ABSTRACT

Respiratory Syncytial Virus Triggers Immune Tolerance through Induction of Tolerogenic Dendritic Cells and Expansion of Regulatory T Cells

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Mentor: John E. Connolly, Ph.D.

Dendritic cells have the ability to control the balance between immunity and tolerance. Upon viral exposