helper differentiation, promoting Th1 vs. Th2 development respectively (87).

III. Microenvironment

Arteries - a novel immunologic niche

Arteries serve a vital function as the conduit for blood, delivering oxygen and nutrients to all tissues. They have a critical role in regulating blood pressure. Life is not

sustainable without functional arteries and arterial disease, mostly in the form of atherosclerosis, is now the major cause of mortality in Western societies. However, recent data have emphasized that the contribution of arteries towards keeping the body functioning is even more essential than previously considered, and far exceeds blood transport and blood pressure regulation. In humans, medium-sized and large arteries are emerging as critical components of the body's defense system, redefining our understanding of how the arterial network interacts with the immune system and how arteries become a target of immune-mediated disease.

Besides building a barrier between circulating immune cells and the tissue space, a function mainly achieved by endothelial cells (EC), macrovessels possess indigenous populations of dendritic cells (DC) (16, 19, 21), which are involved in immune surveillance and regulation of adaptive immune responses. Equipped with the ability to sense danger signals via toll-like receptors (TLR), these DC enable arteries to regulate localized and possibly systemic inflammatory responses (15). This functional ability is a critical pathogenic element in large-vessel vasculitides, such as giant cell arteritis (GCA) and Takayasu arteritis, in which the site of inflammation is the arterial wall itself. In the emerging disease model for GCA, innate immune recognition events facilitated by wall-embedded immune sentinels are considered to be crucially involved in the initiation of vasculitis (6) (Figure 1.3).

An indispensible contribution of the vessel wall provides a framework for the unique tropism for medium-sized and large vessels in GCA. Emerging data suggest that factors shaping the three-dimensional microenvironment in the vessel wall regulate

pathogenic immune responses and may determine the outcome of inflammatory reactions placed within the layers of the vascular wall (17). In GCA, the arterial cellular residents collaborate with recruited cellular components in the execution of diversified effector functions that are supported by the three-dimensional platform of the extracellular compartment. The ensuing tissue remodeling is thus the result of a two-pronged attack driven by specialized subsets of immune cells in combination with a destructive self-inflicted response to injury by the macrovessel (Figure 1.5).

Vascular smooth muscle cells (VSMC)—a cell population prone to respond to injury

Smooth muscle cells are a major component of the vessel wall defining its contractile functions as well as providing precursor cells to heal and repair wall damage. In that process, migratory ability, proliferative expansion and matrix production all contribute to the repair response. In GCA, T cells and macrophages typically accumulate between the medial smooth muscle cell fibers and VSMC are a direct target of inflammatory attack. Specifically, VSMC have been shown to be subjected to oxidative stress in GCA (88, 89), and conversely may also be a source of reactive oxygen species.

VSMC are not only injured by the inflammatory milieu, but they also actively participate in its generation and maintenance. The anatomical separation of the media from the intima is demarcated by the internal elastic lamina (IEL), whose fragmentation represents one of the pathological hallmarks of this disease (90). Apart from its

contractile functions, VSMC have known synthetic functions; and MMP, one of its secretory products, has been purported as the mediator of this IEL degradation. MMP are involved in numerous pathologies including autoimmune and cardiovascular diseases (91), resulting mainly from their ability to degrade components of the extracellular matrix such as collagen and elastin. Specifically, in a clinical study employing histomorphological analyses of GCA patient temporal artery biopsies, a significant association was found between MMP-9 and IEL destruction in diseased vessels (92). The demonstrated ability of VSMC to secrete MMP positions these cells as potential key contributors to the vascular remodeling that characterizes GCA (Table 1.1).

Neoangiogenesis and concentric intimal hyperplasia are additional features of the maladaptive arterial response in GCA that give rise to luminal occlusion and consequently to tissue ischemia that accounts for the clinical symptoms that plague these patients. The vasa vasorum network, conventionally confined to the adventitia in healthy arteries, is found to be extended to the medial and intimal layers of the inflamed artery, a phenomenon that correlates closely with IEL fragmentation as well as with the presence of the multinucleated giant cells from which the disease derives its name. The giant cells and macrophages located at the media-intima junction contribute to the program of tissue injury via the production of proinflammatory cytokines, ROI and MMP (7). The extensive arsenal of these cells includes growth factors such as platelet-derived growth factor and vascular endothelial growth factors (VEGF), both of which drive neovascularization and intimal thickening via their stimulatory effects on VSMC (93).

Endothelial Cell Response

Just as the role of the artery extends beyond its function in hemodynamic regulation, so too does the role of the arterial endothelium extend beyond merely serving as a physical interface between the blood and extravascular tissues. Among their physiological functions, vascular EC regulate inflammatory and immune reactions, as well as vascular remodeling that define the vascular lesions of GCA (94). The medium-sized and large arteries affected by GCA essentially have two populations of EC, and both are likely to have a pathogenic role. However, the EC lining the artery's macrolumen may be less relevant in the events driving GCA than the micro-EC defining the vasa vasorum and outgrowing microvessels supporting the wall remodeling process. EC are major targets of cytokines produced by the inflammatory cells forming the vasculitic lesions including DC, macrophages and CD4 T cells. Notably IL-6, whose systemic levels correlate closely with disease status (95, 96), has potent effects on EC proliferation and tubular formation, and *in vivo* and *ex vivo* models have identified this cytokine as a stimulus for the induction of angiogenesis in large vessels.

Macrophages and giant cells in the lesion are major producers of IL-6 (4), and these tissue-infiltrating cells further contribute to the growth of microcapillaries via the production of VEGF, a stimulant of EC proliferation. Neoangiogenesis serves to perpetuate the inflammatory response given that the vasa vasorum provides the physiologic port of entry for the invading lymphocytes, thereby increasing the number of portals by which the inflammatory reinforcements can access and maintain the ongoing inflammation. EC, however, are not passive targets in the inflammatory response and are themselves responsible for cytokine production in response to paracrine signals.

These signals modulate the EC synthesis of proinflammatory cytokines and adhesion molecules. The outcome of endothelial activation then is a cycle of cytokine production in response to cytokine signaling by vascular infiltrates. The contributory role of the endothelium in the evolution of vascular disease is further supported by studies in bioengineered vessels. In order to address the role of EC on immunomodulatory functions of DC, tubular constructs with or lacking an endothelial monolayer were seeded with DC and compared for their T-cell stimulatory capacity (17). As indicated by enhanced DC activation markers as well as increased T-cell recruitment into the tubular constructs, the results showed an amplification of the immune response mediated by DC and macrophages that correlated with the presence of the endothelial layer. These observations confirm the active involvement of the endothelial layer in pathogenesis and the bi-directionality of the interplay between the vessel wall and the immune cells that maintain chronic inflammatory responses leading to the progressive building of the vascular lesions of GCA.

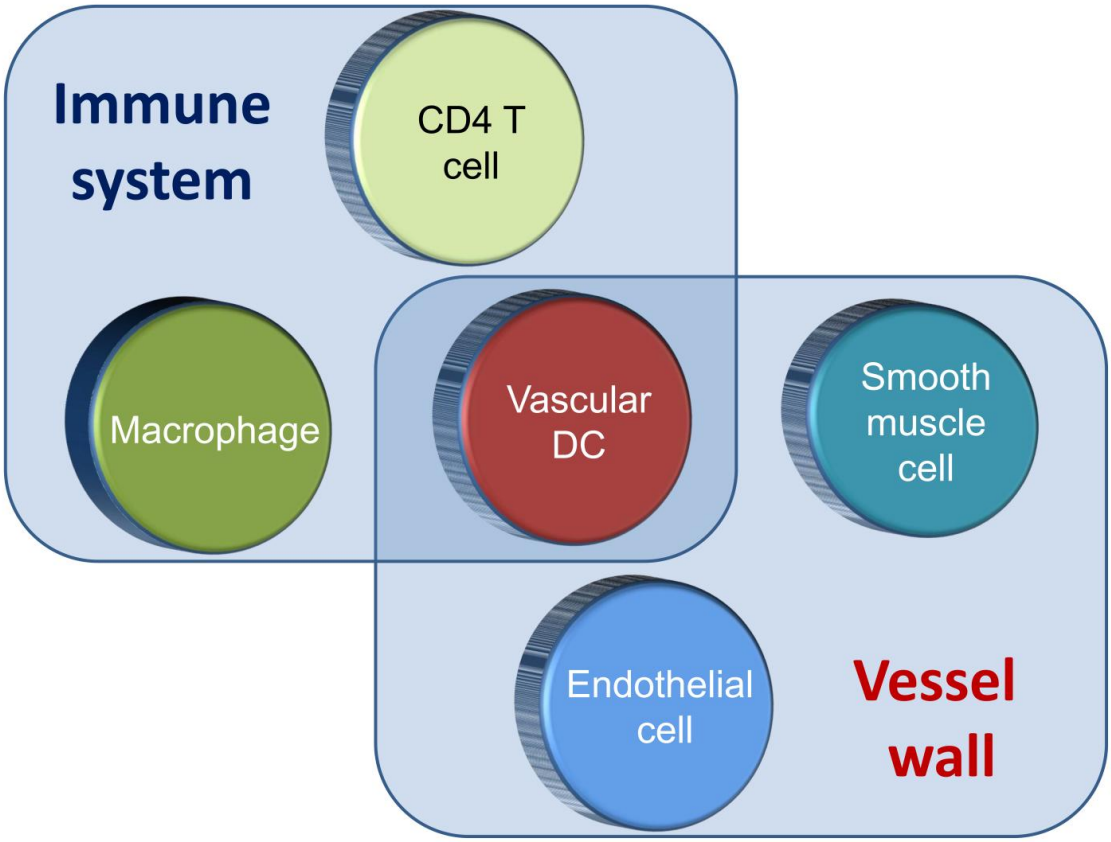

Figure 1.5. Immune cells and vessel wall cells collaborate in mediating vascular damage in GCA. Vascular DC are an indigenous cell population in the arterial wall and respond to danger signals by recruiting T cells and macrophages. Macrophages secrete reactive oxygen intermediates, enzymes and growth factors. Endothelial cells participate in cell recruitment and neoangiogenesis supporting the remodeling of the wall structure. Vascular smooth muscle cells proliferate and migrate to form the hyperplastic and lumen-obstructive intima but also contribute to wall damage by secreting tissue-injurious enzymes.

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Cytokines and GCA

Cytokines are involved in the pathogenesis of most inflammatory diseases; thus it is no surprise that they play a central role in GCA, both in the systemic inflammatory response as well as in the affected blood vessels. Studies have detected increased serum levels of cytokines such as IL-6 in patients with active disease and corresponding decreases in patients demonstrating remission of clinical symptoms following treatment (97). The role of other circulating cytokines remains less well defined. It is cytokine studies of the arterial wall however from which the most insight has been gained thus far regarding the evolution of disease. Temporal artery biopsy studies have provided a tissue cytokine profile which suggests that GCA is a Th1-driven disease in which local IFN-γ production drives disease progression (98). Recently, a novel CD4 T cell subset, Th17 cells, have been described which have been implicated in the pathogenesis of autoimmune disease (99). Indeed, blockade of Th17 development or activity inhibits autoimmune pathology in models of disease such as multiple sclerosis and RA (100, 101). Phenotypic analyses of Th17 cells describe a subset that is enriched for CCR6, the sole receptor for the ligand CCL20 (102). Recent studies showing that $CCR6^+$ T cells play a unique role in initiating and sustaining large vessel vasculitis point to a potential contribution of IL-17 in disease pathogenesis, given the cytokine profile of this cell subset (103). IL-17 is a proinflammatory cytokine and although it has been known to be a product of CD4 T cells for more than 10 years, the recognition of Th17 as a unique subset of helper T cells has only recently been accepted (104, 105). Much of what was initially known regarding IL-17 regulation emerged from murine studies, but more recent studies have begun to dissect the regulation of this cytokine in humans. Of the 6 family members

IL-17 (or IL-17A) and IL-17F play key roles in the activation, recruitment and migration of neutrophils (106). Apart from IL-17, Th17 cells also preferentially produce IL-21, IL-22 and IL-26 (107, 108). Murine Th17 differentiation is driven by IL-6, TGF-β and IL-21 (109, 110), while in human naïve CD4 T cells, differentiation is promoted by IL-6, IL-1β and IL-23, with no demonstrated requirement for TGF-β (111). At the transcriptional level, the Th17 differentiation program is directed by the orphan retinoid nuclear receptor (ROR) γt (112). IL-17 and IL-21 production by Th17 cells is regulated by STAT3 (113) which is activated by IL-6, IL-21 and IL-23 (114). This transcription factor is a positive regulator of RORγt and binds the IL-17 and IL-21 genes, thereby amplifying overall production of IL-17 (115). Although the studies identifying the regulators of human Th17 induction from naïve cells have been somewhat conflicting, it is clear that memory T cells are effective producers of IL-17. TCR stimulation or stimulation with anti-CD3/CD28 alone is sufficient for IL-17 production. Notably within the subset of memory CD4 T cells, single positive IL-17 and double positive IL-17+IFN-γ+ cells have been repeatedly detected, both of which express similar levels of RORγt and T-bet (116). The distinction between the Th17 and Th17/Th1 cells remains unclear as they both demonstrate similar cytotoxic potential and susceptibility to Treg suppression, although phenotypic differences have been identified based on chemokine receptor expression by human cells. The single producers are primarily $CCR6^{+}CCR4^{+}$ T cells, while the double producers express CCR6 and CXCR3 (117). This differential pattern of receptor expression provides clues into potential differences in pathogenic targets that may be linked to unique homing capacities of the two cell populations.

Cell type	Cellular product	Tissue injurious effector functions
Dendritic Cell	Cytokines Chemokines	Recruitment of CD4 T cells to vessel wall Direction of CD4 T cell activation
CD4 T cell	Cytokines Chemokines	Regulation of macrophage, EC and VSMC Regulation of cellular tissue infiltrate VSMC-directed cytotoxicity
Macrophage	PDGF & VEGF MMP ROS	Enzymatic tissue damage Oxidative stress through ROS production Mobilization of VMSC
VSMC	MMP	Induction of neoangiogenesis Wall remodeling Forming hyperplastic intima Destruction of elastic membranes
Endothelial Cell	Cytokines PDGF &VEGF	Generating altered wall matrix Neoangiogenesis Recruitment of inflammatory cells Accessibility to immunoprivileged tissue sites

Table 1. The spectrum of cells and mechanisms involved in vessel wall damage in large vessel vasculitis.

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Autoimmunity: on the losing end of the battle of the co-signals

Dendritic cells represent a diverse population of professional antigen presenting cells and have been shown to be the most potent of these in the execution of this function as it pertains to the initiation of primary immune responses. Their unique ability to prime T cells positions them as key modulators of the immune response. It is this pivotal role in linking the innate and adaptive arms of immunity that underlies their role in the initiation of autoimmune disease such as GCA. The plasticity of the DC facilitates its ability to induce tolerance under steady state conditions, and conversely to generate antigen-specific responses upon pathogen encounter. The signals provided to the DC ultimately dictate the instruction provided to the interacting T cell, which is the cumulative outcome of the accessory signal input. The pathology in GCA, which is the result of a self-inflicted immune response localized within the vessel wall, highlights the need to better understand the components that regulate the induction as well as the breach of tolerance. The divergent DC-driven outcomes are undeniably the result of alterations in the balance of co-stimulatory and co-inhibitory signals. Given the capacity of the TLR to modulate DC function (118), it is likely that the shift in the co-signaling pathways stems from differential patterns of TLR stimulation and consequent downstream changes in the DC differentiation program (119). A dissection of the unique co-signaling molecules induced by differential TLR signaling and the effects on the modulation of T cell effector functions is thus warranted to elucidate the mechanisms underlying autoimmune disease pathogenesis. Therefore, we seek to explore the presence and function of distinctive accessory receptor-ligand pairs in response to differential TLR ligation, with the

knowledge that the information gleaned from such studies would prove to be valuable in the development of potential therapeutic strategies for large vessel vasculitis.

Chapter 2

TLR-mediated induction of negative regulatory ligands on dendritic cells

Abstract

Dendritic cells (DC) shape T-cell response patterns and determine early, intermediate, and late outcomes of immune recognition events. They either facilitate immunostimulation or induce tolerance, possibly determined by initial DC activation signals, such as binding Toll-like receptor (TLR) ligands. Here we report that DC stimulation through the TLR3 ligand dsRNA [poly(I:C)] limits CD4 T-cell proliferation, curtailing adaptive immune responses. CD4 T cells instructed by either LPS or poly(I:C)-conditioned DC promptly upregulated the activation marker CD69. Whereas LPS-pretreated DC subsequently sustained T-cell clonal expansion, proliferation of CD4 T cells exposed to poly(I:C)-pretreated DC was markedly suppressed. This proliferative defect required DC-T cell contact, was independent of IFN-α, and was overcome by exogenous IL-2, indicating T-cell anergy. Coinciding with the downregulation, CD4 T cells expressed the inhibitory receptor PD-1. Antibodies blocking the PD-1 ligand PD-L1 restored proliferation. dsRNA-stimulated DC preferentially induced PD-L1, whereas poly(I:C) and LPS both upregulated the costimulatory molecule CD86 to a comparable extent. Poly(dA-dT), a ligand targeting the cytoplasmic RNA helicase pattern-recognition pathway, failed to selectively induce PD-L1 upregulation, assigning this effect to the TLR3 pathway. Poly(I:C)-conditioned DC promoted accumulation of phosphorylated SHP-2, the intracellular phosphatase mediating PD-1 inhibitory effects. The ability of dsRNA to bias DC differentiation toward providing inhibitory signals to interacting CD4 T cells may be instrumental in viral immune evasion. Conversely, TLR3 ligands may have therapeutic value in silencing pathogenic immune responses.

Introduction

Dendritic cells (DC) are potent antigen-presenting cells (DC) which bridge innate and adaptive immune responses and shape the balance between induction of immunity and tolerance (120). As sentinels, DC alert the immune system; capture, process and present antigens; and transport them to lymphoid tissues where they prime antigen-reactive T-cell clones. Driven by pathogens and inflammatory signals, DC undergo a complex maturation process, which not only leads to enhanced expression of costimulatory molecules and increased formation of stable MHC-peptide complexes but also to cytokine secretion modulating T-cell activation and expansion, synthesis of chemokines and chemokine receptors, and regulation of T-cell and DC trafficking. Through these mechanisms, DC guide lymphocytes along their differentiation process, thus ultimately determining the quantity and quality of the emerging immune response (121, 122).

Microbial signaling through Toll-like receptors (TLRs) is an effective way to induce DC maturation. TLRs are a well-characterized family of pattern-recognition receptors able to detect pathogen-associated molecular motifs. Ten different TLRs have been described in humans; some of them, such as TLRs 1, 2, 4, 5, and 6, preferentially bind bacterial products [e.g. lipopolysaccharide (LPS), flagellin], whereas others such as TLRs 3, 7, 8, and 9 mainly detect nucleic acids (e.g. single- and double-stranded RNA) (27, 123). Intracellular signal transmission of most TLRs involves recruitment of the adapter molecule MyD88. TLR triggering of DC results in activation, as determined by the upregulation of maturation markers (e.g. CD83) and costimulatory molecules (e.g.

CD86, CD80, and CD40). Depending on which TLR is triggered, however, functional differences in DC maturation and in the subsequent DC-induced T-cell responses may occur. For instance, TLR2 preferentially induces DC to produce IL-10, which in turn inhibits a number of pro-inflammatory cytokines. TLR4, on the other hand, is a strong inducer of IL-12p70 and IFN-α (124).

TLR3 mediates responses to double-stranded (ds)RNA and to its synthetic analog polyinosinic: polycytidylic acid [poly (I:C)] (125). dsRNA constitutes the genome of one class of viruses (e.g. Reoviridae) but is also a replication intermediate of most other viruses, assigning TLR3 a key role in anti-viral defense (126). This notion is supported by *in vitro* and *in vivo* studies demonstrating the pivotal position of TLR3 in host responses against a number of viruses (e.g. West Nile, respiratory syncytial, and influenza) (126, 127). Despite effective sensing mechanisms alerting the host to viral infections, many viral pathogens, including HIV, lymphocytic choriomeningitis virus (LCMV), and hepatitis C virus, are capable of evading effective immunity, causing chronic infection (32, 128-131). How DC contribute to this immune failure is incompletely understood, but recent studies characterizing defective CD8 T-cell responses in chronically infected hosts have shed light on virus-induced immune deviation (128). Specifically, insufficient viral clearance has been linked to the induction and maintenance of "exhausted" CD8 cytotoxic T cells (CTL), which may be tolerized by the inhibitory receptor programmed death-1 (PD-1). This type I immunoglobulin receptor belongs to the CD28/CTLA-4 family and transmits co-inhibitory signals through interaction with its ligands PD-L1 (B7-H1) and PD-L2 (B7-DC) (32, 130, 131). Besides its role in impairing anti-viral immune responses, the PD-1/PD-L pathway is a major

immunosuppressive mechanism (128, 129, 132). PD-1/PD-L1 interactions have been implicated in the initiation and the reversal of T-cell anergy (133). Also, PD-1 signaling emerges as a major tolerance mechanism in regulating autoimmune disease. In several murine models, aggravated disease has been reported in PD-1-deficient mice, including autoimmune encephalomyelitis, lupus-like syndrome with arthritis and nephritis (134), and intensified dilated cardiomyopathy (135-137). Finally, the PD-1/PD-L1 pathway is involved in regulating early T-cell fate decisions (138).

CD4 T cells play a critical role in adaptive immunity; however, relatively little is known about the impact of DC TLR triggering on CD4 T-cell responses, especially during the course of viral challenges. CD4 helper T cells contribute to anti-pathogen responses by promoting the generation of functionally mature DC, a process described as DC licensing (139). In the present study, we explored the consequences of TLR3 stimulation of monocyte-derived DC on CD4 T-cell function and compared it with the outcome resulting from TLR4-triggering using LPS. Our results indicate that TLR3-triggered DC, as opposed to those stimulated via TLR4, downregulate CD4 T-cell proliferation. Blockade of the co-inhibitory molecule PD-L1 on TLR3-stimulated DC almost completely restored CD4 T-cell expansion, thus implicating the PD-1/PD-L1 pathway in regulating CD4 T-cell non-responsiveness imposed by TLR3-conditioned DC.

Materials and Methods

Cells

To generate immature monocyte-derived DC (iDC), $CD14^+$ cells were isolated from peripheral blood mononuclear cells using positive selection with magnetic beads (Miltenyi, Auburn, CA) and cultured at a density of $1.5\text{-}2\times10^6$ cells/mL in RPMI (Mediatech, Cellgro, Herndon, VA) supplemented with 1000 U/mL IL-4 and 800 U/mL GM-CSF (both R&D Systems, Minneapolis, MN). Cells were harvested on day 6 and stimulated with poly(I:C) (Sigma Aldrich, St. Louis, MO) or LPS (Escherichia Coli, 0127:B8, Sigma Aldrich, St. Louis, MO) at the concentrations and durations described. All experiments were performed with naked poly(I:C) which is recognized by TLR3 but not by intracellular RIG-I-like receptors. Poly (dA-dT), a ligand for RIG-I-like receptors, was purchased from InvivoGen (San Diego, CA) and used at concentrations between 0.01 and 1 μg/mL. All patients provided informed consent, and biological specimens were handled according to Institutional Review Board-approved protocols.

T-cell proliferation assays

CD4 T lymphocytes were purified from peripheral blood mononuclear cells with magnetic beads (Miltenyi). Cell purity, assessed by flow cytometry, was routinely >95%. iDC were cultured in 96-well round-bottom microtiter plates (Falcon; BD Bioscience, San Jose, CA) (6.5×10^3 iDC/well) in the presence of either 1 μg/mL LPS or 0.5, 5, 50, or 100 μg/mL poly(I:C). In selected cultures T-cell stimulation was facilitated by the bacterial superantigen toxic shock syndrome toxin-1 (TSST-1) (final concentration 0.04 ng/mL; Toxin Technology, Sarasota, FL). After 5 hours, CD4 T cells were added (1×10^5

cells/well). Cultures were pulsed with 1 μCi/well [^{3}H]thymidine on day 2, harvested onto glass fiber filters after 24 hours, and [^{3}H]thymidine uptake was measured using a 1450 MicroBeta scintillation counter (PerkinElmer Wallac, Gaithersburg, MD). Where indicated, cultures were supplemented with 20 ng/mL recombinant human (rh) IL-2 (Chiron, Emeryville, CA). All experiments were performed in triplicates.

To determine the direct effects of poly(I:C) and IFN-α on T-cell proliferation, CD4 T cells were stimulated (100,000 CD4/well) in a 96-well flat-bottom plate coated with 1 μg/mL anti-CD3 (OKT-3, Ortho-Clinical Diagnostics, Raritan, NJ) and 0.5 μg/mL anti-CD28 monoclonal antibody (mAb) (BD Biosciences, San Jose, CA) and cultured with 100 U/mL IFN-α (BD Pharmingen, San Diego, CA), 100 μg/mL poly(I:C), or both. Additionally, supernatants harvested from T cell-DC cocultures containing either untreated DC, LPS-stimulated DC, or poly(I:C)- stimulated DC were transferred onto CD4 T cells driven with anti-CD3/anti-CD28, and proliferation was assessed 3 days later.

Blockade of PD-L1 and PD-L2

Anti-human PD-L1 (clone MIH1) and PD-L2 (clone MIH18) mAbs were purchased from eBioscience (San Diego, CA). iDC were stimulated with poly(I:C) or LPS for 5 hours and incubated with anti-PD-L1 mAb (1 μg/mL), anti-PD-L2 mAb (10 μg/mL), or isotype controls (mouse IgG1, BD Pharmingen, San Diego, CA) for 1 hour before allogeneic CD4 T cells were added. Cultures were pulsed with 1 μCi/well of [^{3}H]thymidine after 48 hours. In the kinetic studies, anti-PD-L1 mAb was added 12, 24, or 48 hours after initiating the DC-CD4 T cell cocultures.

Quantitative PCR/qPCR

iDC were stimulated with poly(I:C) in a 96-well plate for 48 hours. Total RNA was purified using TRIzol (Life Technologies, Gaithersburg, MD) and reverse-transcribed using AMV reverse transcriptase (Roche Molecular Biochemicals, Mannheim, Germany). qPCR were performed using the Mx3000 instrument (Stratagene, La Jolla, CA) in triplicates: 1 μl cDNA was mixed in a total volume of 50 μl SYBR Green Master Mix (5 μl 10 × PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.025% BSA, 0.2 μl 5 U/μl Plt. Taq, 1:20,000 SYBR Green, and 0.1 μM of each primer). The following primers were used: β-actin, 5′-ATGGCCACGGCTGCTTCCAGC-3′ and 5′-CATGGTGGTGCCGCCAGACAG-3′; PD-L1, 5′-AACTACCTCTGGCACATCCTC-3′ and 5′-CACATCCATCATTCTCCCTTTT-3′; CD86, 5′-GATTCGGACAGTTGGAC-3′ and 5′-GTAACCGTGTATAGATGAGC-3′; IFN-α, 5′-ATGCGGACTCCATCTTG-3′ and 5′-CGTGACCTGGTGTATGAG-3′. Results were normalized to 2×10^5 copies of β actin.

Flow cytometry

mAbs used for flow cytometric analysis were phycoerythrin (PE)-conjugated anti-human PD-L1 (MIH1, eBioscience, San Diego, CA), PE-conjugated anti-human PD-L2 (MIH18, eBioscience, San Diego, CA), fluorescein isothiocyanate (FITC)-conjugated anti-human CD83 (HB15e, BD Pharmingen, San Diego, CA), allophycocyanin-conjugated anti-human CD86 (FUN-1, BD Pharmingen, San Diego, CA), FITC-conjugated anti-human CD69 (L78, BD Pharmingen, San Diego, CA), and PE-conjugated anti-human PD-1 (MIH4, BD Pharmingen, San Diego, CA). Cells were analyzed on an LSR II system

(Becton Dickinson, Franklin Lake, NJ). Data analysis was performed with WinMDI software.

Western Blot

iDC were stimulated with poly(I:C) (100 μg/mL) or LPS (1 μg/mL). Where indicated, control IgG1 or blocking anti-PD-L1 antibodies (1 μg/mL) were added after 5 hours of stimulation. Allogeneic CD4 T cells were added 1 hour later, and the cocultures were incubated for an additional 12 or 24 hours. Cells were lysed in cell extraction buffer [10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1mM EGTA, 1mM NaF, 20 mM Na4P2O7, 2 mM Na3VO4, 1% Tritin X-100, 10% glycerol, 0.1% SDS, and 0.5% deoxycholate (BioSource International Inc., Camarillo, CA)] supplemented with 1 mM phenylmethanesulfonyl fluoride and a protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO). Lysates were centrifuged at 14,000g for 10 minutes at 4°C. 30 μg of each supernatant sample was resolved by SDS-PAGE under reducing conditions, transferred to polyvinylidene fluoride membranes, and incubated with rabbit anti-phospho-SHP-2 (Tyr542) antibodies (Cell Signaling Technology, Danvers, MA) overnight at 4°C followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature for 1 hour. Blots were visualized by Immobilon Western chemiluminescent detection system (Millipore Corporation, Billerica, MA, USA). The membranes were reprobed for total SHP-2 by using rabbit anti-SHP-2 antibody (Cell Signaling Technology, Danvers, MA). Densitometry was performed using the ImageJ software (available at http://rsb.info.nih.gov/; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD).

Statistical Analysis

Two-sided Student's t test and Mann-Whitney U test were used, where appropriate. P less than 0.05 was considered to be statistically significant. Results are shown as either mean ± SD or box plots with medians and percentiles, when either parametric or nonparametric tests were used.

Results

DC stimulation with TLR 3 and 4 ligands induces divergent T-cell proliferative responses

To examine how conditioning of DC through different TLR ligands affects CD4 T cells, we compared poly(I:C)- and LPS-stimulated DC for their ability to elicit CD4 T-cell proliferation. *In vitro*-generated iDC were exposed to optimal concentrations of LPS (1 μg/mL) or increasing concentrations of poly(I:C) for 5 hours before allogeneic CD4 T cells were added; T-cell proliferation was assessed 3 days later. LPS-induced DC maturation increased their stimulatory capacity and enhanced T-cell proliferation rates. In contrast, TLR3-stimulated DC were less effective at inducing T-cell expansion than unstimulated DC. The inhibitory effect of poly(I:C)-triggered DC was dose-dependent with maximal effects seen at 100 μg/mL (Fig. 2.1A).

To distinguish whether diminished T-cell proliferation was the result of T-cell death or suppressed responsiveness, cultures were supplemented with exogenous IL-2. IL-2 was sufficient to restore T-cell proliferation and equalized proliferation levels of CD4 T cells instructed by either TLR3- or TLR4-triggered DC (Fig. 2.1B).

To assess whether the anti-proliferative effect of TLR3-conditioned DC was relevant in antigen-driven immune responses, we examined the impact of poly(I:C) stimulation on CD4 T cells responding to the bacterial superantigen TSST-1. As TSST-1 binds to about 10% of all T-cell receptor molecules, T-cell proliferation was enhanced in the presence of this superantigen (Fig. 2.1C). Pre-conditioning of the DC with poly(I:C) inhibited T-cell proliferation under both conditions, alloreactivity and superantigen

reactivity (Fig. 2.1C). These data strongly suggest that TLR3 triggering of DC modifies antigen-specific immune responses by suppressing T-cell expansion.

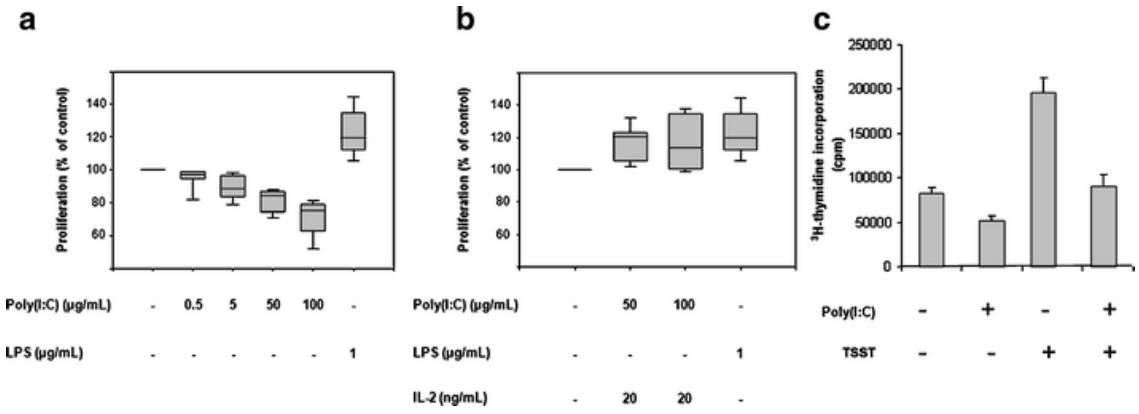

Figure 2.1. TLR3 stimulation reduces the ability of DC to induce T-cell proliferation.

Figure 2.1. TLR3 stimulation reduces the ability of DC to induce T-cell proliferation. **A**. 6.5×10^3 monocyte-derived DC were left untreated (control) or stimulated with poly(I:C) (0.5, 5, 50, or 100 μg/mL) or LPS (1 μg/mL) for 5 h. 1×10^5 allogeneic CD4 T cells were added, and T-cell proliferative responses were measured by [^{3}H]thymidine incorporation after 72 h. Results from seven independent experiments are expressed relative to the T-cell proliferation induced by unstimulated DC (on average 34,326 cpm). *Box plots* display medians, 25th and 75th percentiles as *boxes*, and 10th and 90th percentiles as *whiskers*. Untreated DC versus poly(I:C)-treated (50 μg/mL) DC, P = 0.04. Untreated DC vs. poly(I:C)-treated (100 μg/mL) DC, P = 0.007. Untreated DC vs. LPS-treated (1 μg/mL) DC, P = 0.003. **B**. CD4 T cells were cultured with DC pretreated with poly(I:C) or LPS as described in **A**. IL-2 (20 ng/mL) was added after 48 h, and proliferation was assessed by [^{3}H]thymidine incorporation 24 h later. *Box plots* represent results from five independent experiments. Untreated DC vs. poly(I:C)-treated (50 μg/mL) DC, P = 0.04. Untreated DC vs. LPS-treated (1 μg/mL) DC, P = 0.01. **C.** The effect of poly(I:C) on alloantigen- or superantigen-driven T-cell responses was compared by culturing CD4 T cells with DC that were untreated, loaded with TSST-1, pretreated with poly(I:C) (100 μg/mL), or pretreated with poly(I:C) plus TSST-1 (100 μg/mL and 0.04 ng/mL, respectively). Cultures were initiated at a DC:T-cell ratio of 1:50, and proliferative responses were quantified by [^{3}H]thymidine incorporation after 72 h. Assays were performed in triplicates, and results are presented as mean ± SD. Untreated DC vs. poly(I:C)-treated DC, P = 0.02; TSST-1-loaded DC vs. TSST-1 plus poly(I:C)-treated DC, P = 0.01.

Poly(I:C) and LPS are equally sufficient at inducing DC maturation and cytokine production

Divergent proliferative responses of CD4 T cells raised the question of whether the two classes of TLR ligands initiated distinct DC maturation programs, causing differential expression of costimulatory ligands and opposite effects on T-cell proliferation. We therefore investigated DC cell surface expression levels of CD83 and CD86. DC were stimulated with 100 µg/mL poly(I:C) or 1 µg/mL LPS and at indicated times stained for surface expression of CD83 and CD86. As shown in Fig. 2.2, poly(I:C) and LPS rapidly upregulated expression of CD83 and CD86 on the cell surface with these costimulatory ligands appearing with similar kinetics and similar surface concentrations. Both TLR ligands effectively drove DC into maturation as early as 6 hours after TLR crosslinking. By day 2, ample expression of CD83 and CD86 indicated full maturation of nearly the entire DC population. Thus, poly(I:C)-treated DC were not inferior in providing T-cell costimulatory signals.

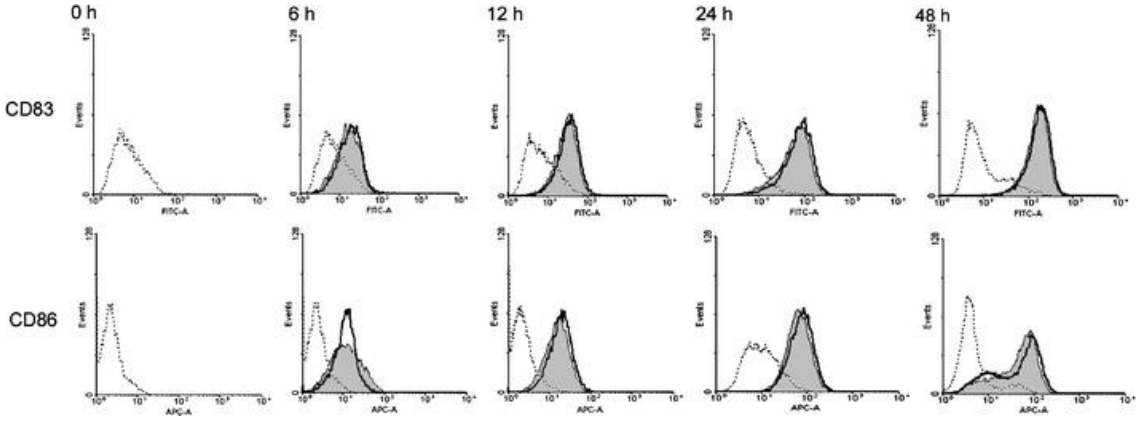

Figure 2.2. TLR3 and TLR4 ligands are equally competent at inducing DC maturation and cytokine production. DC were stimulated with poly(I:C) (100 μg/mL), LPS (1 μg/mL), or left untreated and at the indicated time points stained using anti-CD83 FITC and anti-CD86-PE mAbs. Representative histograms of control cultures (*dashed lines*), cultures including poly(I:C) (*shaded areas*), or LPS (*bold lines*) are shown.

Inhibition of T-cell proliferation is not mediated by soluble factors

Given its role in recognizing viral infections, TLR3 typically elicits IFN-α/ß production when binding dsRNA. IFN-α has been reported to modulate the survival of activated and anergic T cells with sustained responsiveness to IL-2 (140-142). We also considered that the diminished CD4 T-cell proliferation could result from a direct effect of poly(I:C) on T cells (143). Alternatively, a soluble product secreted by TLR3-triggred DC could facilitate T-cell nonresponsiveness.

To assess whether IFN-α was exclusively produced by poly(I:C)-triggered DC and had a direct role in inhibiting T-cell function, we quantified IFN-α transcripts. IFN-α transcription increased in a dose-dependent manner in poly(I:C)-activated DC (Fig. 2.3A), confirming functional intactness of these DC. Interestingly, LPS treatment of DC also augmented expression of IFN-α transcripts to levels almost comparable to those of poly(I:C). This observation brought into question whether IFN-α had a direct role in mediating T-cell nonresponsiveness.

To examine the functional impacts of IFN-α and poly(I:C), T cells were stimulated with immobilized anti-CD3/anti-CD28 Ab and IFN-α (100 U/mL) or poly(I:C) (100 μg/mL), or a combination of both. Neither IFN-α nor poly(I:C) nor their combination affected the proliferative capacity of CD4 T cells (Fig. 2.3B).

To investigate whether TLR-3-activated DC produced soluble factors that suppressed responding CD4 T cells, supernatants were generated by coculturing poly(I:C)- or LPS-stimulated DC and allogeneic CD4 T cells and transferred to CD4 T

cells activated by TCR/CD28 stimulation. Proliferative responses remained unaffected (Fig. 2.3C).

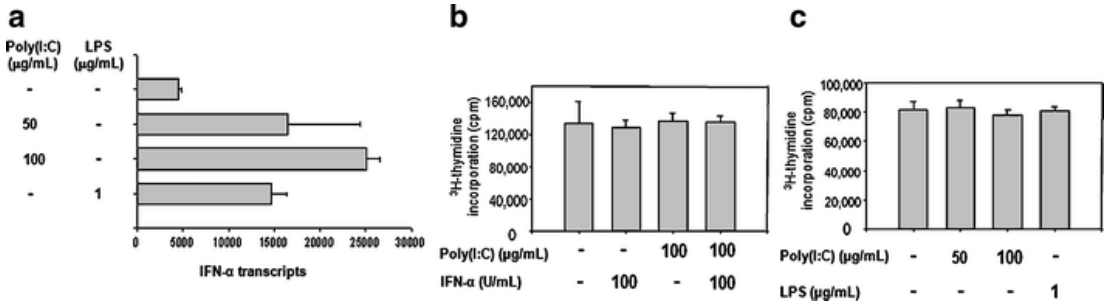

Figure 2.3. Induction of T-cell unresponsiveness by poly(I:C)-treated DC is not dependent on soluble factors. **A.** DC were cultured in medium (control), poly(I:C) (50 or 100 μg/mL), or LPS (1 μg/mL) for 48 h. IFN-α transcripts were measured by quantitative PCR. Data represented are derived from a minimum of three individual experiments. Untreated DC versus poly(I:C)-treated (100 μg/mL) DC, $P < 0.001$; untreated DC versus LPS-treated (1 μg/mL) DC, $P = 0.01$; poly(I:C)-treated (100 μg/mL) DC versus LPS-treated (1 μg/mL) DC, $P = 0.03$. **B.** T cells were stimulated with immobilized anti-CD3 and anti-CD28 Ab in the presence of IFN-α (100 U/mL) or poly(I:C) (100 μg/mL), or a combination of both. Proliferation was measured after 72 h by [^{3}H]thymidine incorporation. **C.** $CD4^+$ T cells were stimulated with untreated DC or DC activated with poly(I:C) or LPS. Supernatants from these cultures were added to fresh anti-CD3/CD28-stimulated $CD4^+$ T cells. Cells were harvested, and proliferation was determined by [^{3}H]thymidine incorporation. Results are expressed as mean ± SD of triplicate measurements and are representative of a minimum of three experiments.

TLR3 ligands preferentially induce expression of the co-inhibitory ligand PD-L1

Similarities in the expression of costimulatory ligands in TLR3- and TLR4-treated DC raised the question of whether the differences in functional outcome of T-cell stimulation were related to differential expression of co-inhibitory ligands. One of the most important receptor-ligand pairs providing negative signals to T cells is the PD-1/PD-L1 pair. PD-1 is typically upregulated on T cells upon activation and could certainly be involved in directing CD4 T cells towards nonresponsiveness(144). We first assessed whether the TLR3 ligand-imposed suppression of T-cell activation was an early event, disrupting early steps of T-cell activation, or occurred during later stages. CD69 surface expression increased as early as after 3 hours. T cells interacting with poly(I:C)- and LPS-conditioned DC showed a similar response in terms of CD69 induction (Fig. 2.4A). There was no difference between poly(I:C) and LPS stimulation until 24 hours, at which point LPS-triggered DC outperformed poly(I:C)-triggered DC in CD69 upregulation. CD69 surface expression steadily declined during the subsequent 24 hours. Thus, the downregulatory function of TLR3-treated DC involved later steps of T-cell activation, affecting sustained T-cell stimulation. Similarly, PD-1 was barely detectable on resting T cells and remained markedly low for the first 12 hours of T-cell stimulation. After 24 hours, PD-1 appeared on the T-cell surface with similar kinetics in TLR3- and TLR4-activated cultures (Fig. 2.4A).

Differential signaling through the co-inhibitory receptor PD-1 by DC populations would require disparate availability of PD-L1 ligand. PD-L1 plays an important role in the induction of T-cell tolerance (41, 144-147). During the initial phase of DC stimulation, FACS was not sensitive enough to detect PD-L1 surface expression (data not

shown). To detect the effect of TLR stimulation on PD-L1 and CD86 transcription, we therefore compared transcript levels in poly(I:C)- and LPS-stimulated DC (Fig. 2.4B). TLR triggering induced a prompt response of DC in terms of PD-L1 transcription. PD-L1-specific sequences were upregulated by poly(I:C) in a dose-dependent fashion with 9-fold to 13-fold increase compared with unstimulated DC. Conversely, LPS stimulation resulted in only a minor increase of PD-L1-specific sequences. In contrast, both poly(I:C) and LPS stimulation proved equally efficient in upregulating CD86 expression (3-fold) compared with baseline. Thus, TLR3-mediated DC differentiation is associated with preferential induction of the co-inhibitory ligand PD-L1.

Because poly(I:C) not only interacts with TLR3 but can also be recognized by cytoplasmic RNA helicases, we wanted to know whether the preferential induction of PD-L1 can be achieved by targeting non-TLR pattern recognition receptors. RNA helicases, such as RIG-I/MDA5 induce the production of type I interferons via the adaptor IPS-1 (148). All of our experiments were performed with naked poly(I:C) which targets TLR3. To stimulate the IPS-1 pathway, we utilized poly(dA-dT) and compared the effects of both polymers on the induction of co-stimulatory and co-inhibitory molecules. DC were stimulated with poly(I:C) (25 and 100 μg/mL) or poly(dA-dT) (0.01 and 1 μg/mL), and transcripts specific for PD-L1 and CD86 were quantified. As shown in Fig. 2.4C, poly(I:C) at both concentrations strongly amplified transcription of PD-L1. In contrast, the IPS-1 stimulator poly(dA-dT) had no or only a minimal effect on the activation-induced upregulation of PD-L1 sequences. The difference between both stimulators was maintained for CD86. Poly(I:C) functioned as a potent inducer of CD86 whereas poly(dA-dT) enhanced CD86 transcription by less than 2-fold (Fig. 2.4D).

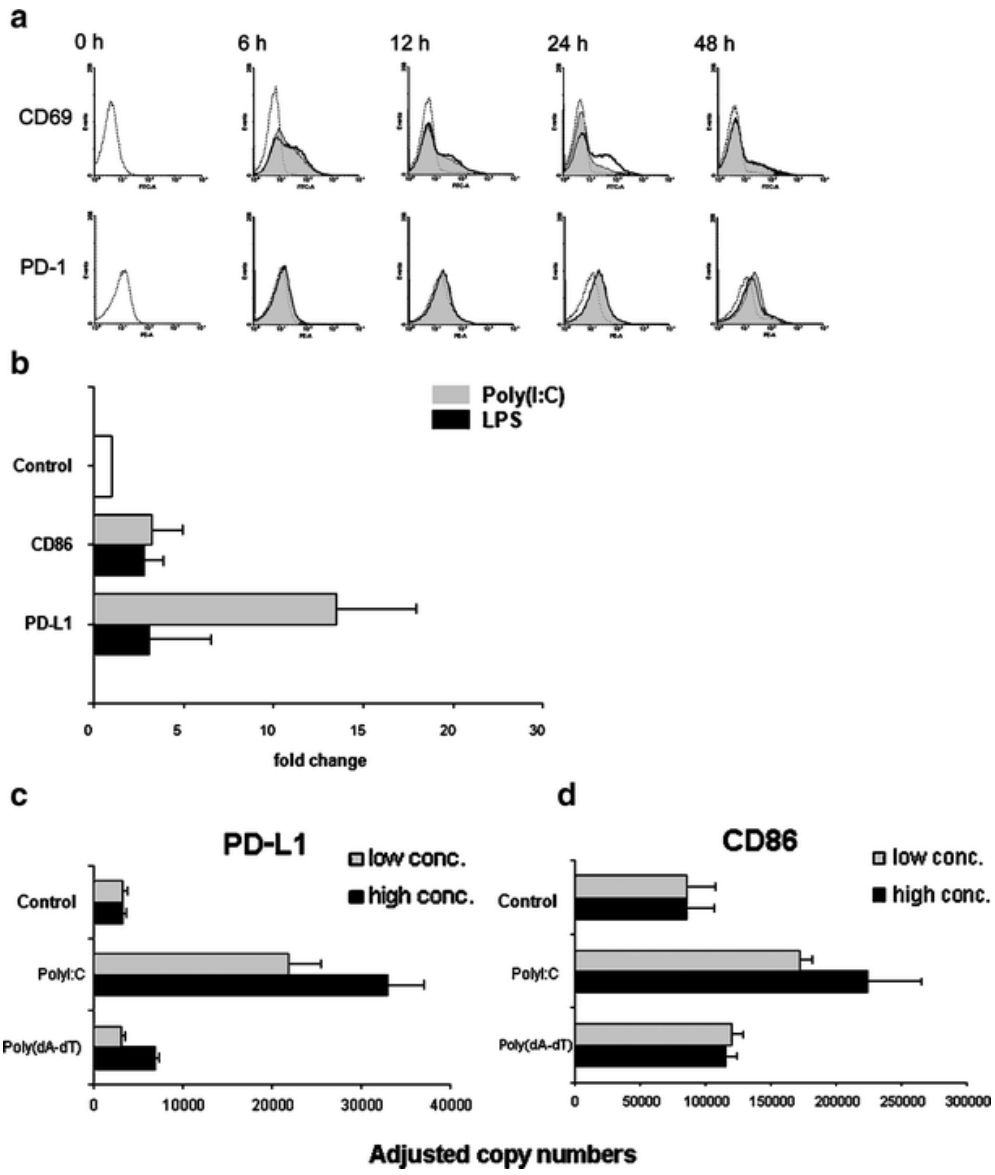

Figure 2.4. Poly(I:C) stimulation preferentially induces the co-inhibitory ligand PD-L1.

Figure 2.4. Poly(I:C) stimulation preferentially induces the co-inhibitory ligand PD-L1. A. Freshly isolated T cells were cocultured with TLR-stimulated DC [poly(I:C) 100 μg/mL and LPS 1 μg/mL], and expression of the activation markers CD69 and PD-1 on CD4 T cells was determined by flow cytometry at the indicated time points. Representative histograms of control cultures (*dashed lines*), cultures including poly(I:C) (*shaded areas*), or LPS (*bold lines*) are shown. Early activation events are indistinguishable, but after 24 h, LPS-triggered DC mediate stronger CD69 expression than poly(I:C)-stimulated DC. Induction of the co-inhibitory receptor PD-1 expression was similarly efficient by poly(I:C)- and LPS-stimulated DC and peaked at 24 h. **B.** Dendritic cells were stimulated with TLR ligands poly(I:C) or LPS, and mRNA was harvested after 6 h. Expression of PD-L1 and CD86 transcripts was assessed by quantitative PCR. Results are presented as mean ± SD relative to transcription level of unstimulated DC and are representative of three independent experiments. *CD86* Control vs. LPS-stimulated (1 μg/mL) DC, $P = 0.04$; poly(I:C)-stimulated (100 μg/mL) DC vs. LPS-stimulated (1 μg/mL) DC, $P > 0.05$. *PD-L1* Poly(I:C)-stimulated (100 μg/mL) DC versus LPS-stimulated (1 μg/mL) DC, $P = 0.01$. **C.** and **D.** DC were stimulated with poly(I:C) or the RIG-I-like receptor–ligand poly(dA-dT) for 4 h, and transcript levels for PD-L1 (*left*) and CD86 (*right*) were quantified by qPCR. Two concentrations of each ligand were used: 25 and 100 μg/mL of poly(I:C) and 0.01 and 1 μg/mL of poly(dA-dT). Representative results from one of two independent experiments are shown as mean ± SD. *PD-L1* Control vs. poly(I:C)-treated DC (25 μg/mL), $P = 0.007$; control vs. poly(dA-dT)-stimulated DC (0.01 μg/mL), $P = 0.4$. *CD86* Control vs. poly(I:C)-treated DC (25 μg/mL),$P < 0.01$;control vs. poly(dA-dT)-stimulated DC (0.01 μg/mL), $P = 0.07$.

TLR3 mediated induction of PD-L1 is not reversed by TLR4 stimulation

These experiments confirmed that the differential induction of PD-L1 by poly(I:C) stimulation was mediated through TLR3. The selective enhancement of PD-L1 transcription by poly(I:C) raised the question of whether under conditions of combined stimulation of TLR3 and TLR4 ligands, PD-L1 induction was maintained, providing ideal conditions for suppressed T-cell expansion in hosts infected with both viral and bacterial microorganisms. To mimic these conditions, we stimulated DC with poly(I:C) in the absence and presence of LPS. Under all stimulation conditions, PD-L1 transcripts were induced to a similar level, indicating that LPS could not reverse the selective induction of co-inhibitory molecules on the surface of the instructing DC (Fig. 2.5).

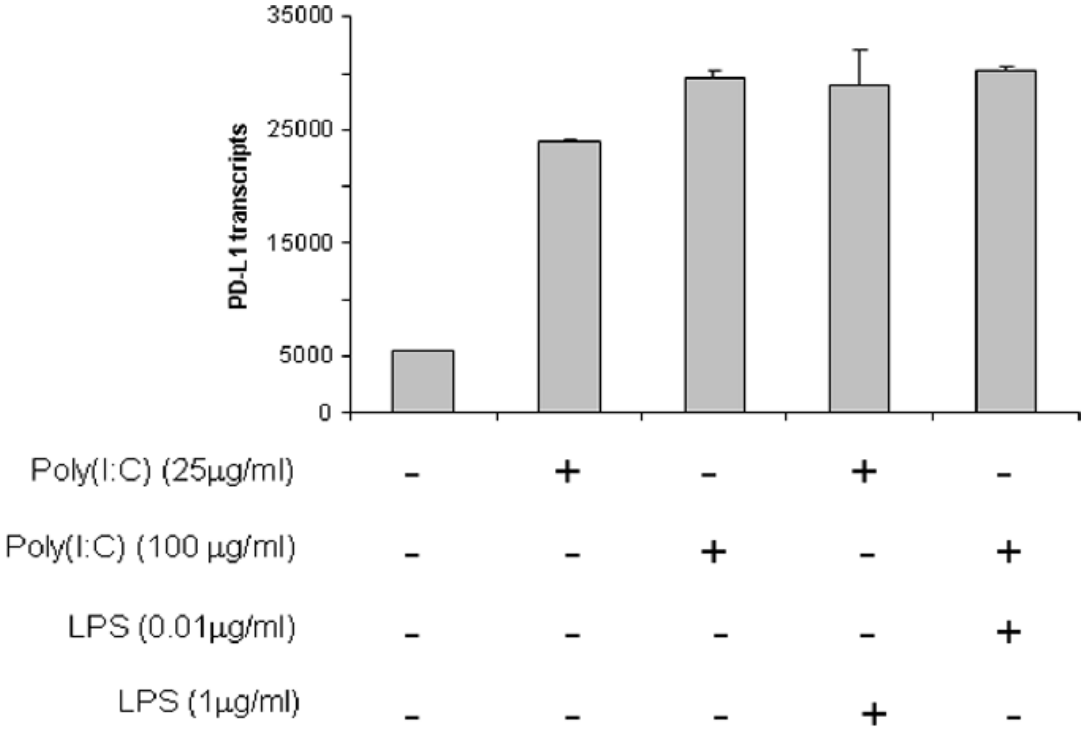

Figure 2.5. TLR4 stimulation cannot reverse the TLR3-mediated induction of the inhibitory ligand PD-L1. DC were stimulated with poly(I:C) alone or with a combination of poly(I:C) and LPS. After 4 h, cells were harvested, and PD-L1 transcripts were quantified by real-time PCR. Results are expressed as mean ± SD of triplicate cultures.

Blockade of PD-L1 restores the proliferative response of CD4 T cells instructed by TLR3-conditioned DC

To dissect the role of the PD-1/PD-L1 pathway in modulating the outcome of DC-T cell interactions, we used anti-PD-L1 and anti-PD-L2 mAbs to block access to PD-1. DC triggered with either poly(I:C) or LPS for 5 hours were incubated with blocking mAb or isotype control antibodies before CD4 T cells were added. Fig. 2.6A (*left panel*) shows that anti-PD-L1 effectively reversed the dose-dependent inhibition of T-cell responses by poly(I:C)-treated DC and led to a 2-fold increase in T-cell proliferation. Preventing PD-1 ligation resulted in T-cell proliferation levels comparable to those of untreated cultures. In contrast, anti-PD-L2 could not counteract the suppression of T-cell proliferation (Fig. 2.6A, *right panel*). Simultaneous addition of both blocking antibodies had no additive effect (data not shown). There was only a modest increase in T-cell proliferation if anti-PD-L1 was added to cocultures containing either LPS-preconditioned DC or DC that had not been exposed to TLR ligands.

To determine the kinetics of underlying inhibitory signaling mechanisms after PD-1 engagement on T cells, PD-L1-blocking mAb was added at different time points after initiation of the T cell-DC cocultures (Fig. 2.6B). Disruption of PD1-PD-L1 interaction even after 12 hours was sufficient to prevent negative signaling in T cells. Blocking access to PD-L1 after 24 hours resulted in partial restoration and after 48 hours had no effect, demonstrating that PD-1-mediated suppression of T-cell signaling occurred during a defined window of DC-T-cell interaction. These findings are consistent with the surface expression studies showing that PD-1 on T cells was not fully expressed until after 12 hours. The kinetics of the antibody-blocking studies confirmed that negative

signals provided by TLR3-conditioned DC were particularly important in dampening sustained TCR signaling but did not interfere with initial events.

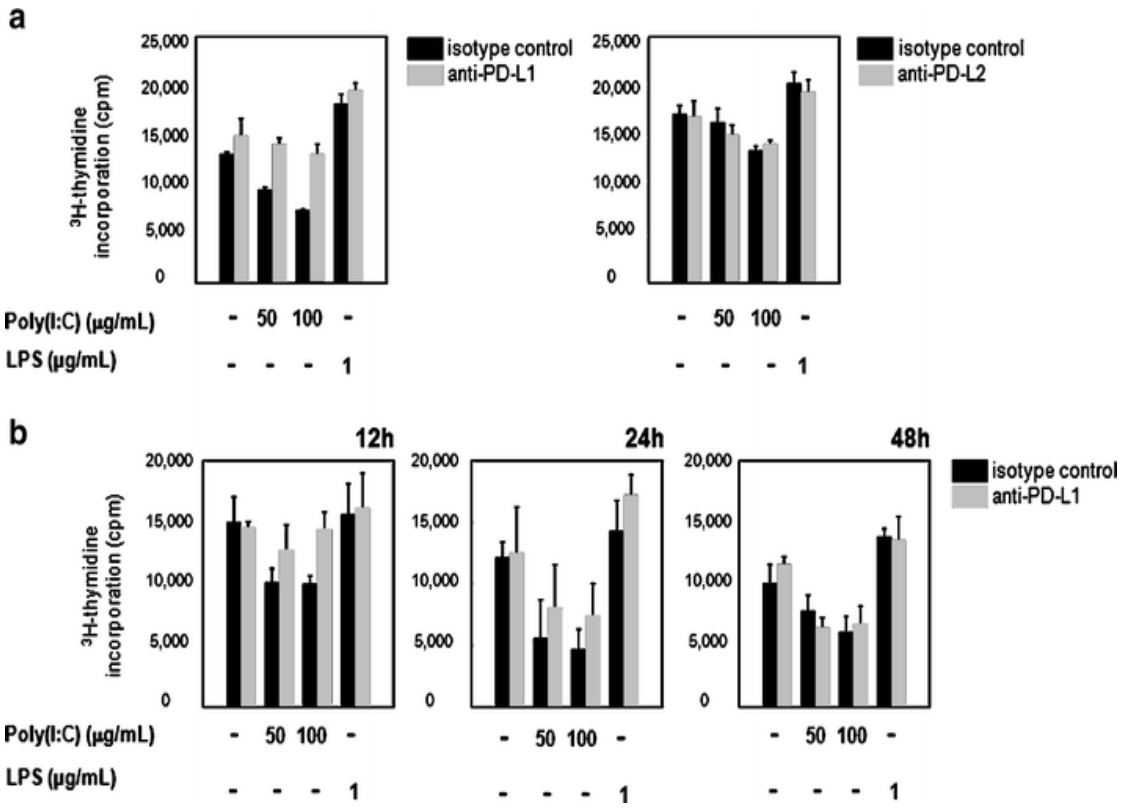

Figure 2.6. Blockade of PD-L1 restores CD4 T-cell proliferation.

Figure 2.6. Blockade of PD-L1 restores CD4 T-cell proliferation. **A.** DC were stimulated with poly(I:C) or LPS for 5 h and incubated with anti-PD-L1- (1 μg/mL; *left panel*) or anti-PD-L2-blocking (10 μg/mL; *right panel*) mAb or the appropriate isotype control for 1 h. The 1×10^5 CD4 T cells were added, and proliferation was assessed by [^{3}H]thymidine incorporation 72 h later. One representative experiment of three is shown. *Left panel*: Unstimulated DC versus poly(I:C)-stimulated (100 μg/mL) DC, $P = 0.0003$; unstimulated DC versus LPS-stimulated (1 μg/mL) DC, $P = 0.008$; poly(I:C)-stimulated (100 μg/mL) DC versus mAb-blocked poly(I:C)-stimulated (100 μg/mL) DC, $P = 0.01$. *Right panel*: Unstimulated DC versus poly(I:C)-stimulated (100 μg/mL) DC, $P = 0.03$; unstimulated DC versus LPS-stimulated (1 μg/mL) DC, $P = 0.009$. **B.** Anti-PD-L1 blocking mAb was added at different time points after initiation of T cell–DC interaction, and T-cell proliferative responses were measured as described above. One representative experiment of three is shown. *12 h* Unstimulated DC versus poly(I:C)-stimulated (100 μg/mL) DC, $P = 0.04$; poly(I:C)-stimulated (100 μg/mL) DC versus mAb-blocked poly(I:C)-stimulated (100 μg/mL) DC, $P = 0.03$. *24 h* Unstimulated DC versus poly(I:C)-stimulated (100 μg/mL) DC, $P = 0.01$. *48 h* Unstimulated DC versus poly(I:C)-stimulated (100 μg/mL) DC, $P = 0.05$

dsRNA-conditioned DC modulate CD4 T-cell responses by inducing SHP-2 recruitment

If indeed PD-1 crosslinking is the critical step through which TLR3-conditioned DC instruct T cells to enter a state of hyporesponsiveness, then T cells exposed to poly(I:C)-pretreated DC should have evidence for PD-1 signaling. Engagement of both the TCR and PD-1 receptor is required for phosphorylation of an immunoreceptor tyrosine-based switch motif (ITSM) in the cytoplasmic domain of PD-1. Association of Src homology region 2 domain-containing phosphatase-2 (SHP-2) with the ITSM and phosphorylation-dependent stimulation of its tyrosine phosphatase activity are proposed to mediate inhibitory effects of PD-1 (32, 149, 150). Cell lysates were prepared after 24 hours of DC-CD4 T-cell coculture, and immunoblot analyses for total SHP-2 and phospho-SHP-2 were performed. Results are given in Fig. 6 and show that at the number of cells included, SHP-2 is nearly exclusively detected in T cells and only minimally in DC. Exposure to poly(I:C)-treated DC caused marked phosphorylation of SHP-2 (Fig. 2.7A, lane 4; D), whereas LPS-treated DC did not transmit a signal to T cells enhancing SHP-2 phorphorylation (Fig. 2.7B, lane 2; D). No such effect was observed when DC were left untreated. Generation of phospho-SHP-2 required 24 hours of coculture. Immunoblotting of cell extracts harvested after 12 hours did not show enhancement of SHP-2 phosphorylation under any of the culture conditions (data not shown). SHP-2 phosphorylation induced by TLR3-triggered DC could be partially blocked by anti-PD-L1 blocking antibody (Fig. 2.7C, lanes 1 and 2; D). This observation was consistent with reinstitution of T-cell proliferation in the presence of this antibody. Taken together, these results indicated that TLR3-activated DC modulate TCR-mediated signals by PD-1

engagement, and that crosslinking of PD-1 by its ligand PD-L1 has functional relevance in DC-T cell interactions.

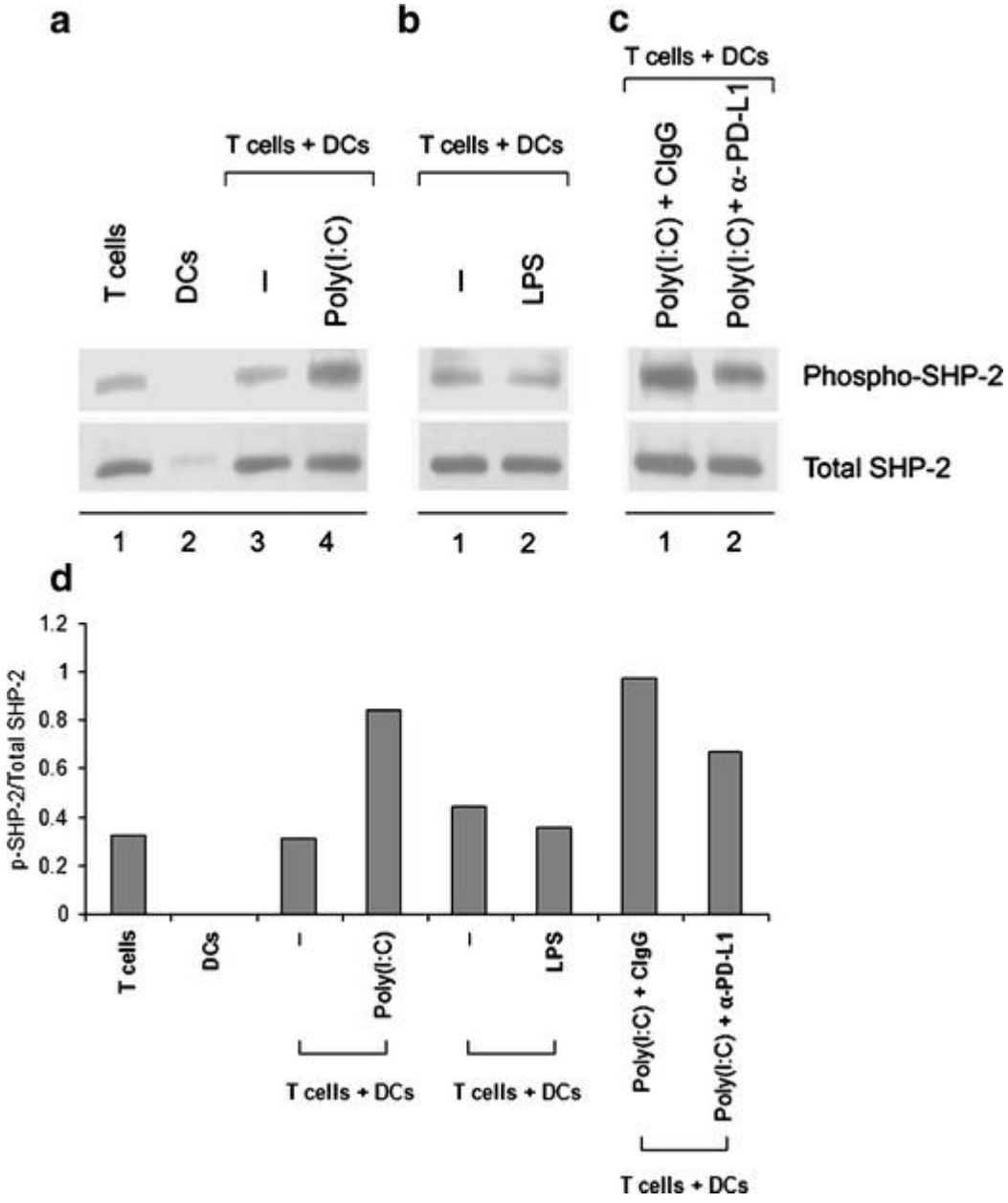

Figure 2.7. TLR3-stimulated DC enhance SHP-2 phosphorylation in CD4 T cells downstream of PD-1/PD-L1 interaction.

Figure 2.7. TLR3-stimulated DC enhance SHP-2 phosphorylation in CD4 T cells downstream of PD-1/PD-L1 interaction. **A**, **B.** CD4 T cells were cocultured with unstimulated (**A**, lane *3* and **B**, lane *1*), poly(I:C)-pretreated (**A**, lane *4*), or LPS-pretreated (**B**, lane *2*) DC for 24 h, lysed, and cell extracts were resolved on SDS-PAGE acrylamide gels. Proteins were transferred onto PVDF membranes and membranes probed for phospho-SHP-2 (*upper row*). Membranes were stripped and reprobed for total SHP-2 (*lower row*). Lanes *1* and *2* in **A** are T cells and DC cultured alone. **C.** CD4 T cells were cocultured with DC as in **A**, lane *4*, but with the addition of either control-IgG1 (lane *1*) or anti-PD-L1 blocking mAb (lane *2*) and probed for phospho and total SHP-2. Results in **A**–**C** are representative of three independent experiments. **D.** Quantification of the phospho-SHP-2/SHP-2 ratios was performed using the ImageJ software (NIH).

Discussion

DC hold a central position in alerting the host immune system to infection and in coordinating early and late immune responses. They are known as the most effective DC in T-cell priming, but remain critical even during chronic immune reactions. The present study shows that the nature of the initial DC stimulus is crucial in preparing the DC for the induction of T-cell responses. Indeed, ligands activating TLR3 induced DC maturation but also conditioned the DC to inhibit CD4 T-cell proliferation. This activity of the TLR3 ligand poly(I:C) contrasted with the robust effectiveness of the TLR4 ligand LPS in generating stimulatory DC that drove CD4 T cells into expansion. The inhibitory effect of TLR3-pretreated DC involved intermediate steps in T-cell activation while early events were spared, guiding mechanistic studies towards receptor-ligand pairs that were by themselves T cell-activation dependent. Indeed, T-cell non-responsiveness was at least partially reversible by blocking access to PD-1, an inducible co-inhibitory receptor appearing on T cells after activation. CD4 T cells primed with TLR3-conditioned DC displayed a typical signature of phosphorylated SHP-2, a phosphatase inhibiting TCR-mediated signals.

The synthetic analog of double-stranded RNA poly(I:C) mimics a molecular pattern associated with viral infection. Poly(I:C) interacts with two types of pattern recognition receptors, TLRs and the RIG-I-like receptors (RLRs) (148). Experiments probing the effects of the RLR stimulator poly(dA-dT) revealed that only poly(I:C) had the ability to selectively induce the co-inhibitory molecule PD-L1 (Fig. 2.4), assigning this mechanism to the TLR3 receptor. Thus, biologic consequences of accessing a particular pattern recognition system may be profound, and viral RNAs recognized

through either endosomal TLR3 or cytoplasmic RNA helicases may modulate innate and subsequent adaptive immune responses in a very unique fashion.

TLR3 is expressed by a variety of different cell types (myeloid and monocyte-derived DC, fibroblasts, epithelial cells, NK cells, and some T-cell subpopulations) (123, 151)and has been postulated to be a key player in anti-viral immunity (127). Immune responses against viruses require an intricate orchestration of innate signals provided by DC and adaptive virus-specific responses (152). Evidence suggests that MHC class I-restricted recognition of virus-associated antigens by cytotoxic CD8 T cells is a critical pathway in eliminating virus-infected cells and preventing further spread of the infection (152). CD4 T cells are thought to play an important auxiliary role in coordinating anti-viral adaptive immunity (153). Our data indicate that the PD-1/PD-L1 pathway is employed by DC for suppression of adjacent CD4 T cells after licensing by TLR3 stimuli. Such DC remain capable of initiating the early T-cell activation cascade and produce type I interferons, which may contribute to anti-viral immunity. This bypass of CD4 helper T-cell inhibition might have evolved as a mechanism to prevent overwhelming immune stimulation. In the course of a viral infection, the host organism needs to detect and clear the pathogen, which involves not only broad DC stimulation, but also the expansion of self-reacting T cells. In this context, the concept has been put forward that autoimmune disease is precipitated by antiviral immunity. In view of the potentially detrimental outcome of immune activation, downregulation of CD4 T-cell help by DC after detection of the viral agents emerges as a powerful and targeted mechanism to prevent immunopathology. On the other hand, viruses may ultimately escape host immunity by exploiting such a pathway intended for maintenance of

peripheral tolerance (128). Data presented here suggest that anti-viral immune responses controlled by CD4 T cells may be effectively compromised by triggering of intracellular TLR3 receptors with reprogramming of DC away from T-cell priming functions towards suppression of CD4 T cells. This hypothesis is consistent with recent reports demonstrating that wild-type mice are more susceptible to West Nile Virus-mediated brain inflammation than TLR3-deficient mice (154). The role of TLR3-activated DC *in vivo* and their modulatory function during the course of viral infection warrant validation from studies focusing particularly on the contribution of CD4 T cells.

T cells exposed to TLR3-conditioned DC did not undergo cell death but remained IL-2-responsive, a feature typically seen in anergic T cells. Also, blocking with anti-PD-L1 antibodies reversed their hyporesponsive state. Phenotyping of activated CD4 T cells in cocultures confirmed that TLR3-conditioned DC did not instruct T cells to die but to become hypoproliferative instead. T-cell cell cycle entry was comparable in cocultures driven by LPS and poly(I:C)-treated DC, but the frequencies of CD69-expressing CD4 T cells was eventually higher if DC were conditioned with LPS. In essence, CD4 T cells exposed to TLR3-triggered DC entered the activation program but could not maintain it. Upregulation of PD-1 receptors was intact, making these CD4 T cells sensitive to inhibitory signals. The cytoplasmic domain of PD-1 contains both an immunoreceptor tyrosine-based inhibitory motif (ITIM) and a switch motif (ITSM). The ITSM is considered to be important in mediating the inhibitory PD-1 function by the recruitment of SHP-2 (32, 149). Subsequent phosphorylation of SHP-2 induces its phosphatase acitivity, thus mediating the negative signals of PD-1 by dephosphorylating and inactivating effector molecules downstream of TCR and CD28, such as ZAP-70 and

phosphatidylinositol 3-kinase (PI3K) (41, 149, 155). This eventually leads to impaired IL-2 synthesis, inhibition of glucose metabolism, and T-cell cycle arrest (150, 156).

Our results confirm that SHP-2 is the key adaptor in the inhibitory PD-1 signaling cascade which was activated by poly(I:C) stimulation. However, this also raises the question as to why LPS-pretreated DC were not equally capable of initiating a similar process. First, we have found that poly(I:C) stimulation resulted in preferential upregulation of PD-L1 transcription, biasing the signaling potential of the differentiating DC towards inhibition versus stimulation. TLR4 binding obviously enhanced CD86 and, to a lesser degree, PD-L1, and functionally skewed the balance towards amplification of TCR-mediated signals. Of note, it has been shown that the upregulation of costimulatory molecules by poly(I:C) is only partially dependent on the TLR3-TRIF-signaling axis, but also occurs in the absence of the adapter TRIF, whereas LPS always requires the presence of TRIF (157). Second, in blocking LPS-treated cultures with anti-PD-L1 mAb, we did observe minor enhancement of T-cell proliferation. As the fate of T-cell stimulation ultimately depends on the integration of positive and negative signals, LPS-conditioned DC favor providing amplificatory signals, which outweigh incoming inhibitory signals. The signaling studies shown in Fig. 6 support this notion. However, we cannot rule out the possibility that ligation of PD-1 may lead to a number of different scenarios. The ITSM motif on the cytoplasmic domain of PD-1 may interact with different adapter molecules, depending on the activating stimulus T cells receive; as a consequence, PD-L1 could transmit either positive or negative signals, or bind to a putative costimulatory receptor other than PD-1 (130, 158-160).

Data presented here have multiple implications which are also of clinical relevance. If TLR3 mediates anti-viral responses and the downregulation of CD4 T-cell proliferation induced by TLR3-triggered DC represents a viral escape mechanism, then blocking PD-1/PD-L1 interaction could be useful in enhancing anti-viral responses. In murine models, PD-1 and its ligands as critical contributors to the exhaustion of CD8 T-cell memory responses have been implicated in the chronicity of a number of viral infections (128, 161). In HIV-infected individuals, impaired virus-specific CD8 T cells overexpressed PD-1, and the frequency of such T cells has been suggested as a marker of disease progression and response to HAART therapy (162, 163). Blocking PD-1 in the LCMV model was sufficient to recover CD8 T-cell proliferation and to improve their antiviral activity (162-165). Similar findings were obtained in other human chronic viral infections such as hepatitis C virus infection (166). PD-L1 was also found to be upregulated in patients with chronic HBV infection and induced defective HBV-specific T-cell responses (167).

Besides its role in anti-viral immunity, TLR3 has a number of critical functions in regulating adaptive immune responses and functional activities of nonimmune cells. TLR3 stimuli can augment antitumor immunity by directly triggering apoptosis of human breast cancer cells. Evidence has been provided that the PD-1/PD-L1 axis plays a role in cancer immunology. In patients with renal cell carcinoma, PD-L1 was found to be expressed by tumor cells and tumor-infiltrating lymphocytes, and the extent of PD-L1 expression correlated with tumor aggressiveness, thus suggesting that this could represent a mechanism through which tumors evade host immunity (132). Notably, experimental studies in mice implanted with human ovarian cell carcinoma demonstrated that the

transfer of T cells preconditioned with PD-L1-blocked DC effectively reduced tumor growth (129). Conversely, poly(I:C) has been explored as an adjuvant to enhance the specific anti-tumor immune response against peptide-based vaccines (168, 169). Our findings suggest that poly(I:C) may mediate divergent effects, functioning as an immunostimulator under certain conditions but impairing immune responses, especially when deviating DC maturation toward PD-L1 high expressors.

It is expected that dsRNA-derived immune stimulation also has profound effects in inflammatory autoimmune diseases, such as experimental inflammatory arthritis (170). The suppression of T-cell proliferative capacity in our studies is in line with a previous report in which poly(I:C) was used as an adjuvant component in experimental autoimmune encephalomyelitis and failed to induce T-cell proliferation (171). In that disease model, poly(I:C) can be viewed as a potent immunoregulatory agent, significantly limiting disease severity and progression driven by CD4 T cell-mediated damage. Mechanistic studies in animal models have suggested that the anti-inflammatory action of dsRNA in experimental autoimmune encephalomyelitis could be due to an upregulation of type I interferons leading to increased PD-L1 expression together with suppression of CD4 T cells (172). In these studies, blockade of PD-1/PD-L1 resulted in disease acceleration (135, 173). Loss of peripheral tolerance is a sine qua non condition in the development of autoimmunity, but impairment of central tolerance mechanisms might also contribute. The PD-1/PD-L1 axis has a well-documented role in maintaining peripheral tolerance(174). However, recent evidence points towards an involvement in central tolerance mechanisms; blockade may prevent T-cell anergy in lymphoid organs upon tolerogen recognition (133).

In conclusion, recognition of dsRNA by DC results in a complex program of differentiation that can support both immunostimulatory and immunosuppressive pathways. DC conditioned by TLR3 ligands support early activation events in CD4 T cells but curtail their expansion by favoring the induction of co-inhibitory ligands. T cells exposed to such DC display an anergic phenotype, and their inability to clonally expand is associated with accumulation of phosphorylated SHP-2. Thus, SHP-2 emerges as a critical mediator in T-cell anergy, allowing for fast reversal of T-cell nonresponsiveness. Two interventions, supplying exogenous IL-2 as well as preventing PD-1 ligation, restored proliferation in the present studies. Therapeutic targeting of the PD-1/PD-L1 pathway should be explored in attempts to enhance anti-viral immune responses but may also prove useful in modulating CD4 T-cell pathogenic immune responses in the setting of autoimmune disease.

Chapter 3

Blocking the NOTCH Pathway Inhibits Vascular Inflammation in Large Vessel Vasculitis

Abstract

Giant cell arteritis (GCA), a granulomatous vasculitis, primarily affects the aorta and its branches. Vessel wall inflammation causes wall destruction or luminal occlusion resulting in life-threatening complications, including blindness, stroke and aortic aneurysm. The only established therapy is chronic treatment with high doses of corticosteroids. In search for novel therapeutic targets we analyzed inflamed temporal arteries for the expression of molecules sustaining vasculitis. Notch and its ligand Jagged1 were abundantly expressed, and cleavage of Notch intracellular domain (NICD) in wall-infiltrating T cells indicated ongoing activation of the Notch pathway. We devised two strategies to block Notch pathway activation; γ-secretase inhibitor treatment, preventing nuclear translocation of the NICD, and competing for receptor-ligand interactions through excess soluble ligand, Jagged1-Fc. In human arteries engrafted into immuno-deficient mice, Notch pathway disruption had strong immunosuppressive effects, inhibiting T cell activation in early and established phases of vasculitis. Notch inhibition was particularly effective in downregulating Th17 responses, but also markedly suppressed Th1 responses. Diminishing Notch signaling depleted T cells from the mural infiltrates, implicating Notch-Notch ligand interactions in regulating T cell retention and survival in the inflamed microenvironment. Modulating the Notch signaling cascade emerges as a promising new strategy for immunosuppressive therapy of large vessel vasculitis.

Introduction

Medium and large-sized arteries are vital structures which are highly susceptible to wall destruction or luminal stenosis when attacked by inflammatory immune responses. Vascular inflammation occurs in the setting of atherosclerosis but in a much more aggressive way in the primary vasculitides. Given the critical role of the aorta and its branches in blood supply, clinical consequences of medium and large vessel vasculitis are often severe and life threatening (1). The most frequent vasculitis manifesting in the walls of large blood vessels are two variants of giant cell arteritis (GCA); temporal arteritis affecting the elderly and Takayasu arteritis occurring in the young (175). This autoimmune syndrome is characterized by intramural and perivascular granulomatous lesions that destroy the vascular wall structure or induce occlusion of the lumen through fast and concentric outgrowth of neointima (93). GCA of the aorta leads to aneurysm formation and obstruction of primary branching points. GCA of the subclavian artery, the carotids, the vertebral artery, the ophthalmic artery and the temporal artery impairs blood flow and causes tissue ischemia. Clinical manifestations include blindness, stroke, “pulseless” disease and aortic aneurysm. Vascular inflammation is almost always combined with a systemic inflammatory syndrome, which presents as fever, malaise, anorexia, failure-to-thrive and polymyalgia rheumatic (176).

While the etiology of GCA remains unresolved, studies of underlying disease mechanisms have demonstrated that vascular lesion formation is mediated by a maladaptive immune response, characterized by in situ activation of CD4 T cells (177). In the vessel wall CD4 T cells receive activating signals from a population of tissue-resident vascular dendritic cells (vDC) (6). The characterization of T cell receptors in

distinct inflamed arteries has strongly supported the notion that recognition of disease-relevant antigens is ultimately driving the pathogenic immune responses (8). Three elements are now considered as critically involved in shaping the outcome of antigen encounter; the strength of the antigen:TCR signal, the microenvironment, and accessory signals derived from the antigen-presenting cell (DC). DC surface receptors have the capacity to co-stimulate or co-inhibit signals mediated through the TCR and to ultimately shape the outcome of the T cell activation cascade.

The current project has examined how interfering with Notch-Notch ligand interactions affects T cell activation in vasculitis, with the goal of targeting such interactions for therapeutic interventions. Notch is best known for its role in regulating lineage decisions and differentiation during postnatal development (178). The detection of Notch1 mutations in over 50% of T cell-acute lymphoblastic leukemias has highlighted a contributory role in cancer development (179)and has emphasized that the Notch pathway may have a central position in regulating T cell functions. Notch encodes a transmembrane receptor with conserved extracellular and intracellular domains (52). Signal transduction via the receptor is initiated by ligand binding which leads to two proteolytic cleavage processes. The first is mediated by ADAM family metalloproteases, while the second is catalyzed by γ-secretase (180). The latter cleavage event liberates the Notch intracellular domain (NICD) facilitating its translocation to the nucleus, where it induces the transcription of target genes that include Hes (Hairy enhancer of split) and other Hes related proteins (HERP) (50).

Although the role of Notch is well defined in lymphocyte development, and despite the fact that the expression pattern suggests a key function in peripheral T cells, little is

known regarding its specific effects on peripheral immunity. In mammals 4 Notch receptors (Notch1-4) and five ligands (Jagged1 and 2, Delta1, 3 and 4) have been identified thus far. Notch 1 and 2 appear to be constitutively expressed in naïve CD4 T cells while expression of all 4 receptors increases following antigen stimulation. The Notch ligands Delta 1, Jagged 1 and 2 but not Delta 3 or 4 have been detected in primary human T cells (74). However, how Notch-Notch ligand interactions influence the function of mature T cells remains incompletely understood. Experimental data suggest cross-talk between the TCR signaling cascade and the canonical Notch signaling pathway (181). Colocalization and functional studies suggest involvement of Notch in TCR mediated activation of peripheral T cells (75, 76). Decreased proliferation, cytokine production and viability, induced by pharmacological Notch inhibition (78), point to a role in the amplification of adaptive immune responses. In keeping with its contribution to early cell fate decisions, Notch has also been assigned a role in regulating Th1 vs. Th2 divergence (85, 87, 182), and has been implicated in driving regulatory T cell differentiation (80, 183, 184).

Current therapeutic approaches in large vessel vasculitis rely on non-specific immunosuppression, specifically high doses of corticosteroids given over prolonged time periods, sometimes over decades. A lack of knowledge of causative antigens limits the possibilities for an antigen-specific approach. Given the chronicity of the disease process, coupled with the central role of CD4 T cells, interfering with in situ T cell activation in the unique microenvironment of the vessel wall emerges as an attractive strategy. T cell function in large vessel vasculitis can be studied in a preclinical model in which vessel wall inflammation is induced by adoptively transferred human T cells in human arteries

engrafted into SCID mice. In this disease model, disrupting the Notch-Notch ligand pathway has profound implications, suppressing T cell activation, cytokine production and accumulation of intramural T cell infiltrates. Blockade of the Notch pathway using the γ-secretase inhibitor (GSI) *N*-[*N*-(3,5-difluorophenacetyl)-l-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT), and the soluble Notch ligand Jagged1-Fc, both inhibit CD4 T cell responses in the vasculitic lesions. Immunosuppression through Notch blockade is effective during the early and established phase of vascular inflammation, opening the possibility for a novel therapeutic intervention in treating GCA.

Material and Methods

Tissues and Cells

Temporal arteries affected by GCA were derived from patients undergoing diagnostic biopsies. Normal human temporal, distal subclavian and axillary arteries and aorta were collected from surgical waste or from early postmortem tissues. Arteries were used if free of atherosclerotic lesions and rejected with any signs of wall remodeling. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood obtained from healthy donors and patients freshly diagnosed with GCA using a Ficoll gradient (Mediatech, VA). CD4 T cells were purified using positive selection with CD4 microbeads (Miltenyi Biotec). Monocyte-derived DC were generated from $CD14^{+}$ monocytes isolated by positive selection with human CD14-specific microbeads (Miltenyi Biotec, Auburn, CA) by 6-day culture supplemented with 1000U/ml GM-CSF and 800U/ml IL-4. All protocols were approved by the Emory University Institutional Review Board and informed consent was obtained.

Antibodies and Reagents

Antibodies to human CD4-FITC and CD25-DC were purchased from BD PharMigen (San Diego, CA); anti-human Notch1-PE (clone 527425) was obtained from R&D systems. Anti-Notch1 (ab27526;Abcam) was used for extracellular detection of Notch1. Antibodies against cleaved intracellular domain of Notch1 (ab8925;Abcam) were used for flow cytometry and immunohistochemistry. Goat-anti rabbit-Alexa 488 was purchased from Stem Cell Technologies. The recombinant Jagged1-Fc fusion protein was

manufactured by R&D systems. DAPT and LPS (Escherichia coli, 0127:B8) were obtained from Sigma-Aldrich (St. Louis, MO).

CFSE Dilution Assays

Purified CD4 T cells were labeled with 5μM CFSE (Molecular Probes, Eugene, OR) and quenched by the addition of fetal calf serum. CFSE loaded T cells were incubated with 10μM DAPT reconstituted in DMSO or the equivalent dose of carrier DMSO 30 mins prior to initiation of cultures. T cells were activated in flat bottom 96 well plates coated with α-CD3 (1μg/ml) and α-CD28 (1μg/ml) Abs. For cellular co-culture assays T cells were stimulated in round bottom 96 well plates with monocyte derived DC activated with 1μg/ml LPS (Sigma), in the presence of 1μg/ml OKT3. DC were added to the T cells at a ratio of 1:10. On day 3, cells were harvested and proliferation was measured flow cytometrically by loss of CFSE fluorescence.

Proliferation Assay

T cells were cultured in flat bottom 96 well plates, at a density of $2x10^5$ cells/well, with plate bound α-CD3/CD28 (1μg/ml) Abs and varying doses of Jagged1-Fc (0.25-10μg/ml) or isotype control IgG. Cells were incubated for 48 h, pulsed with 1μCi of [^{3}H]thymidine and 24 h later proliferation was determined by [^{3}H]thymidine incorporation measured by liquid scintillation counting. Results are expressed as mean counts per minute of triplicate cultures $\pm$ SD.

Flow Cytometric Analysis

For cell surface expression analysis, PBMC were stained with the relevant primary antibodies or appropriate isotype controls for 30 mins at 4°C. For detection of intracellular molecules cells were fixed and permeabilized using Cytofix/Cytoperm (BD Biosciences). Permeabilized cells were incubated with appropriate anti-Notch1 antibody (ab8925; diluted 1:200) or isotype control (30 mins; 4°C) followed by incubation with FITC-conjugated secondary Ab (1:400) for 30 mins at 4°C. Cells were washed and data acquired using the BD/LSRII instrument and FACSDIVA software (BD Biosciences). Data analysis was performed with FlowJo software (Tree Star, Ashland, OR).

Immunohistochemistry

OCT embedded arteries were used to prepare slides as previously described. Briefly, 7μM tissue sections were blocked with 5% normal sheep serum, followed by incubation with the primary antibodies: rabbit anti-human CD3 (1:200) or rabbit anti-human Notch1 (ab8925; 1:200) overnight at 4°C. The tissue was then incubated sequentially with biotin-conjugated secondary antibody and Vector ABC Elite peroxidase complex for 30 mins each. Chromagen,3,3’ diaminobenzidine (DAB) was used for visualization of binding and slides were counterstained with hematoxylin. Isotype matched primary antibodies were used for control stains. Paraffin embedded temporal artery sections were de-waxed and immunostained with rabbit anti-human Notch1 (ab27526; 1:100) overnight at 4°C. Detection of antibody binding was performed as described above. For immunofluorescent analysis, OCT embedded tissue sections were stained overnight at 4°C with primary antibodies, and then incubated with secondary antibodies: goat anti-rabbit-Alexa 488 and

rabbit anti-mouse-Alexa 564 for 30 mins. Tissue sections were visualized by mounting in DAPI containing Vectashield (Vector Laboratories) and images from two fluorescent channels were merged using ImageJ software. All images were captured using an Olympus BX41(DP70BSW CCD camera) fluorescent microscope with 10x, 20x, and 40x apochromatic objectives (N.A. 0.4, 0.75. 0.9) (Olympus Co.)

Human Artery–SCID Mouse Chimeras

Axilliary artery sections were implanted subcutaneously on the lower dorsal midline of NOD.CB17 Prkdc (SCID) mice (Jackson Laboratory, Harbor, ME). On day 7 post-implantation mice received an IP injection of LPS (3µg/mouse) or PBS. Twenty-four hours later, PBMC ($4x10^7$ cells/mouse) pre-incubated for 30 mins with 1mg of DAPT or the vehicle DMSO, were adoptively transferred into the chimeras via IV injection. Mice treated with soluble ligand received an adoptive transfer of CD4 T cells pre-incubated with 100 µg of Jagged1-Fc or control IgG on day 8, followed by IP administration of 100µg of Jagged-1 Fc or control IgG on days 9 and 10. Arterial grafts were harvested on day 15 and shock frozen for RNA isolation and PCR analysis, or OCT embedded for immunostaining. For DAPT therapy of established disease, engrafted mice were injected with LPS on day 7, followed by adoptive cell transfer on day 8, and administered 1mg doses of DAPT by IP injection on days 12 through 15. Tissue was harvested on day 20 and processed appropriately for analysis. All animal experiments were done in compliance with protocols approved by the Emory University Institutional Animal Care and Use Committee.

Quantitative RT- PCR

RNA from shock frozen tissue was purified using Trizol, or extracted from human cell pellets using the Invitrogen PureLink RNA Mini kit. RNA was reverse transcribed (Roche) and amplified. Expression was analyzed by real time PCR using the MX3000 system. Specific PCR primers are listed in Supplemental Table 1. Relative RNA was normalized to $2x10^5$ copies of β-actin.

Statistical analysis

Data were analyzed using the Student's t test. Statistical significance was considered to be $p < 0.05$.

Supplemental Table 1. Human primer pairs for qPCR

Gene	Sense	Antisense
β-actin	5-ATGGCCACGGCTGCTTCCAGC-3	5-CATGGTGGTGCCGCCAGACAG-3
IFN-γ	5-ACCTTAAGAAATATTTTAATGC-3	5-ACCGAATAATTAGTCAGCTT-3
IL-17	5-AGGCCATAGTGAAGGCAGGAATCA-3	5-ATTCCAAGGTGAGGTGGATCGGTT-3
TCR	5-CCTTCAACAACAGCATTATTATTCCAG-3	5-CGAGGGAGCACAGGCTGTCTTA-3
CCR6	5-GGCGACTAAGTCATTCCG-3	5-CTCCGAGACAGTCTGGTAC-3
Smoothelin	5-TTGGACAAGATGCTGGATCA-3	5-CGCTGGTCTCTCTTCCTTTG-3
Notch1	5'GGTCAATGCGAGTGGC-3	5'GGCAGCAAGGCTACTGTG-3
Jagged-1	5-GCTGCCTTTCAGTTTCGCCTGG-3	5-GCAGAACTTATTGCAGCCAAAGCC-3
Hes-1	5-TGG GTG CCA AGC ACT GC-3	5'-TCG TGA CCA CCT TGT TTT TCT G-3

Results

Notch expression in the vascular lesions of Giant Cell Arteritis

In GCA granulomatous vessel wall infiltrates are composed of highly activated macrophages and T cells. Many of the genes overexpressed in the lesions have been attributed to specialized macrophage functions (88). T cells recruited into the intramural lesions produce IL-2 and IFN-γ but it is unknown whether specialized T cell subsets mediate the inflammation and how co-stimulatory receptor-ligand pairs modulate function of vasculitis T cells. To examine whether Notch1 and its major ligand Jagged1 are expressed in GCA vessel wall lesions, temporal artery specimens from patients with typical histomorphologic findings were analyzed. Real time-PCR studies of tissue extracts from GCA affected arteries detected 6-fold greater Notch1 expression than that measured in normal arteries. Also, the Notch ligand Jagged1 was expressed with high abundance and was detected at up to 10-fold higher transcript levels in diseased vessels. (Fig. 3.1A). Cellular analysis of tissue sections revealed dense infiltrates penetrating deep into the medial layer, with almost all mononuclear cells being positive for the T cell marker CD3 (Fig. 3.1C and 3.1F). Notch1 receptor expression was a feature of the majority of intramural lymphocytes (Fig. 3.1D). Staining for the cleaved intracellular domain of Notch1 (NICD) (Fig. 1G) showed that the role of the Notch1 receptor in GCA arteries extended beyond that of a mere cellular marker, and that activation of the Notch pathway is a feature of the tissue-invading lymphocyte population. Notch activation in T cell infiltrates was confirmed at the single cell level by co-staining for NICD and CD3, which showed colocalization at the cell surface (Fig. 3.1H). The widespread NICD detection provided confirmatory evidence that cells participating in the vessel wall

infiltrates encounter Notch ligands in situ, resulting in the cleavage of the intracellular domain.

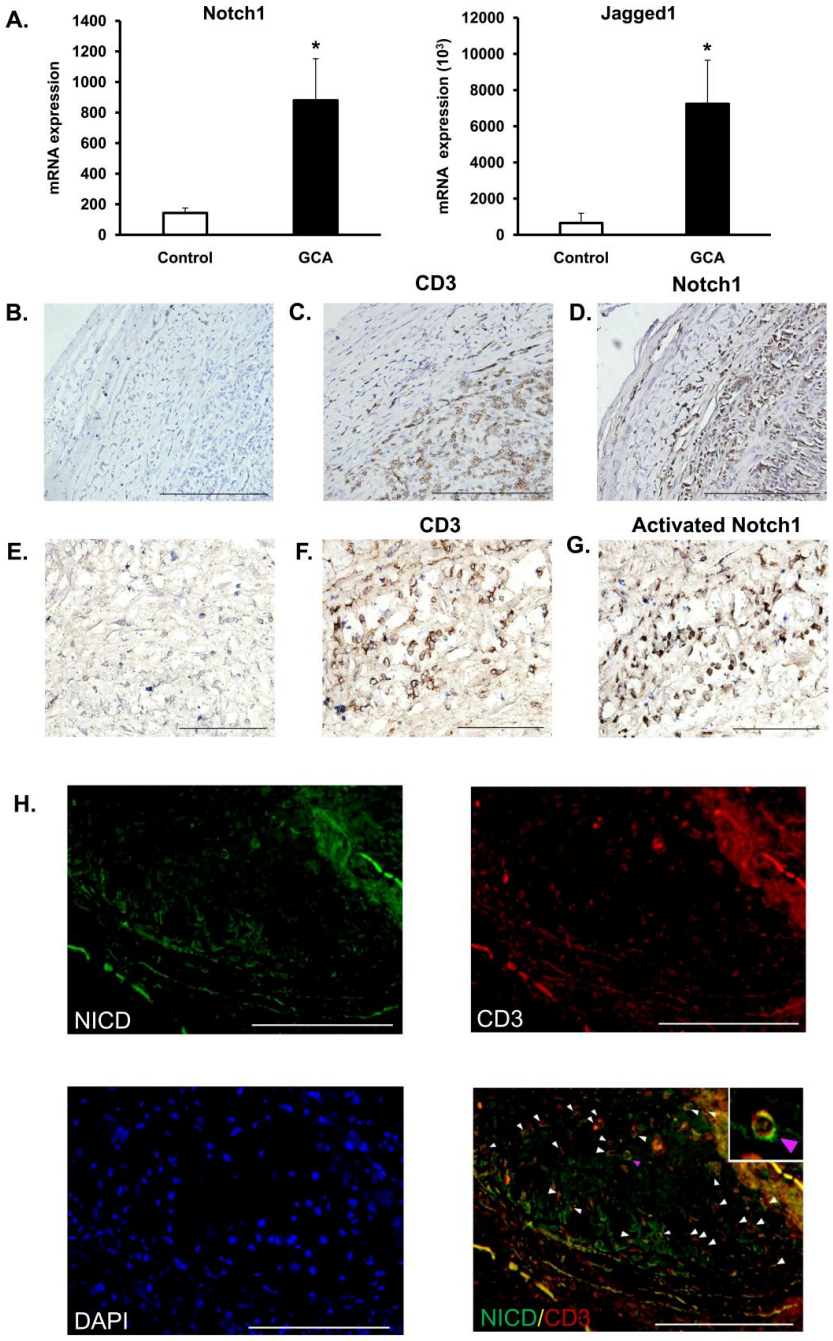

Figure 3.1. Activated Notch1 is abundant in GCA arteries.

Figure 3.1. Activated Notch1 is abundant in GCA arteries. RNA was isolated from diagnostic biopsies of temporal arteries, which either had no evidence for inflammation (Control) or showed granulomatous infiltrates typical of GCA (GCA). A. Expression levels of transcripts for Notch1 as well as the Notch ligand Jagged1 were quantified by real time PCR. Data shown are the mean $\pm$ SEM of 4 non-inflamed arteries and 4 GCA arteries. Paraffin-embedded temporal artery specimens affected by GCA were stained with isotype control (B), anti-CD3 (C) or anti-Notch1 (D) antibodies. Immunostaining of frozen temporal artery sections with isotype control antibodies (E), anti-CD3 antibodies (F) or antibodies to the cleaved intracellular domain of the Notch1 receptor (G) was performed to detect activation of the Notch signaling pathway in tissue-infiltrating T cells. Original magnification, x200 (B-D) Scale bar = 200µm; x400(E-G). Scale bar = 100µm. (H). Activated Notch1 and CD3 were visualized by immunofluorescence using Alexa 488-labeled (upper left-green) and Alexa 546-labeled (upper right-red) antibodies respectively. DAPI staining was used for visualization of nuclei (bottom left-blue) and colocalization of NICD and CD3 resulted in a yellow cellular stain (bottom right and inset). Magnification, x200. Scale bar = 200µm.*$p<0.05$.

Notch expression on circulating T cells in GCA

GCA is an HLA Class II restricted disease and independent vasculitic lesions from GCA-affected arteries harbor identical T cell receptors (8); strongly supporting the notion that the wall-invading infiltrates are ultimately driven by antigen recognition. However, it is not known where antigen is first encountered. Also, vascular inflammation is almost always combined with a syndrome of intense systemic inflammation, characterized by strong upregulation of acute phase reactants (185). We therefore explored whether Notch expression is limited to the vascular lesions or is also present in circulating immune cells. PBMC were collected from patients freshly diagnosed with vasculitis. Cell extracts were examined for the expression of Notch1 and Jagged1 transcripts. Induction of the Notch target gene Hes-1 was evaluated as evidence for ongoing Notch pathway signaling (Fig. 3.2). When compared to PBMC from healthy age-matched control individuals, PBMC from GCA patients contained 20-fold higher levels of Notch1 transcripts (Fig. 3.2A). Similarly, Jagged1 sequences were abundantly expressed in the GCA samples and barely detectable in the control donors. Ongoing signaling through the Notch pathway was confirmed by the upregulation of Hes-1 (1200 transcripts in controls; 3700 transcripts in GCA patients) (Fig. 3.2A). In essence, both Notch receptors and Jagged1 ligands are highly induced in PBMC from GCA patients and Notch-derived signals are constantly reaching the nucleus to sustain Hes-1 expression.

In the vascular lesions Notch1 appeared on lymphocytes. To evaluate whether circulating T cells spontaneously express high levels of Notch1 receptors flow cytometric

analysis was applied. Essentially, in GCA patients the entire population of peripheral CD4 T cells was strongly positive for Notch1 (Fig. 3.2B).

To test whether the phenotypic changes in circulating GCA CD4 T cells were associated with functional changes, expression patterns of two major T-cell cytokines were assessed. Samples of PBMC harvested from untreated GCA patients contained high levels of both IFN-γ and IL-17 sequences, demonstrating activation of T cells committed to the Th1 and the Th17 pathway (Fig. 3.2C). The concentrations of IFN-γ-specific sequences were more than twice as high in the patient-derived samples compared to the control samples. The differences were even more pronounced for IL-17. A 4-fold increase in the levels of IL-17 transcripts distinguished PBMC from GCA patients from those of healthy controls.

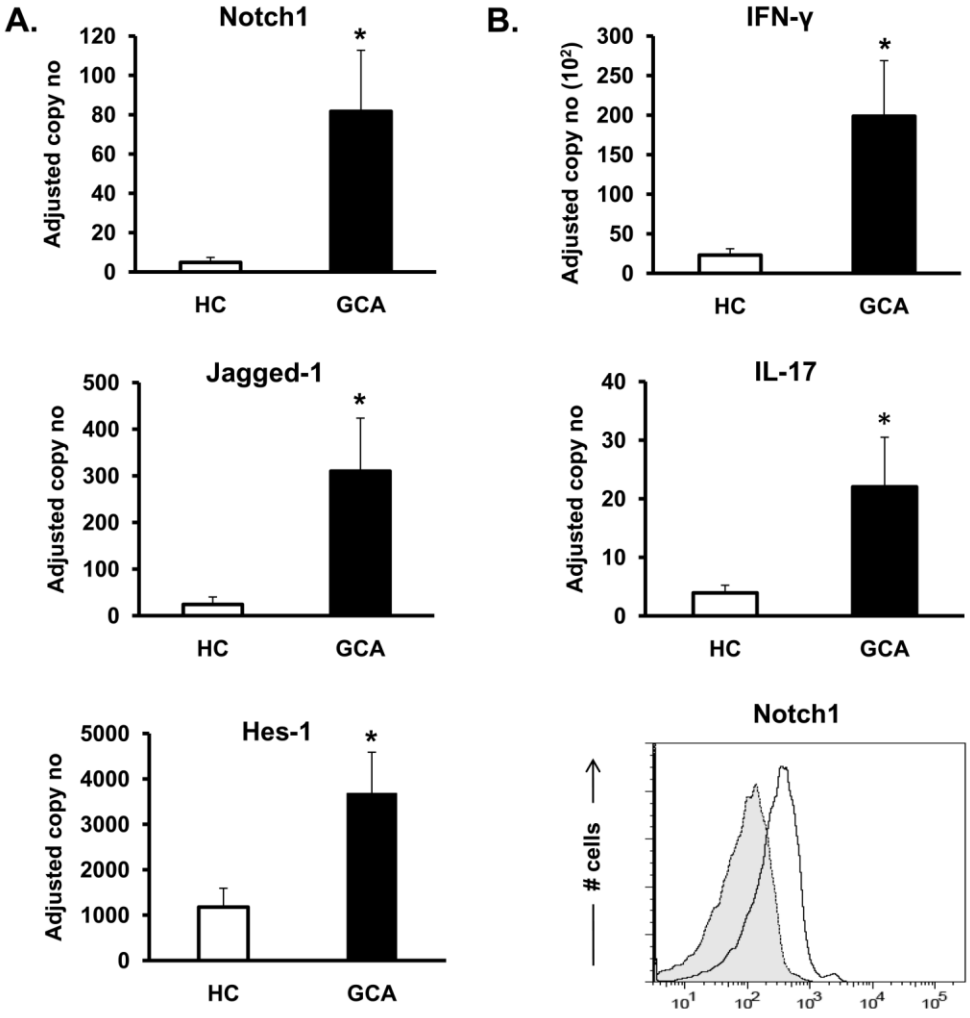

Figure 3.2. Expression of Notch1 receptor and the pro-inflammatory cytokines IL-17 and IFN-γ by peripheral T cells in GCA. A. Peripheral blood mononuclear cells (PBMC) were isolated from 8 GCA patients and 8 healthy controls (HC). A. Notch1, Jagged-1 and Hes-1 transcripts were quantified by real time PCR. B. Concentrations of IL-17 and IFN-γ specific transcripts were measured by quantitative RT-PCR. Data are shown as the mean ± SEM. C. PBMC were isolated from patients with untreated GCA and stained for CD4 and Notch1 receptor. Surface expression of Notch1(black line) on CD4 T cells was determined by flow cytometry. Isotype control is represented by shaded histogram.

T cell activation enhances Notch expression by CD4 T cells

To examine which signals could mediate the upregulation of Notch1 expression on peripheral and tissue-residing T cells in GCA, we studied the impact of TCR stimulation on Notch1 expression. Stimulation with anti-CD3/CD28 promptly doubled the levels of Notch1 transcripts in T cells (Fig. 3.3A). This increased transcriptional activity was reflected in a correlate increase in surface expression as measured by flow cytometry (Fig. 3.3B). Notch receptor expression increased in a time-dependent manner. By 24 hrs post-stimulation, levels had increased about 4-fold, reaching almost 8-fold higher levels by 48 hrs (Fig. 3.3C). This kinetic response demonstrated that changes in Notch1 receptor expression are an integral component of the T cell activation program and that Notch-Notch ligand interactions may not be as relevant early after antigen recognition but days after TCR ligation.

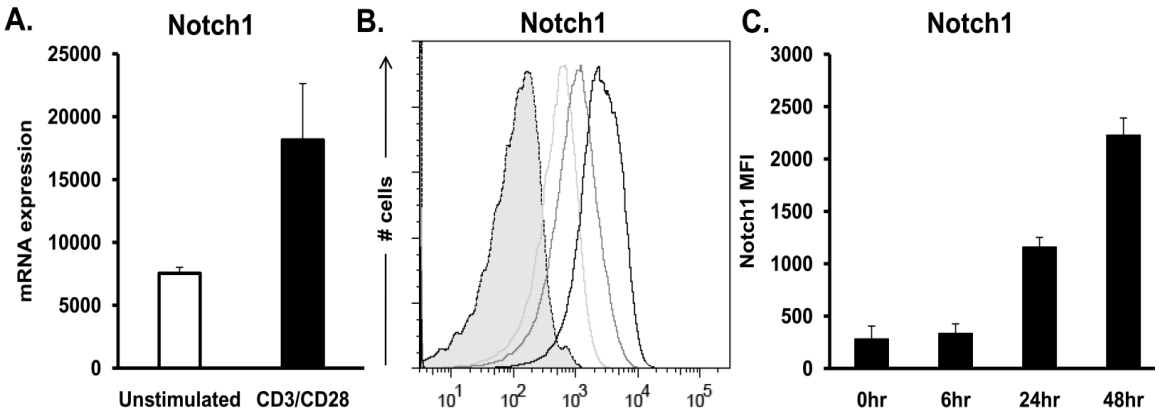

Figure 3.3. T cell activation enhances Notch1 surface expression. A. CD4 T cells purified from PBMC of healthy donors were stimulated with immobilized anti-CD3/CD28 mAbs. The cells were harvested at 24 h and Notch1 expression was analyzed by real time PCR. B. Alternatively, cell surface expression of the Notch1 receptor was quantified by flow cytometry on CD4 T cells stimulated with anti-CD3/CD28 (1µg/ml) for 0 (light grey line), 24 (dark grey line) or 48 h (black line). Shaded histogram represents isotype control. Representative data from one of six independent experiments are shown. C. Cell surface expression of Notch 1 on CD4 T cells was measured as mean fluorescence intensity 0, 6, 24, or 48 h post stimulation. Data shown are the mean $\pm$ SEM from 6 independent experiments.

Notch blockade inhibits CD4 T cell function

In order to determine whether the presence of the Notch1 receptor on T cells translated into functional consequences, we inhibited Notch activity using the γ-secretase inhibitor DAPT. T cell activation was initiated by triggering of the CD3 and CD28 receptors. As expected, TCR cross-linking resulted in vigorous T cell proliferation. CFSE dilution studies revealed that by day 3 more than 50% of cells had entered the cell cycle. Cell cycle kinetics were unaffected by DMSO which was used as a vehicle for the enzyme blocker (53.2% of the cells had divided) (Fig. 3.4A). Cells stimulated in the presence of the Notch inhibitor showed a 25% decrease in proliferation with only 39% of cells progressing through the cell cycle (Fig. 3.4A). The CFSE dilution studies demonstrated that interference with the enzymatic activity of γ-secretase stopped cells from entering the cell cycle and delayed cell cycle progression with only a small fraction of cells having entered a 3rd cell cycle.

To confirm that DAPT was efficient in disrupting the Notch signaling cascade we assessed the accumulation of NICD, a process directly dependent on enzymatic activity of the γ-secretase complex(180). Treatment with DAPT was sufficient to abrogate the accumulation of NICD in T cells undergoing activation with intracellular NICD levels diminished after short–term and prolonged T cell activation (Fig. 3.4B and data not shown). To investigate whether the effects of DAPT on Notch signaling extended downstream of Notch receptor cleavage, the induction of the Notch target gene Hes-1, was measured. In DAPT treated cells there was an 80% reduction in the level of this transcription factor (Fig. 3.4C), providing additional confirmation that the γ-secretase inhibitor was actively targeting the Notch pathway.

To more closely approximate the in vivo activation of T cells and mimic conditions encountered by T cells interacting with wall-residing dendritic cells, we explored the impact of Notch inhibition in T cell-dendritic cell coculture assays. In control cultures 63% of the T cells upregulated the activation marker CD25 and a similar proportion of cells underwent activation in the presence of DMSO as a vehicle (Fig. 3.4D). Inhibiting intracellular Notch cleavage by DAPT reduced T cell activation with only 47% of the T cells acquiring CD25 expression. The CFSE dilution analysis confirmed that a high proportion of T cells never entered the cell cycle if Notch cleavage was prevented.

In essence, Notch-Notch ligand interactions provide co-stimulatory signals, amplifying the process of T cell activation and regulating T cell expansion.

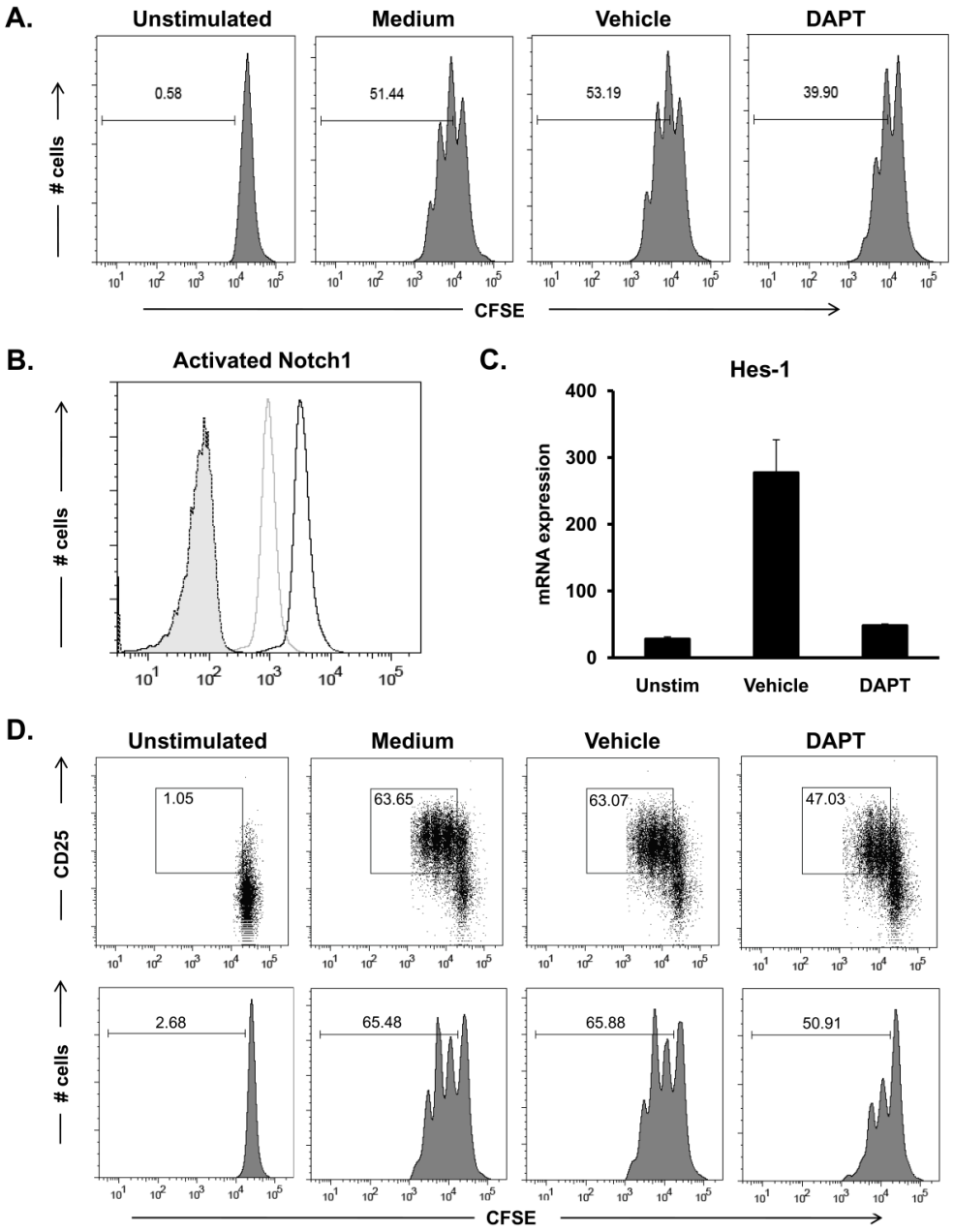

Figure 3.4. Notch blockade dampens T cell responses.

Figure 3.4. Notch blockade dampens T cell responses. A. Freshly isolated CD4 T cells were stimulated with immobilized anti-CD3/CD28 mAbs (1µg/ml) in the presence or absence of the γ-secretase inhibitor DAPT (10µM) or vehicle control. CFSE dilution was evaluated by flow cytometry at 72 h using forward and side scatter gates. B. CD4 T cells were stimulated for 24 h with anti-CD3/CD28 (5µg/ml) in the presence of DAPT (light grey line) or vehicle (black line) and analyzed for expression of the cleaved intracellular domain of the Notch1 receptor. Shaded histogram = isotype control. C. RNA was extracted from cells stimulated as described and analyzed by real time RT-PCR for Hes-1. D. T cells labeled with CFSE were cultured with LPS (1µg/ml) stimulated DC loaded with OKT3 (1µg/ml), in the presence or absence of DAPT or vehicle. On day 3 cells were stained for CD4 and CD25 (upper panels) and proliferation was assessed by flow cytometry using CD4 gates (upper and lower panels). Numbers shown indicate the number of cells that have divided. Data shown are representative of at least 6 independent experiments.

Inhibition of Notch signaling restricts early CD4 T cell infiltration in the arterial wall

In order to investigate the potential contribution of the Notch pathway in vascular inflammation we utilized a global Notch inhibitor in a human-artery SCID mouse chimera model. In this disease model human arteries are implanted into SCID mice and wall-residing dendritic cells are conditioned by injecting pathogen-derived molecular patterns, specifically TLR-4 stimulating lipopolysaccharides (6). Subsequently, adoptive transfer of allogeneic human T cell leads to accumulation of cellular infiltrates in the arterial wall, starting at 24 hours and progressing over the next 3 to 5 days.

To study the role of Notch signaling during the early phase of vasculitis, human T cells were co-administered with DAPT or vehicle (Fig. 3.5). To monitor the composition and the activity of tissue-infiltrating cells, tissue extracts of explanted arteries were analyzed for transcript levels of TCR and Hes-1. Notch inhibition had a profound effect on the invasion of T cells into the vessel wall layers. The density of T cell infiltration was measured by immunohistochemical quantification of CD3 T cells in tissue sections. As a control, one set of chimeras did not receive LPS and in such animals human T cells failed to enter the arterial structure (Fig. 3.5A). LPS injection followed by T cell infusion resulted in dense T cell infiltrates, penetrating throughout the wall; about 50 cells were found per high-powered field (Fig. 3.5A). Co-administration of DAPT inhibited the process of T cell recruitment/retention, and only 10 cells/hpf were detected in the human grafts. Loss of T cells in the intramural infiltrates correlated with a 45% reduction of TCR transcript levels, which were similar in non-inflamed arteries, and DAPT-treated arteries, both significantly lower than in arteries treated with vehicle (Fig. 3.5B).

To test whether disrupting Notch cleavage could successfully paralyze the Notch pathway we determined the expression levels of the Notch target gene Hes-1. Mice infused with DAPT-treated human T cells produced 80% less Hes-1 in the arterial grafts than mice with fully developed vascular infiltrates (Fig. 3.5C). In essence, interfering with Notch cleavage and translocation had profound implications for T cells invading the artery wall and essentially disrupted the process of T cell recruitment/retention.

Gene expression patterns in the explanted grafts were in keeping with visualization of tissue-infiltrating T cells (Fig. 3.5 D-F). Explanted arteries from animals that had never received LPS had no sign of inflammation. Adoptively transferred human T cells infiltrated deeply into the vessel wall in blood vessels exposed to LPS. This process of recruiting and retaining T cells in the vascular wall structure was successfully disrupted if the T cells were co-administrated with DAPT, blocking the cleavage of intracellular Notch and essentially disabling the Notch pathway.

The prevention of T cell accumulation in the vessel wall essentially abrogated inflammatory activity. Tissue IFN-γ and IL-17 production (Fig. 3.5G) were markedly suppressed if the Notch pathway was blocked, decreasing 30% and 90% respectively.

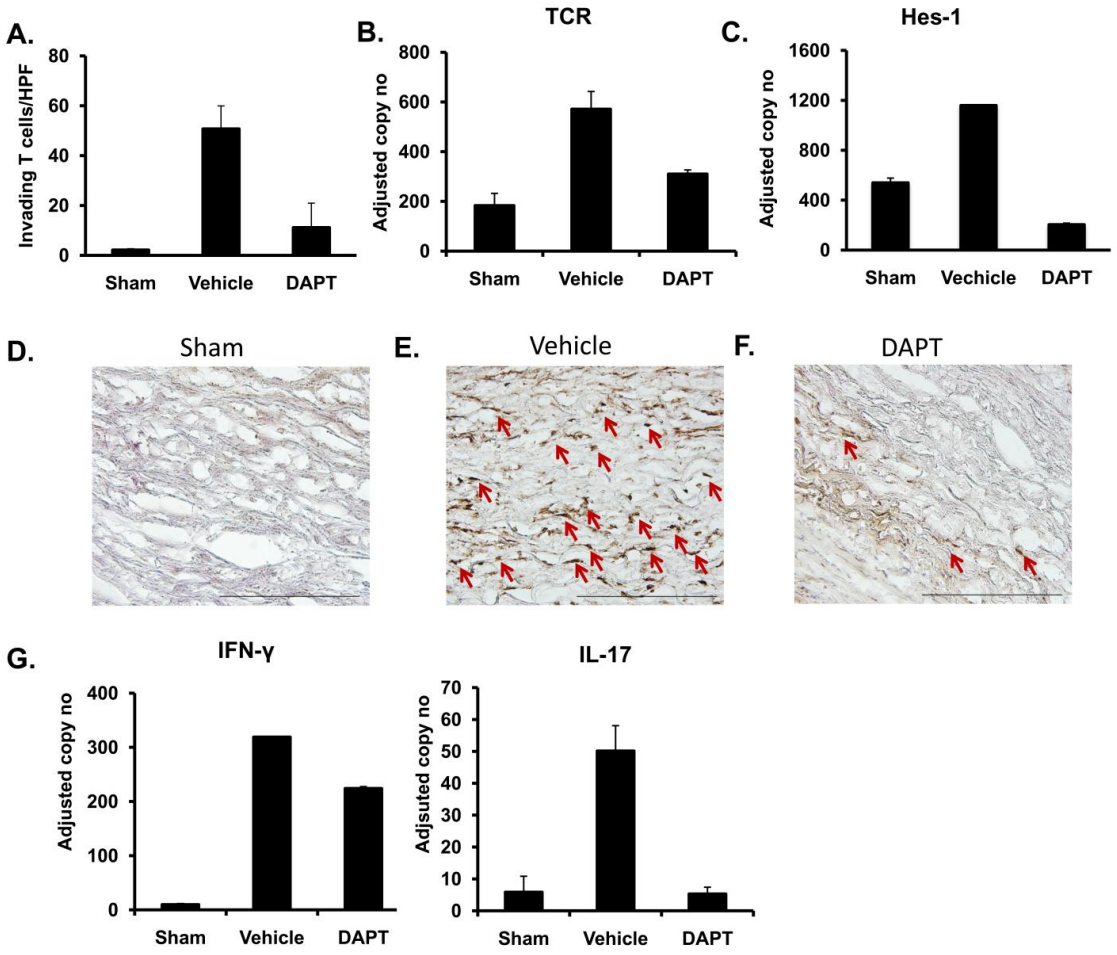

Figure 3.5. γ-secretase treatment suppresses vessel wall inflammation.

Figure 3.5. γ-secretase treatment suppresses vessel wall inflammation. Axillary arteries free of atherosclerosis or inflammation from 5 donors were implanted into SCID mice. On day 7 vessel wall dendritic cells were activated by injecting the chimeras with LPS (3μg/mouse), or sham treated with PBS. 24 h later DAPT- or vehicle- pretreated CD4 T cells were adoptively transferred by intravenous injection. Arterial tissue was explanted 1 week later and human T cell infiltrates were identified by staining with anti-CD3 antibodies (A, brown stain). Vessel-infiltrating T cells were enumerated in at least 10 randomly chosen high-powered fields. TCR transcripts (B) and Hes-1 transcripts (C) in the tissue were quantified by RT-PCR. Immunohistochemical detection of $CD3^+$ cells in arteries retrieved from sham- (D), vehicle- (E) or DAPT- (F) treated chimeras. Magnification x200. Scale bar = 200μm. G. Inflammatory responses were measured by quantifying mRNA expression for IFN-γ and IL-17 in explanted tissue grafts. Results are shown as mean $\pm$ S.D. of triplicate measurements and are representative of at least 5 independent experiments.

Jagged1-Fc treatment is protective against vessel wall inflammation

In an alternative yet complementary approach we utilized another strategy to prevent activation of the Notch pathway by occupying the Notch1 receptor with the exogenous decoy ligand Jagged1-Fc. Immunosuppressive effects of the soluble Notch ligand were first explored in in vitro systems. Triggering of CD4 T cells by cross-linking the TCR induced the activation marker CD25 on essentially the entire T cell population within 24 hours. In the presence of exogenous soluble Jagged1 Fc protein, T cell activation was curbed with a minor population acquiring surface expression of CD25 (Fig. 3.6A). To evaluate the potential of the decoy ligand to modulate T cell expansion, proliferation of CD4 T cells was measured 3 days after T cell stimulation by thymidine incorporation. A control Fc protein was able to improve the proliferative expansion, likely by interacting with inhibitory Fc receptors. Jagged1-Fc protein impaired T cell proliferation in a dose-dependent manner (Fig. 3.6B). At doses of 5-10 µg/ml Jagged1-Fc T cell proliferation reached only 50% of that in untreated T cells. CFSE dilution 3 days post-stimulation provided corroborating evidence of the inhibitory effect of the soluble Notch ligand, as stimulation in the presence of 5µg/ml Jagged1-Fc significantly diminished cell cycling by 75% (Fig. 3.6C)

In vivo therapeutic effects of Jagged1-Fc were probed in the human artery-SCID chimera model. Vascular inflammation was induced in engrafted human arteries by first conditioning the wall-embedded DC with the pathogen-derived motif LPS and then injecting human T cells. Transferred human T cells invaded the vessel wall; forming a transmural infiltrate. To explore immunosuppressive effects of Jagged1-Fc, 100µg of the soluble ligand was administered concurrent with the adoptive transfer of human T cells,

and two additional doses of Jagged1-Fc were given 24 and 48h later. Control animals received human IgG at a dose of 100μg/mouse. Treatment with Jagged1-Fc over a 3 day course effectively inhibited Notch-dependent signaling as indicated by the marked reduction of Hes-1 sequences by over 70% in explanted human arteries from Jagged1-Fc treated chimeras (Fig. 3.6D). The loss of Notch target gene induction was accompanied by a 38% loss of TCR sequences, indicating that fewer T cells were accumulating in the arterial wall (Fig.3.6E). Administration of Jagged1-Fc suppressed in situ production of both, IFN-γ (70%) and IL-17(90%) (Fig.3.6F), thus essentially eliminating the major inflammatory drivers of the vasculitic process.

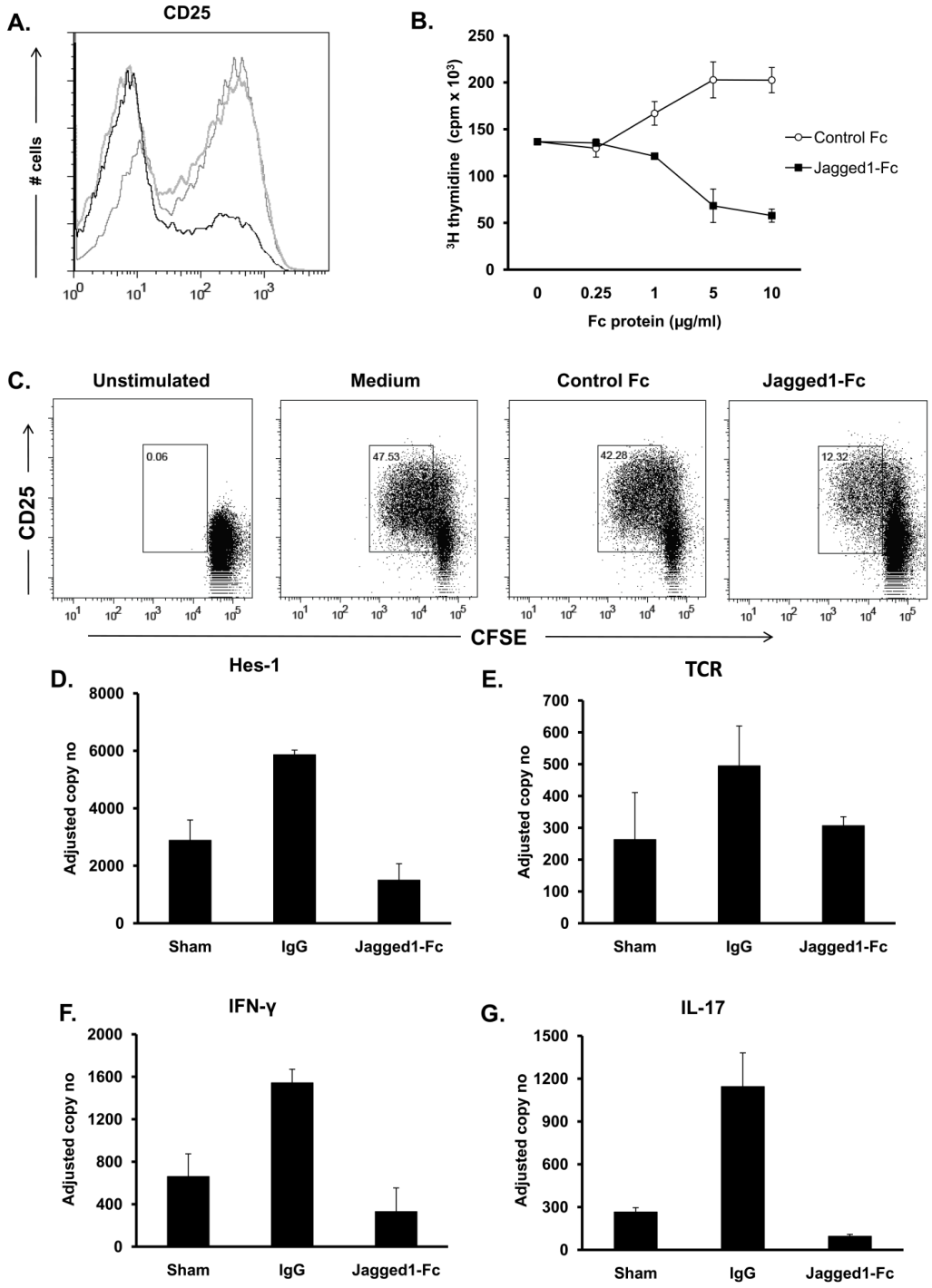

Figure 3.6. Soluble Jagged1 ligand inhibits T cell activation and abrogates vessel wall inflammation.

Figure 3.6. Soluble Jagged1 ligand inhibits T cell activation and abrogates vessel wall inflammation. T cells purified ex vivo from human PBMC were stimulated with immobilized αCD3/CD28 mAbs (1μg/ml) and graded doses of Jagged1-Fc or control IgG-Fc. A. Flow cytometric analysis was applied to assess CD4 T cells expressing the activation marker CD25 (light grey line). Jagged1-Fc (black line) or isotype control (dark grey line) were added at a dose of 5μg/ml. B. T cell proliferation was measured at 72 hours by [^{3}H]thymidine incorporation. Data are shown as mean of triplicate wells $\pm$ SD and are representative of at least 3 independent experiments. C. Isolated CD4 T cells were labeled with CFSE and incubated with αCD3/CD28 antibodies in the presence or absence of Jagged1-Fc or control Fc for 72 h. Cells were stained with antibodies to CD25 and proliferation was analyzed by flow cytometry. The numbers shown indicate the number of divided cells relative to total CD4 gated events. D. CD4 T cells pre-incubated for 30 min with Jagged1-Fc or control Fc were adoptively transferred to SCID-chimeras on day 8 and mice were administered additional doses of Jagged1-Fc or control Fc 24 and 48 h later via IP injection. Tissues explanted a week later were analyzed by real time PCR for Hes-1 (E) and tissue expression of TCR (F). G. Real time PCR was used to quantify IFN-γ and IL-17 transcripts in explanted tissue grafts. Representative data from one of at least five independent experiments are shown as mean $\pm$ S.D. of triplicate measurements.

Notch blockade ameliorates established vascular inflammation

Treatment with either DAPT or Jagged1 Fc protein resulted in a marked decline of tissue-infiltrating T cells and suppression of T cell cytokine production. To address the question of whether blockade of the Notch pathway could be successfully applied after T cells had already established intramural infiltrates and were receiving activating signals in the specialized microenvironment of the vessel wall, we inhibited Notch-derived signals in established vessel wall inflammation. For these experiments human arteries were engrafted into SCID mice and conditioned with LPS. Human T cells were infused one day later. Over the next 24 to 48 hours T cells accumulate in the human vessel where they undergo in situ activation. The cytokine production profile in such arteries is that of combined IFN-γ and IL-17 production. The subset of CCR6+CD4 T cells plays an important role in forming panarteritic infiltrates (103). Damage to the vessel wall can be demonstrated through a switch of the gene transcription profile. Specifically, vascular smooth muscle cells in the inflammatory milieu lose production of smoothelin as an indication of their transition from a contractile to a non-contractile phenotype.

Preventing the cleavage of the intracellular domain of Notch by administering the γ-secretase inhibitor DAPT 96 hours after the induction of vascular inflammation caused a marked reduction in the tissue production of both TCR (53%) (Fig. 3.7A) and the T cell subset marker CCR6 (80%) (Fig. 3.7B). Thus, Notch inhibitor treatment at the peak of vascular disease evolution was highly effective in thinning the T cell infiltrate. DAPT therapy essentially eliminated CCR6 transcripts from the lesions (Fig. 3.7B) indicating the depletion of a T cell subset implicated in the formation of transmural inflammatory infiltrates. The suppression of T cell function was associated with a decline in both IFN-γ

and IL-17 (Fig. 3.7C and 3.7D). Again, as seen in earlier experiments, DAPT appeared to be more effective in abrogating tissue IL-17 than tissue IFN-γ (86% vs. 62%). Confirmatory evidence for blockade of Notch-dependent activation came from quantification of the Notch target gene Hes-1 which was profoundly suppressed after injection of DAPT (Fig. 3.7E). Vascular damage quantified by the levels of smoothelin production was intense in the arteries invaded by the T cells; VSMC literally ceased transcribing this gene diminishing smoothelin levels by 98% of that in normal tissue (Fig. 3.7F). DAPT was able to partially reverse the smoothelin loss, restricting the reduction of smoothelin transcripts to 45% of the undamaged tissue grafts, indicative of protection of VSMC from the inflammatory injury.

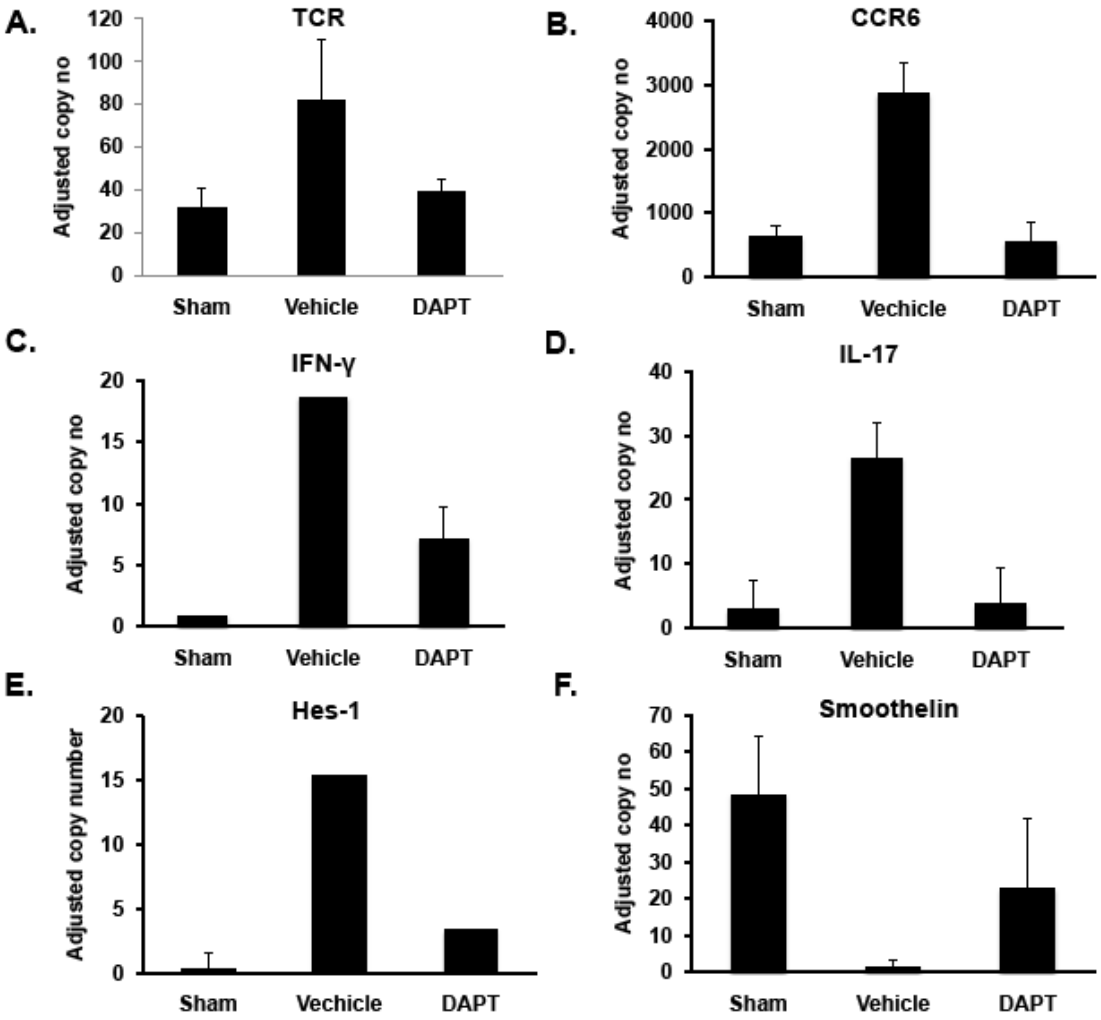

Figure 3.7. Notch inhibition attenuates ongoing T cell inflammatory responses in the vessel wall.

Figure 3.7. Notch inhibition attenuates ongoing T cell inflammatory responses in the vessel wall. Human artery-SCID chimeras were created as described in Fig. 5. On day 7 post-artery implantation, vascular dendritic cells were activated by injecting the chimeras with LPS (3μg/mouse), or sham treated with PBS. 24 h later allogeneic CD4 T cells were adoptively transferred into the chimeras via IV injection. 5 days after initiating vessel wall inflammation 1 mg DAPT or vehicle was administered by IP injection, with 3 subsequent doses given 24, 48 and 72 h later. Grafts were explanted on day 20 post-implantation and markers of human CD4 T cell infiltration in the arteries were quantified by RT-PCR for TCR (A) and CCR6 (B) transcripts. Inflammatory burden and in situ T cell activation was evaluated by measuring IFN-γ (C) and IL-17 (D) in arterial grafts by RT-PCR. E. Hes-1 expression in tissue grafts was measured to assess ongoing Notch signaling in the tissue infiltrating cells. F. Smooth muscle cell damage was assessed by quantifying the expression of smoothelin, a marker of smooth muscle cell viability and function. Data are shown as mean $\pm$ S.D. of triplicate measurements and are representative of 5 independent experiments.

Discussion

The Notch signaling pathway is mostly recognized for its role in determining cell fate, especially during development, tissue homeostasis and stem cell maintenance (178). As an evolutionarily conserved mechanism this pathway has been implicated in transducing signals between neighboring cells, emphasizing its participation in the 3-dimensional structuring of tissue sites. Here we report that the Notch pathway is of disease relevance in large vessel vasculitis, regulating the activity of vessel-wall infiltrating T cells that orchestrate tissue damage in this specialized microenvironment. Blocking Notch signaling has profound effects on T-cell dependent immune responses and suppresses the tissue production of pro-inflammatory cytokines, in particular IL-17. Both early and later stages of vasculitis are sensitive to disrupting Notch-Notch ligand interactions suggesting that this pathway can be developed into a target for novel immunosuppressive interventions in inflammatory vasculopathies.

GCA is a prototypic arteritis that is characterized by a strict tissue tropism for the aorta and its branches. In GCA affected arteries, CD4 T cell invasiveness, cytotoxicity and in situ cytokine production lie at the core of the pathogenic process (1). Dendritic cells serve as critical antigen presenting cells. Initiation of vessel wall inflammation requires DC activation and DC depletion abrogates chronic vasculitis (6). Thus, vasculitic T cells are dependent upon continuous instructions provided by DC. DC not only sustain T cell activation in the vessel wall microenvironment (17), they also shape the overall architecture of the disease process. DC-stimulatory signals delivered by TLR4 ligands induce panarteritis whereas exposure to TLR5 ligands results in a periarteritic pattern (103). DC are known to express Notch ligands, including Jagged and Delta ligands, and

differences in DC activation pathways have been associated with the induction of distinct T cell differentiation trajectories (87). Microbial components have been shown to regulate expression of Notch ligands in murine DC, and the observation of similar alterations in Notch ligands upon TLR triggering of human DC (data not shown) strongly supports the model that sensing of pathogen-derived patterns (21) and Notch-Notch ligand signaling are interconnected biologic events (186). In the current study blockade of the Notch signaling pathway was sufficient to markedly reduce the T cell infiltrate in the vessel wall, both during early and late stages of vasculitis. Immnohistochemical studies revealed that Notch blockade particularly affected the density of the wall-invading cell populations, with thinning of the T cell infiltrate, essentially leaving only few T cells in the vessel wall. This anti-vasculitic effect may either result from preventing T cell recruitment or from impairing T cell survival in the tissue niche. Given that Notch blockade had similar inhibitory effects in in vitro cultures it is unlikely that the Notch pathway affects only T cell migration, as T cell cytokine production was profoundly reduced. Also, kinetics of Notch receptor induction by T cell activation pointed towards a delayed role of Notch in the T cell activation program, not to early events. In a number of systems the Notch pathway has been associated with the regulation of lymphocyte activation and proliferation (187-189). This may also be the mechanism underlying the loss of T cells from the vasculitic infiltrates. Alternatively, Notch could hold an active role in regulating local T cell apoptosis in keeping with one of its described functions (190), a process crucially important in determining the size of the T cell pool and the density of tissue infiltrates. Finally, it is possible that Notch interferes with survival signals delivered to the wall-infiltrating cells. In that context it would be

important to know where the Notch-triggering signals derive from and whether this is unique for the microenvironment of the arterial wall layers.

The contribution of cytokines in GCA is well appreciated, both in the systemic inflammatory response as well as in the affected blood vessels (191). Despite the tissue-specific tropism that defines GCA, circulating conditions have been shown to be reflective of disease status in the patients, as indicated by increased serum levels of IL-6 in patients with active disease compared to that of patients following successful treatment (97). Temporal artery biopsy studies have provided a tissue cytokine profile suggesting that GCA is a Th1-driven disease in which local IFN-γ production drives disease progression (177). This coupled with the implication of Th17 cells in the pathogenesis of autoimmune disease (13) led us to investigate the circulating expression of these inflammatory cytokines. Upregulation of inflammatory T cell cytokines in the circulation went hand in hand with persistent activation of the Notch signaling pathway, as indicated by the induction of the Notch target gene Hes-1. The differential effects of Notch blockade on IFN-γ versus IL-17 production suggested a formidable role of Notch-Notch ligand interactions in shaping the T cell's commitment to a particular cytokine profile. Independent of the approach taken to suppress canonical Notch signaling, IL-17 production appeared more sensitive and IFN-γ more resistant. A preferential contribution of Notch signaling in Th17 cells is supported by in vitro studies in which Notch inhibition consistently is more effective in inhibiting IL-17 production (data not shown). A selective role of Notch-Notch ligand interaction in regulating the function of certain T cell subsets is supported by the finding that regulatory T cells appear to be unaffected by Notch blockade. Neither in the in vitro cultures nor in the SCID chimeras have we seen a

suppressive effect of Notch blockade on the development and presence of regulatory T cells (data not shown). The outcome of Notch signaling is cell-type specific and the emerging data support the notion that T cell cytokine production displays a differential dependence on the Notch pathway.

Given the current restriction of therapeutic options in large vessel vasculitis to high dose corticosteroids, a dissection of the intricacies of the unique signals that regulate and sustain the ensuing self-propagating inflammation is warranted for the development of more targeted therapy. Overall, our in vivo studies are strongly suggestive for a role of Notch triggering in the initiation of vascular wall inflammation. Disrupting Notch activation via GSI-inhibition or using decoy ligands has profound immunosuppressive effects, findings that support our hypothesis that modulating the Notch pathway may provide novel therapeutic opportunities in GCA. Precedence for the potential use of γ-secretase inhibitors in the amelioration of human disease exists in the field of cancer therapy in the treatment of tumors induced by aberrant Notch activation (63). Also, formulations for their use in the treatment of Alzheimer's disease have already entered clinical trials.

Chapter 4

Notch Signaling Preferentially Regulates the IL-17 pathway

Introduction

CD4 T cells are a heterogeneous population classified into subsets based on the unique cytokine production profiles generated in antigen-specific responses. Conventionally T helper cells have been stratified into two distinct groups: Th1 or Th2 cells (192). Recently, however, Th17 cells have emerged as an additional subpopulation. Th1 cell differentiation is driven by IL-12 and this subset is defined by IFN-γ production. Th2 development is regulated by one of its major cytokine products, IL-4. The distinction between the Th1 and Th2 subsets extends beyond a divergence in their cytokine profiles (193), as their unique phenotypes translate into functional differences such that Th1 cells are key regulators of cellular immune responses, while Th2 cells aid in the promotion of humoral immunity.

The CD4 T cell lineage has now expanded to include IL-17 producing T cells, known as Th17 cells (99, 107). IL17A and IL17F are among the major cytokine products (194) and while these cytokines have been shown to be important in host responses against extracellular bacteria and fungi (109), overproduction of these cytokines has also been implicated in the pathogenesis of numerous human inflammatory disorders that include multiple sclerosis, rheumatoid arthritis (RA), lupus, Crohn's disease and asthma (195-197). The contribution of Th17 cells to autoimmune pathology is supported by the inhibition of inflammatory responses in animal models of disease when the Th17 subset is selectively eliminated (198).

Studies aimed at investigating the cytokines and signaling pathways involved in the regulation of Th17 differentiation have revealed the transcription factor RORγt as the master regulator in shaping the development of this T cell lineage (199). As in other functional T cell subsets RORγt is directed by the co-operation of a distinct group of cytokines. In humans the specific cytokine milieu requirements differ from those identified in the mouse, foregoing the need for TGF-β demonstrated in the mouse (200, 201), and instead showing dependence on IL-1β, IL-6 and/or IL-23 for Th17 development (117). In keeping with the reciprocal regulation of Th1 vs. Th2 differentiation by IL-4 and IFN-γ, both of these cytokines negatively regulate Th17 differentiation (116).

An exploration of specific phenotypic characteristics, including cell surface expression of unique molecular markers, has provided insight into the role of Th17 cells. These investigations identified CCR6 as a significant functional marker for the IL-17 producing cells (13). RORγt + T cells isolated from human peripheral blood are contained within the CCR6+ CD4 memory T cell compartment. Expression of CCR6 confers them with the migratory capacity that facilitates their recruitment into inflammatory sites, such as the inflamed joints in RA (14). The sole known ligand for CCR6 is the chemokine CCL20 (102). The IL-23 receptor has been identified as an additional marker of IL-17 producing memory T cells (202). The cytokine IL-23, one of many DC products, aids in the expansion and maintenance of this cellular subset (203).IL-6, IL-21 and IL-23 all serve to promote IL-17 production through the activation of the transcription factor STAT3 via tyrosine phosphorylation (117).

Recent studies have highlighted a significant contribution of the Th17 cell subset to the pathogenesis of GCA (Deng et al, Circluation, in press). IL-17 levels are markedly elevated in the peripheral blood of untreated patients and Th17 cells constitute a key component of the inflammatory response. In humanized mice engrafted with human aorta, transferred human Th17 cells cause vessel wall inflammation. These findings are supported by the data in Chapter 3 (Fig3.2) in which gene expression profiling detected elevated levels of IL-17 in GCA patient samples when compared to healthy controls. In experiments testing the inhibitory effects of Notch pathway blockade the γ-secretase inhibitor, DAPT, preferentially suppressed the in vivo production of IL-17 vs. IFN-γ. These data let us to postulate that the Notch pathway plays a role in the skewing of T cell responses, particularly as it applies to the generation of Th17 responses. In a preliminary set of experiments we have investigated potential differences in the contribution of Notch signaling to Th17 vs. Th1 cytokine production.

Methods

Cells

Human peripheral blood mononuclear cells (PBMC) were purified from heparanized blood obtained from healthy donors by a Ficoll gradient. $CD14^+$ cells were positively selected using CD14 MicroBeads (Miltenyi) and the purified $CD14^+$ monocytes were cultured at a concentration of $2x10^6$cells/ml in RPMI supplemented with 800U/ml GM-CSF and 1000U/ml IL-4. Dendritic cells were harvested on day 6. CD4 T cells were prepared from PBMC by positive selection following incubation with anti-CD4 magnetic beads or by negative selection using a CD4 T cell isolation kit (Miltenyi). Alternatively, CD4 T cells were isolated from whole human blood using the RosetteSep system from StemCell Technology (Vancouver, British Columbia, Canada). CD45RA+ and CD45RO+ CD4 T cells were isolated from purified CD4 T cells by positive selection using anti-human CD45RA or CD45RO magnetic beads, respectively.

Antibodies and reagents

Antibodies to human CD4-PE, IFN-γ-FITC, CD45RA-FITC and recombinant human IL-6 were purchased from BD Biosciences (San Diego, CA); anti-human IL-17-DC, human IL-6, IL-17, IL-23 and IFN-γ ELISA kits were obtained from ebioscience; Jagged1-FITC, anti-human Notch1-PE (clone 527425) was obtained from R&D systems. DAPT and LPS (Escherichia coli, 0127:B8) were purchased from Sigma-Aldrich (St. Louis, MO).

Co-culture assay

For cellular co-culture assays CD4 T cells were stimulated in round bottom 96 well plates with monocyte derived DC activated with 1µg/ml LPS (Sigma), in the presence of 1µg/ml OKT3. DC were added to the T cells at a ratio of 1:2. CD4 T cells were incubated with 10µM DAPT reconstituted in DMSO or the equivalent dose of carrier DMSO 30 mins prior to initiation of cultures.

Flow Cytometric Analysis

For cell surface expression analysis, cells were stained with the relevant primary antibodies or appropriate isotype controls for 30 mins at 4°C. For detection of intracellular cytokines cells were stimulated for 4 hrs with phorbol 12-myristate 13-acetate (PMA) and ionomycin in the presence of brefeldin A. Cells were fixed and permeabilized using Cytofix/Cytoperm (BD Biosciences) and incubated with anti-IL-17 and anti-IFNγ antibodies or isotype control (30 mins; 4°C). For signaling molecule detection cells were stimulated with IL-6, anti-CD3/CD28 (1 µg/ml each) or anti-Notch1 (5µg /ml), fixed in BD Cytofix buffer, permeabilized with BD Perm Buffer III, and stained with anti-pSTAT3 antibodies. Cells were washed and data acquired using the BD/LSRII instrument and FACSDIVA software (BD Biosciences). Data analysis was performed with FlowJo software (Tree Star, Ashland, OR).

ELISA

Human IL-6, IL-17, IL-23 and IFN-γ were measured in the supernatant of the DC-T cell co-cultures using human cytokine multiplex kits. Supernatants were incubated for 2 h at room temperature in 96 well plates pre-coated overnight with cytokine capture antibodies. Bound biotin-conjugated cytokine detection antibodies were analyzed using Avidin-HRP and colorific reactions analyzed at an OD of 450.

Statistics

Data were analyzed using the student's t test

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Results

Abrogation of Notch signaling preferentially diminishes IL-17 production

Our studies using the human artery-SCID chimera in Chapter 3 outlined a contributory role for Notch signaling in the evolution of vascular inflammatory responses. Global attenuation of vessel wall inflammation was achieved utilizing the Notch pathway inhibitor, DAPT. Unexpectedly, in the course of these investigations we noted a differential impact of Notch inhibition on IL-17 vs. IFN-γ production, in which IL-17 production was more significantly impaired by the removal of the Notch signal. Our interest in characterizing the contribution of this pathway to the execution of T cell effector function observed in vivo, led to an exploration of the T cell cytokine profile in the presence or the absence of Notch signaling. To this end CD4 T cells were co-cultured with LPS stimulated DC in the presence or absence of the Notch inhibitor, DAPT, and subsequently analyzed for cytokine expression by flow cytometry. Consistent with the afore mentioned results, in vitro expression studies revealed a diminished cytokine response by the CD4 T cell following treatment with the γ-secretase inhibitor. Notably, the frequency of IFN-γ producing cells was only diminished by 10-15% whereas IL-17-producing cells decreased by about 30-40% (Fig 4.1A& 4.1C). Despite the variations in the baseline IL-17 levels expressed by CD4 T cells isolated from healthy individuals, the significance of the diminishing effect of the γ-secretase inhibitor on IL-17 production from one patient to another remained consistent (Fig 4.1B). Cytokine analysis of supernatants confirmed these observations. DAPT treatment diminished IL-17 concentrations up to 50% (Fig 4.1D), while having no significant impact on the IFN-γ levels measured in the cultures. This data implicates the Notch pathway as a key

regulator of the IL-17 production by T cells, while its more minimal effects on IFN- γ expression point to a contributory albeit less major role in the regulation of this latter cytokine. These studies, for the first time, suggested a differential effect of the Notch signaling on T cell commitment to either the Th1 or Th17 arm of the adaptive immune system.

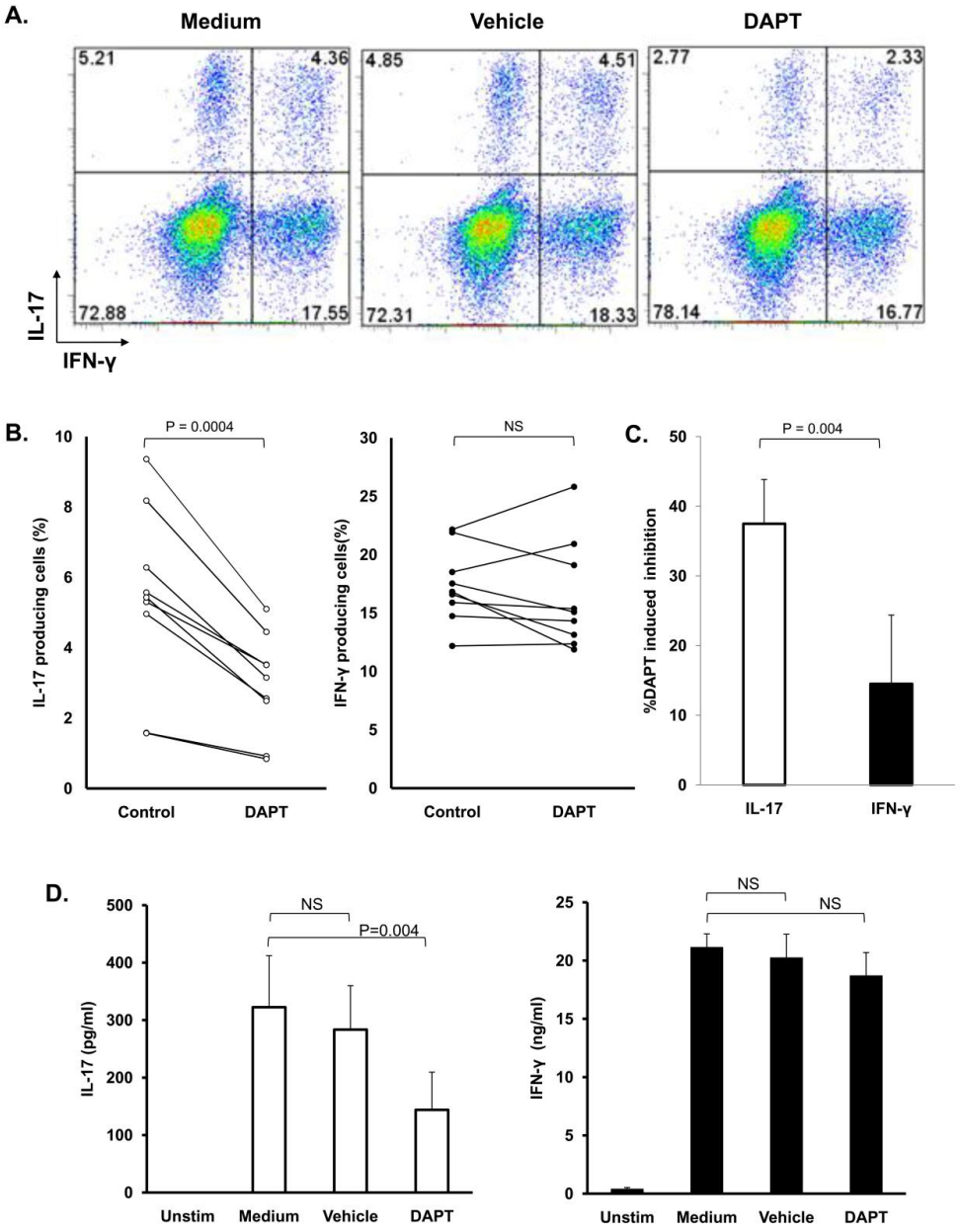

Figure 4.1. IL-17 and IFN-γ production by CD4 T cells are differentially affected by Notch inhibition.

Figure 4.1. IL-17 and IFN-γ production by CD4 T cells are differentially affected by Notch inhibition. A.T cells were cultured with LPS (1μg/ml) stimulated DC loaded with OKT3 (1μg/ml), in the presence or absence of DAPT or vehicle (DMSO). On day 5 frequencies of IL-17 and IFN-γ producing cells were measured after stimulation with PMA plus ionomycin in the presence of Brefeldin A for 5h. Cells were then stained for CD4 and intracellular IL-17 and IFN-γ and analyzed by flow cytometry using CD4 gates. Data are representative of at least 9 independent experiments. **B**. Flow cytometry was used to determine the percentage of cells producing IL-17 and IFN-γ in cells from 9 different donors.**C**. Summary of the inhibitory effect of γ -secretase treatment on the frequencies of IL-17 and IFN-γ –producing T cells. **D**. ELISA was used to assess IL-17 and IFN-γ in 72h culture supernatants of cells from 5 donors.

Notch signaling regulates IL-17 production by memory CD4 T cells

As the Notch pathway inhibitor was present in the cultures throughout the entire culture period it was unclear whether the Notch pathway affected the differentiation of naïve T cells into Th17 cells or interfered with the function of committed Th17 cells. To address this question we next sought to compare the effects of Notch inhibition on cytokine production in naïve vs. memory CD4 T cells. We first examined whether Notch receptors are expressed differentially on naïve and memory T cells (Fig 4.2A). Flow cytometry assigned Notch1 expression to both resting naïve and memory CD4 T cells, and T cell activation using anti-CD3/CD28 stimulation induced Notch receptor upregulation by both subsets (Fig 4.2A). Notably, however, the memory population consistently displayed higher Notch1 expression levels than the naïve subset attaining at least 4-fold higher levels at 24hr post-stimulation. These differential expression patterns may translate into divergent contributions of Notch to naïve and memory T cell responses.

Having evaluated receptor expression, we next investigated the impact of Notch inhibition on IL-17 production by naïve vs. memory CD4 T cells. $CD45RA^+$ and $CD45RO^+$ CD4 T cells were isolated from the peripheral blood of the same donor and cultured with LPS stimulated DC in the presence or absence of the Notch inhibitor, DAPT. The flow cytometry results indicated minimal IL-17 induction in the naïve cell subset (<1%), despite the abundant induction of IFN-γ producing Th1 cells (15-30%) (Fig 4.2B). Conversely, there was consistent IL-17 production among the memory cells (5.92%) that remained unaltered in the presence of the vehicle control (5.91%) (Fig 4.2B). Introduction of the Notch inhibitor significantly reduced IL-17 expression by 50%

(3.1%). Comparing frequencies of IFN-γ- and Th17-producing cells in cultures with and without the Notch pathway inhibitor revealed similar results as seen in

unseparated T cell populations (45% and 10% respectively) (Fig 4.2C& 4.2D). In essence, the Notch pathway appeared to regulate the function of already committed Th17 cells.

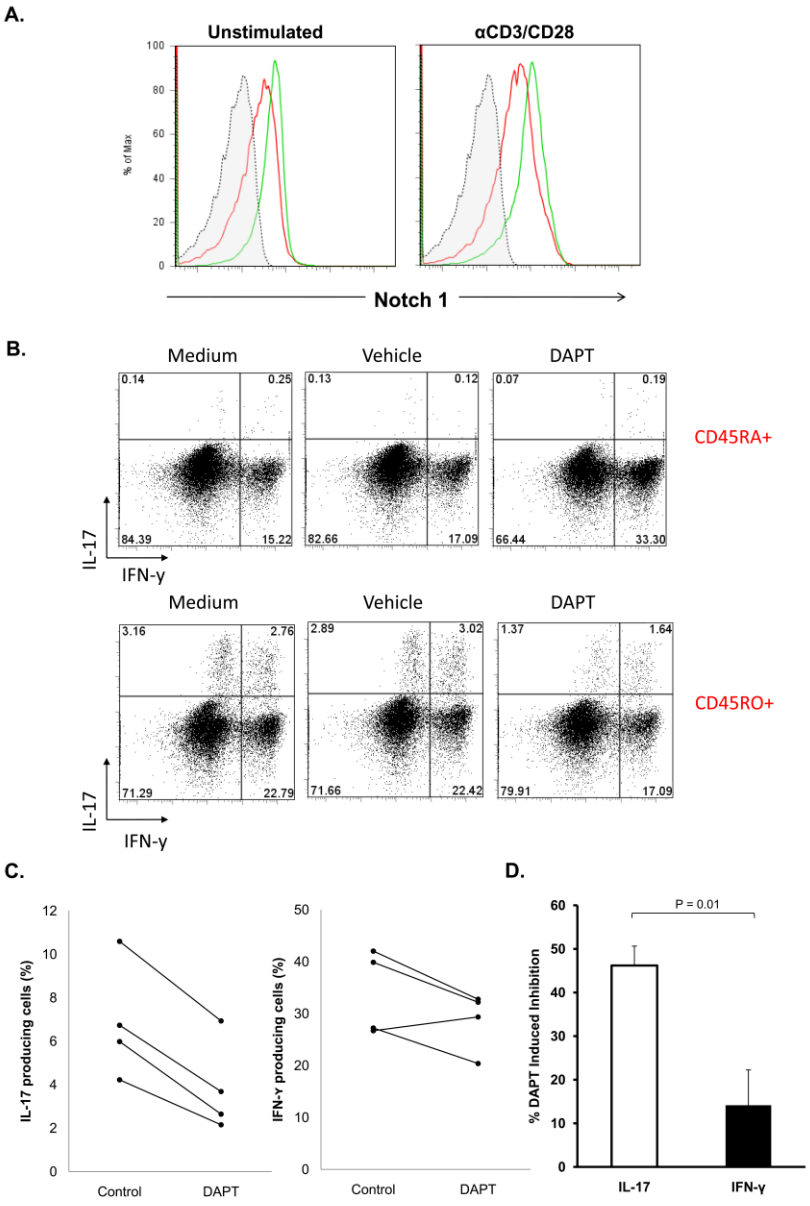

Figure 4.2. Notch signaling regulates CD4 T cells committed to the Th17 pathway.

Figure 4.2.Notch signaling regulates CD4 T cells committed to the Th17 pathway. **A**.CD4 T cells purified from PBMC of healthy donors were stimulated with immobilized anti-CD3/CD28 (1µg/ml) mAbs. Cell surface expression of the Notch1 receptor was assessed by flow cytometry on naïve ($CD45RA^+$ -red line), and memory ($CD45RA^-$ -green line) T cells stimulated for 0, 24 or 48 h. Shaded histogram represents isotype control. Representative data from one of three independent experiments are shown. **B**. Naïve ($CD45RA^+$) and memory ($CD45RO^+$) cells were purified from CD4 T cells and independently cultured with LPS (1µg/ml) stimulated DC loaded with OKT3 (1µg/ml), in the presence or absence of DAPT or vehicle (DMSO). On day 5 IL-17- and IFN-γ-producing T cells were analyzed after stimulation with PMA plus ionomycin in the presence of Brefeldin A for 5h. Cells were then stained for CD4 and intracellular IL-17 and IFN-γ in naïve (upper panel) and memory (lower panel) T cells was assessed by flow cytometry using CD4 gates. Data are representative of at least 4 independent experiments. **C**. Flow cytometry was used to determine the percentage of cells producing IL-17 and IFN-γ in memory cells from 4 different donors. **D**. Summary of the inhibitory effect of γ -secretase treatment on the frequencies of IL-17 and IFN-γ -producing T cells.

Dendritic cell maturation and function are unaffected by Notch inhibition

Given the indispensable role of the DC in dictating T cell responses, we explored the possibility that the inhibitory effects on T cell activity resulted from a Notch inhibitor-mediated disruption of DC function. The ability of DC to regulate T cell mediated responses is tightly correlated with their maturation status. In its transition from immature antigen-capturing cell to mature antigen-presenting cell DC acquire surface markers that include MHCII and CD83. LPS is a potent stimulator of DC maturation and thus the TLR4 ligand was used to assess the impact of Notch inhibition on DC development. CD83 expression was significantly enhanced (Fig 4.3A), being upregulated by more than 90% of the cells following TLR4 triggering, a response that was maintained even in the presence of the γ-secretase inhibitor (89.83%). This data confirmed that the decreased T cell cytokine response could not be attributed to a mere failure of the DC to complete its developmental transition. Alternatively, we wondered whether blockade of the Notch signaling pathway might impact the effector functions of DC, as IL-17 production is regulated by several major cytokine products released by DC, including IL-6 and IL-23. To verify that the impaired IL-17 production detected in the DC-CD4 co-culture assays was not a result of diminished Th17-promoting cytokines, IL-6 and IL-23 levels were measured by ELISA in supernatants from these cultures (Fig 4.3B). LPS stimulation enhanced the IL-6 production, leading to a greater than 2-fold increase in the concentration detected in LPS stimulated cultures (1300pg/ml) as compared to those without TLR stimulation (600pg/ml) (Fig 4.3B). Notably, the introduction of DAPT into the cultures had no significant effect on the IL-6 levels measured following TLR4 stimulation, which were similar in vehicle and DAPT cultures (1200pg/ml). Similarly,

Notch blockade did not suppress the LPS-induced production of IL-23 (Fig 4.3B). IL-23 release was indistinguishable in cultures containing vehicle versus those receiving DAPT. DMSO, the vehicle for DAPT seemed to activate DC to enhance IL-23 production. Thus, it seems unlikely that a DAPT-mediated reduction in DC-secreted Th-17 differentiating cytokines can account for the dampened IL-17 responses.

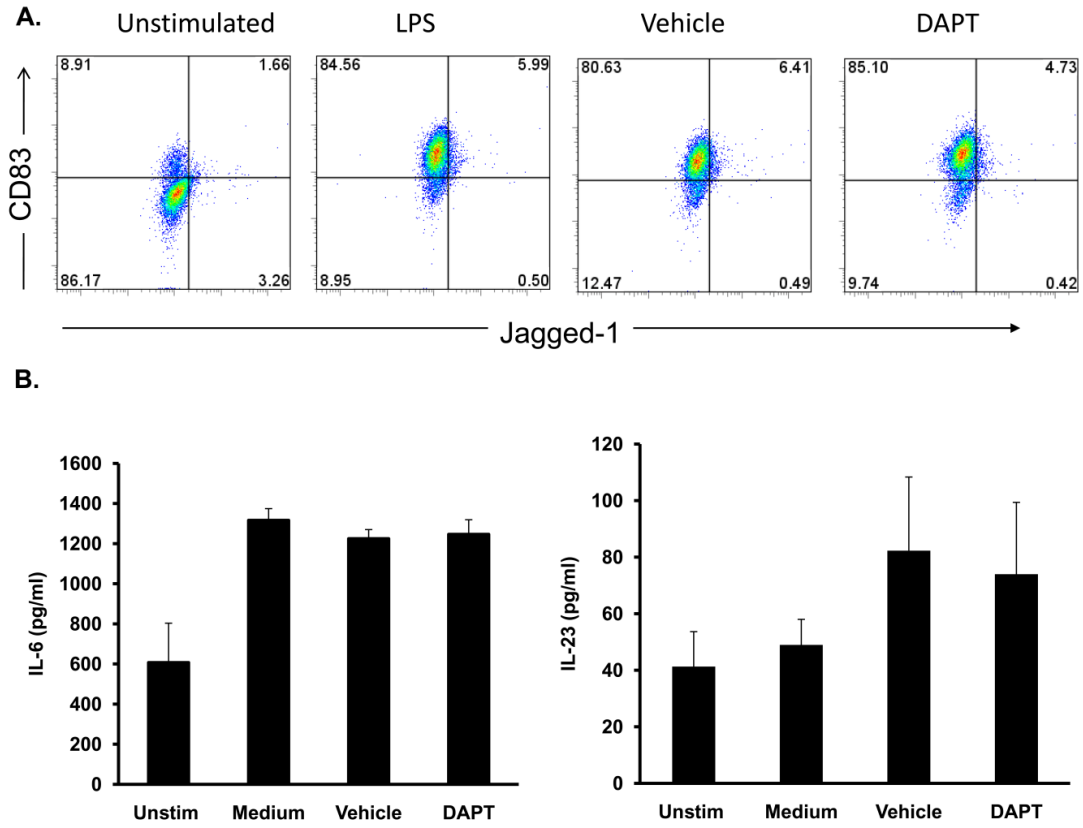

Figure 4.3. Dendritic cell function is unaffected by γ-secretase inhibition. A. DC were stimulated with LPS (1μg/mL) in the presence or absence of DAPT or vehicle (DMSO). After 24 hrs cells were stained with anti-CD83 FITC and anti-Jagged1-PE mAbs and surface expression was analyzed by flow cytometry. **B**. Freshly isolated T cells were cultured with LPS (1μg/ml) stimulated DC loaded with OKT3 (1μg/ml), in the presence or absence of DAPT or vehicle (DMSO). ELISA was used to assess IL-6 and IL-23 in 72h culture supernatants of cells from 5 different donors.

Notch1 mediated signaling enhances STAT3 activation

We explored the possibility that the Notch pathway interferes with Il-17-inducing cytokines and turned our attention to one of the key regulators of IL-17 production, STAT3. Stimulation by Th17-regulating cytokines, such as IL-6, leads to activation of the cytokine receptor associated Janus family kinases (JAKs), with subsequent activation of STAT3 via tyrosine phosphorylation Y705. Activation of the STAT protein is followed by dimerization and translocation to the nucleus where it regulates gene expression(204). To evaluate the effect of Notch signaling on STAT3 activation CD4 T cells were stimulated by cross-linking with an anti-Notch1 antibody.

First, CD4 T cells were stimulated with IL-6 and after 15 mins accumulation of phosphorylated STAT3 (pSTAT3) was quantified by flow cytometry. Incubation with IL-6 induced tripling in the phosphorylation status of the STAT3 protein (Fig 4.4A& 4.4B). Antibody-mediated triggering of the Notch receptor for 15 minutes had no effect on STAT3 activation. To rule out a delayed effect of the Notch pathway we explored STAT3 activation following 24h of Notch1 stimulation. As observed at 15 mins, IL-6 stimulation effectively enhanced STAT3 phosphorylation (Fig 4.4C & 4.4D), while an independent Notch1 signal had no apparent effect on STAT3 activation.

Given the reported dependence of Notch signaling on signals transmitted though the TCR(75), we next assessed the impact of Notch activation in the presence of ongoing anti-CD3/CD28 stimulation. Indeed, while anti-CD3/CD28 stimulation alone was ineffective at inducing STAT3 activation, Notch1 stimulation delivered concurrent with anti-CD3/CD28 antibody stimulation, enhanced STAT3 phosphorylation up to 3-fold as compared to unstimulated T cells (Fig 4.4E & 4.4F). The failure to elicit a similar

response using an isotype antibody, suggested that the observed effects were Notch1 specific.

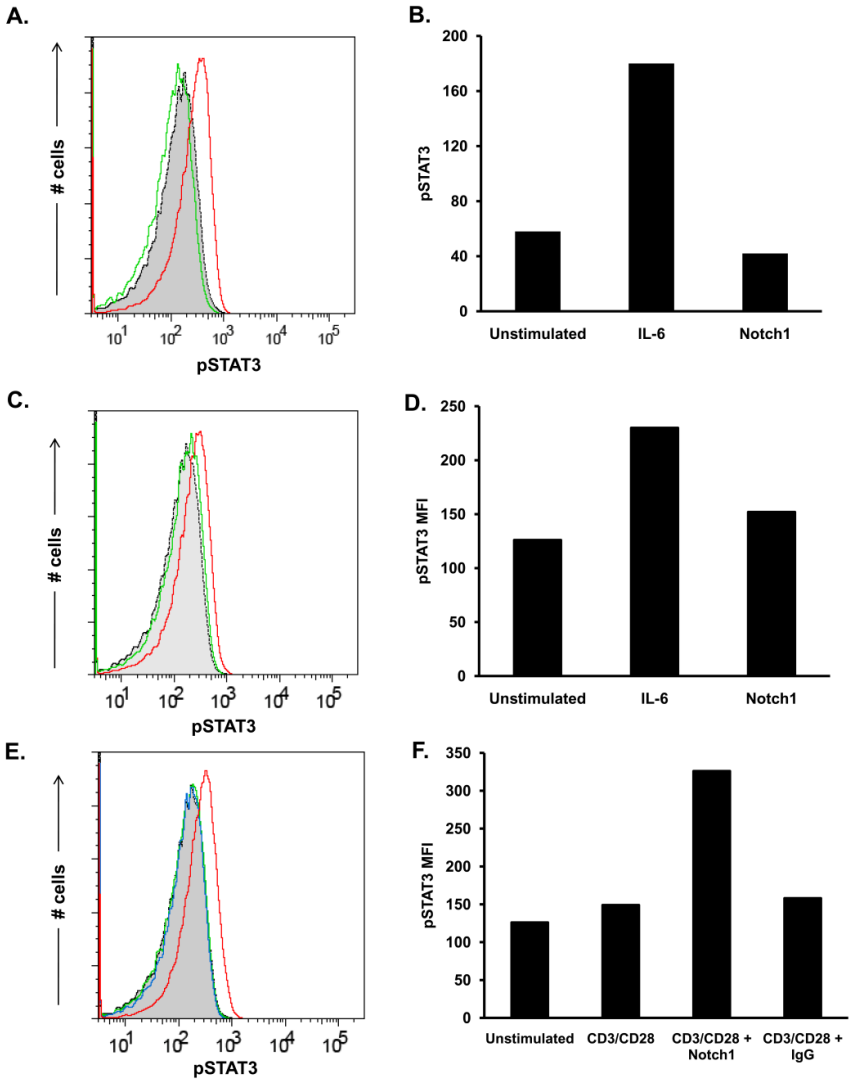

Figure 4.4. Notch1 co-signaling enhances activation of the STAT3 pathway.

Figure 4.4. Notch1 co-signaling enhances activation of the STAT3 pathway. A. Purified CD4 T cells were left unstimulated (shaded histogram) or stimulated for 15 min using 10 ng/ml IL-6 (red line), anti-Notch1(5μg/ml) (green line). pSTAT3 levels in T cells were determined by phosflow and representative histograms from at least 3 donors are shown. **B**. pSTAT3 expression was measured as mean fluorescence intensity 15 min following stimulation. **C**. CD4 T cells purified from PBMC of healthy donors were left unstimulated (shaded histogram) or stimulated for 24hr with IL-6 (red line) or anti-Notch1 mAb (green line). pSTAT3 levels in T cells were determined by phosflow **D**. pSTAT3 expression was measured as mean fluorescence intensity 24h following stimulation. **E**. CD4 T cells were left unstimulated (shaded histogram) or stimulated in plates coated with anti-CD3/CD28 (green line), anti-CD3/CD28 plus anti-Notch1 (red line), or anti-CD3/CD28 plus isotype control (blue line) antibodies. pSTAT3 levels in T cells were determined by phosflow.**F**. pSTAT3 concentrations were measured as mean fluorescence intensity 24h following stimulation.

Discussion

Using pharmacological inhibition we have provided evidence supporting a unique role of the Notch signaling pathway in IL-17 production by human CD4 T cells. The preliminary data point to a preferential contribution of Notch in the execution of Th17 subset specific functions. Disruption of Notch signaling has comparatively minimal effects on the Th1 signature cytokine, IFN-γ. These findings are particularly significant when placed within the context of the information gleaned from a humanized mouse model in which we have discerned mechanisms of vessel wall inflammation. In the human artery-SCID chimeras, inflammation in the engrafted human arteries correlates closely with the production of T-cell cytokines, IL-17 and IFN-γ. Here, we have identified Notch receptor ligation as a signal that biases CD4 T cells towards the IL-17 pathway; blockade of the Notch pathway preferentially inhibits IL-17 production, opening the possibility for a novel therapeutic intervention in treating large vessel vasculitis. The differential susceptibility of the Th1 and Th17 subsets to γ-secretase inhibition undoubtedly has important implications for targeting distinct T cell effector functions within the local environment of the vessel wall.

Despite the demonstrated effect of Notch inhibition on IL-17 production, the mechanism underlying the inhibitory effect remains unclear. Since DC are involved in directing T cell lineage decisions (205, 206) we examined whether interference with the Notch signaling pathway primarily altered DC function, with selective inhibition of IL-17 production as a secondary effect. Suppression of the Notch signal had no significant consequences on DC maturation, and the production of Th17 differentiating cytokines remained equally unaffected. Subset-specific analyses identified the memory T cell as the

T cell subset most affected by modulation of Notch signaling, supporting involvement of the Notch pathway in our studies at a stage in IL-17 production that occurs post-Th17 differentiation. The current model proposes that in human CD4 T cells TCR occupancy is sufficient for induction of IL-17 production by memory cells. Just as there is conflicting data regarding the cytokine requirement for human Th17 differentiation, so too does the question of which cytokines regulate IL-17 production by human T cells remain unresolved.

In humans differentiation of naïve T cells towards the Th17 lineage has been shown to be stimulated by various cytokine combinations that include but are not limited to, IL1-β+ IL-6 (207), IL1-β + IL-23 (202, 208) and IL-23 alone (209). While much emphasis has been placed on identifying the cytokines that regulate Th17 differentiation from naïve cells, far fewer studies have focused on the cytokine mediated regulation of IL-17 production by memory CD4 T cells. In humans IL-23 has been shown to induce IL-17 production and to enhance its production via expansion of IL-17 producing T cells in vitro. Another study has also reported an enhancement of IL-17 secretion in humans by memory T cells in response to IL1- β and IL-23 stimulation. IL-6 has also been shown to induce IL-17 production by memory CD4 T cells (114). While IL-21 is not required for Th17 differentiation, as a key product released by Th17 cells it has been implicated as a positive autocrine regulator of IL-17 production (210, 211). IL-21 induces its own expression and also that of the IL-23 receptor, thereby actively facilitating induction and expansion of IL-17 producing cells (212, 213). While the data set is preliminary and a direct role of IL-21 in the Notch-mediated inhibition of IL-17 production has not yet been investigated it is possible that Notch blockade suppresses IL-21 production and indirectly

diminishes Il-17 secretion. In our experiments Notch inhibition had to be present for 24 hours before Il-17 production was negatively affected; compatible with an indirect effect on an Il-17 regulating cytokine. Here Il-21 is a prime candidate and its role has to be examined by appropriately designed experiments.

The central role of STAT3-dependent signaling events in regulating Th17 cells (99) led us to explore whether the Notch pathway interferes with the STAT3-regulated signaling cascade. Indeed, our preliminary data suggest that a Notch-mediated signal modulates STAT3 phosphorylation. Notch receptor triggering alone did not change the accumulation of pSTAT3. TCR cross-linking could not induce pSTAT3. However, a combination of both signals resulted in enhanced levels of pSTAT3. The contribution of Notch to STAT3 signaling showed both temporal dependence, as well as a dependence on the co-delivery of T cell activation signals, as the provision of isolated Notch signals was insufficient to generate an enhanced pSTAT3 response. This serves to highlight not only the significance of the context within which Notch signals are provided, but also supports a possible role of Notch as a co-stimulatory molecule; acting with a delayed kinetic as outlined in Chp 3.

Additional experiments are needed to further dissect how the Notch pathway and STAT3 activation are connected (214). This could be achieved via an assessment of the impact of Notch signaling on STAT3 target gene expression, and a similar investigation of Notch blockade on STAT3 dependent genes. Gene expression profiles generated in Notch stimulated CD4 T cells could provide clues towards potential modulations in STAT3 inducible genes as compared to T cells stimulated in the absence of Notch signaling. The results from these studies could provide corroborating evidence for

involvement of the Notch pathway in STAT3 signaling events. IFN-γ which is relatively resistant to the effects of Notch inhibition is considered a STAT1 target gene. The STAT1 pathway could thus serve as a control for Notch-independent signaling.

Finally, the question remains how in vitro observations translate into in vivo events. And, it would be important to know whether complex interactions between different signaling cascades have relevance for human disease. The humanized mouse provides an ideal model for recapitulating human immune responses in vivo, including those relevant to disease. We have successfully applied this model to test the role of Notch-initiated signaling in early and late phases of vessel wall inflammation. Explanted human arteries could well be analyzed for gene expression profiles generated in the absence and presence of Notch-mediated signals. γ-secretase inhibitors can be analyzed in this model system in which induction and maintenance of vasculitis can be examined separately. Our preliminary experiments would encourage the exploration of the STAT3 pathway. This can be accomplished through different approaches, including pharmacologic inhibition, gene knockdown and antibody-mediated strategies. The ultimate benefit of the humanized mouse model lies in the information it provides on the activity of the disease process. One of the candidate molecules that should be explored is IL-21 and its potential role in enhancing IL-17 production. In the end, though, combined therapeutic interventions will have to be developed which not only allow suppression of the disease-relevant Th17 pathway but also abrogate Th1 immunity. Only combined suppression of both T-cell lineages will permit induction of disease remission in large vessel vasculitis.

Chapter 5

Summary

Blood vessels, in particular the large arteries, are vital organs and inflammatory diseases attacking vascular integrity are associated with life-threatening complications. Therapeutic management of vasculitides is currently limited to non-specific immunosuppression (215, 216), as the underlying disease mechanisms are insufficiently understood. A better understanding of pathogenic immune responses compromising arterial function is emerging for the large vessels vasculitides, which target the aorta and its major branches. Work presented here has focused on discerning mechanisms of disease in giant cell arteritis and utilizing improved understanding of relevant immune responses to optimize immunotherapy of this serous autoimmune syndrome.

The emerging disease model of GCA identifies it as a T-cell mediated disease with immune-mediated damage resulting in arterial wall remodeling, intimal hyperplasia, luminal stenosis and tissue ischemia. In the case of the aorta, vasculitis may result in dissection, aneurysm or rupture. The response-to-injury program of the blood vessel is a concerted action between the immune system and wall-resident cells, involving the release of growth and angiogenic factors from macrophages and giant cells and the migration and hyperproliferation of vascular smooth muscle cell (217). Innate immune cells, specifically, dendritic cells positioned in the vessel wall, have been implicated in the earliest steps of vasculitis (218). Pathogen-derived molecular patterns are capable of activating vascular DC and initiating adaptive immune responses (21). Experiments presented here support the notion that two T cell lineages, Th1 and Th17 cells participate in the disease process. Activation of T cells in the disease lesions is complex and involves

signals mediated through the Notch pathway. This finding draws attention to blocking Notch signaling as a novel approach to treating vasculitis.

Notably, the pattern of the emerging vessel wall inflammation is determined by the initial insult. Ligands to toll-like receptor (TLR) 4, such as lipopolysaccharides, facilitate the recruitment of CD4 T cells that invade deep into the wall and distribute in a panarteritic pattern (11). This pattern of vasculitis is sensitive to blocking CCR6-CCL20 interactions, providing indirect evidence for the participation of $CCR6^{+}$Th17 cells. Conversely, ligands for TLR5 condition vascular DC to support perivasculitic infiltrates (11). In essence, both innate and adaptive immune reactions collaborate to render the arterial wall susceptible to inflammatory damage. Unique features of the tissue microenvironment, including specialized DC, shape the course of the inflammatory response. Disease-relevant T cells derive from the Th1 and the Th17 subsets. It remains to be determined how vessel-specific antigen-presenting cells communicate with the two T cell lineages. Th17 cells appeared to depend more on Notch-dependent signals, which attests to the multiplicity of signaling pathways involved in vascular inflammation.

As medium-sized and large arteries are not exposed to the external world, the question arises about what benefit the host derives from sensing immune danger signals in the arterial wall. In essence, networks of DC in the vascular wall could potentially increase the risk of attracting tissue-damaging immune responses given their potency in the initiation of adaptive immune responses. The versatility of the DC, however, is likely the key feature that explains what appears to be a symbiotic relationship with the vessel wall, as it is equally competent in the mediation of immune tolerance and protection from damaging inflammation. The execution of this latter function is essential for the

regulation of self-reactivity (26), and in healthy arteries the DC likely comprise a subset of steady state DC that patrol this peripheral checkpoint and defend the vessel against any potential attack by autoreactive T cells.

Given that the artery acts as a conduit for the blood, which serves as a major pipeline for the dissemination of both self- and foreign-antigens, the location of the DC in the proximal adventitia means that these resident cells are optimally positioned for sampling their environment, via the vasa vasorum, which are restricted to the adventitial layer of the vessel wall. These arterial wall sentinels may have an intrinsically high threshold for mounting a danger response within the confines of a microenvironment that has evolved to minimize collateral damage as a selective advantage to maintain the integrity of this non-regenerative and vital organ system.

Experiments confirming the in vivo relevance of observations made in in vitro systems relied upon a humanized mouse model in which normal human arteries were studied. This may impose limitations as DC-T cell interactions in vasculitic arteries may involve even more receptor-ligand pairs and additional signals specific for the afflicted host. However, previous experiments utilizing inflamed arteries from patients with GCA have stressed the central role of DC and their pinnacle contribution to T cell activation. Depletion of DC in the chimeras engrafted with GCA-affected temporal arteries lead to rapid and profound suppression of vasculitis with inhibition of T cell and macrophage function (6, 219).A critical role of DC in regulating vascular inflammation was confirmed in a second experimental model in which we implanted non-inflamed human arteries into SCID mice and initiate vessel wall inflammation by adoptive transfer of alloreactive human T cells (219). Intriguingly a prerequisite of vascular inflammation is the

activation of wall-resident DC with pathogen-derived motifs, such as lipopolysaccharide (LPS) (15). Arteries from different vascular beds express a unique pattern of TLRs (21) raising the question as to whether stimulation of different TLRs impacts the inflammatory response in a predictable way. This question prompted the exploration of TLR3 and TLR4 as two approaches for shaping T cell responses. Emphasis was placed on the induction of co-stimulatory molecules induction, and the impact of initial DC activation on CD4 T cell effector functions. The results of this investigation highlight the essential role of the initial triggering event in the evolution of an adaptive immune response, which as demonstrated is in large part dictated by the induction of positive or negative regulatory ligands on the DC (220). The biasing of TLR4 stimulated DC towards amplification of T cell responses provides greater insight into the previously reported requirement for the TLR4 ligand- mediated breach of tolerance in our model of large vessel vasculitis.

Recent work by Deng et al, demonstrated selectivity for functional T-cell subsets driving large-vessel vasculitis. Phenotyping of panarteritic T cells in temporal artery specimens from GCA patients confirmed the dominance of $CCR6^+$ T cells (11). Most importantly, the recruitment of $CCR6^+$ T cells could be linked to the stimulatory conditions of wall-residing DC, assigning to them a true checkpoint function in attracting T cells and assembling the population of T cells mediating vasculitis. The distinct features of TLR4- and TLR5-induced vasculitis, which correspond to two distinct histomorphological patterns of disease in patients with GCA, re-emphasize that the nature of the original trigger is crucial in directing pathogenic immune responses in large-vessel vasculitis.

Given the critical role of vascular DC in initiating and sustaining vasculitic immune responses, and because such responses are due to the integration of multiple accessory signals, we hypothesized that TLR4 ligation leads to the initiation of unique co-signaling pathways in addition to those of the B7-CD28 superfamily. Such co-stimulatory signals could contribute to the propagation of the aberrant CD4 T cell responses in the unique environment of the vessel wall and would provide useful targets for the development of novel therapeutic interventions. Pilot experiments analyzing gene expression pattern lead to the Notch pathway and a detailed analysis of Notch-dependent immune functions in the inflamed artery.

Our studies are the first to examine the role of the Notch signaling pathway in the pathogenesis of large vessel vasculitis. We show that in human arteries affected by GCA, Notch receptors and Notch ligands are abundantly expressed. Importantly, the receptor expression correlates with active Notch signaling as indicated by the widespread detection of the cleaved intracellular domain in cellular infiltrates of the tissue lesions, demonstrating that the Notch pathway is indeed activated in GCA affected arteries. Notch receptors are expressed on resting and, increasingly, on activated T cells. Expression by naïve and memory CD4 T cells suggests that both T cell subsets should be amenable to Notch-targeted therapy, providing opportunities for immunosuppression during early and chronic stages of vasculitis.

Evidence from murine studies shows Notch signaling may be required for TCR-mediated activation and proliferation in peripheral T cells (187). We investigated factors regulating Notch expression in human CD4 T cells and consequences of interfering with Notch-Notch ligand interactions for their downstream functions. Data presented here

show that Notch1 is constitutively expressed, and is induced in response to T cell activation in a time-dependent fashion. The DAPT mediated inhibition of NICD induction, coupled with reduced transcription of the Notch responsive target gene Hes-1, demonstrate the functional competence of the activation induced Notch receptor. Although based on generalized expression patterns Notch ligands are typically assigned to the DC, ligands are also found on CD4T cells, thus autocrine signaling could account for the active Notch signaling observed in the DC-free system (221).

Multiple T-cell effector functions including IFN-γ and IL-17 production, as well as tissue invasiveness are relevant in arterial injury. While the impact of IFN-γ in driving macrophage differentiation and subsequent inflammation in the arterial wall is well understood (7), the significance of IL-17 in the evolution of vascular lesions remains to be elucidated. In fact, despite the implication of IL-17 in the pathogenesis of autoimmune disease demonstrated using multiple models, the precise function of this Th17 specific cytokine remains poorly understood (222). Similarly, the potential distinction between the IL-17 producers and the IL-17+IFN-γ+ double producers requires further clarification (13). IL-17 producers and double producers are equally affected by Notch signaling abrogation and are both distinctly more susceptible to Notch blockade than the sole IFN-γ producers. These data raise the question of whether these populations represent distinct subsets or a cellular subset continuum in which Th17 cells are in a constant state of flux between the single and dual producing phenotypes. A better understanding of the unique cell subsets involved in the initiation and maintenance of these responses will facilitate the development of more targeted therapeutic approaches.

The administration of the γ-secretase inhibitor DAPT in our human-SCID chimera model of large vessel vasculitis successfully and effectively abrogated the pro-inflammatory response in the vascular wall. While the use and efficacy of γ-secretase inhibitors in the treatment of other human diseases is well-documented, challenges remain that may preclude their use in clinical applications. Unfortunately, therapy utilizing this class of drugs has been associated with toxic side effects, such as GI dysfunction. These adverse effects pose a challenge and potential obstacle in the clinical use of these inhibitors in the treatment of chronic diseases such as GCA. The associated cytotoxicity is not surprising, however, for several reasons. The Notch signaling pathway is instrumental in normal cellular physiology and the interruption of Notch processing and signaling by γ-secretase inhibition could account for the side effects noted in Alzheimer's disease and anti-tumor therapy. Also, the possible non-selectivity of the γ-secretase inhibitors for the γ-secretase complex and the pool of known substrates broaden the potential for unforeseen side effects. The greatest challenge then lies in establishing a treatment regimen that optimizes the efficacy while minimizing the toxicity of the γ-secretase inhibitors. It is possible however, in instance of disease characterized by enhanced activation of the Notch pathway as in GCA, that this balance might be able to be achieved through modifications in dosing and duration of treatment, thus reducing complications that may arise from more chronic γ-secretase blockade.

Alternatively, the reported ability of soluble forms of Notch ligands to suppress Notch activation poises them as attractive candidates for use in applications requiring repression of T cell responses, based on the contribution of this pathway to the execution of effector functions demonstrated herein. These ligands are thought to inhibit active

Notch signaling via competition with endogenous cell-bound ligands for Notch receptor binding (223, 224). The potential utility in the treatment of autoimmune disorders is highlighted by the dampening of the local tissue immune response in the Jagged1 Fc treated chimeras. Soluble Notch ligands have been utilized in a mouse model for multiple sclerosis, in which Jagged1 Fc treatment proved to be protective from disease (188). Despite the clear evidence showing the inhibition of tissue inflammation accompanied by a diminished T cell infiltrate, the question remains to be addressed as to what extent this effect is due to regulation of T cell recruitment versus T cell survival. It is plausible that Notch signaling may confer the activated T cells with a reduced susceptibility to apoptosis, such that the interruption of this signal results in decreased T cell survival rates in the tissue.

Our findings support the characterization of the Notch receptor as a molecule with co-stimulatory capacity. The subset-specific receptor density suggests flexibility in Notch receptor usage that likely translates into unique functional outcomes for a T cell in the development of an antigen-specific response. Given that T cell responses ultimately derive from the processing of cumulatively integrated signals, the incorporation of the Notch signal may aid in tailoring such responses via modifications in surface receptor expression. In the absence of CD28 ligation the T cell demonstrates a high TCR occupancy requirement for T cell activation (225). This dependence on CD28 co-stimulation for optimal T cell activation accounts for one of the major mechanisms of peripheral T cell tolerance, in which a T cell is rendered anergic upon TCR engagement in the absence of CD28 co-signaling (32). Evidence of cross-talk between the TCR and Notch proffers threshold modification as a potential niche for the receptor in adaptive

immunity. Alterations in Notch receptor distribution may serve as a tuning fork through which modulations in the T cell activation threshold could be more precisely regulated, in collaboration with CD28 co-stimulation. Notch signaling then would be implicated in a role that extends beyond cell fate determination, and instead spans from T cell lineage commitment to decisions mediating the disparate outcomes of T cell tolerance vs. T cell activation. In the execution of this latter function the role of the Notch ligand expressing DC emerges at the forefront, given the requirement of ligand binding for initiation of Notch signaling. This places the DC in a cardinal position from which it can effectively assimilate, and subsequently translate, environmental cues into directed adaptive immune responses by altering both the concentration and the family of ligands expressed at the cell surface. Although there are no demonstrated restrictions on the ability of ligands from either the Delta or Jagged family to bind any of the 4 Notch receptors, differences in affinities or familial proclivities of a given ligand for a particular receptor broaden the spectrum of biological functions that can be coordinated. We have shown that sensing of pathogen associated molecular patterns (PAMPs) can be synthesized by the DC and the information thus gained can, in turn, be transformed into correlate changes in Notch ligand expression, emphasizing the collaboration of the microenvironment in the evolution of antigen-driven responses. In our disease model for large vessel vasculitis, LPS breaks the immunoprivilege of the arterial wall thereby permitting the initiation of vasculitis. Given the demonstrated modulatory effects on Notch ligand expression, the LPS induced breach of tolerance may very likely involve the promotion of active Notch signaling in the tissue by providing increased access of stimulatory Notch ligands to the T cell. The functional heterogeneity within a localized vascular territory implies that the T

cell response is the result of the integration of unique signals received from the DC, orchestrating the generation of divergent T cell responses, driven by dynamic environmental cues. The inhibition of T cell invasion and cytokine production in the SCID chimera following Notch signaling blockade strongly implicates the Notch pathway as a critical modulator of T cell function in the emerging vasculitic response.

These observations should reinvigorate the discussion about how pathogen-derived motifs render the artery susceptible to inflammation. It is clear that TLR ligands can modulate the function of DC, both by inducing stimulatory and inhibitory receptors. Classifying patients with large vessel vasculitis based on the nature of the innate and adaptive immune responses in the artery could provide valuable clues towards disease instigators and, consequently, in the development of specialized treatment and management strategies.

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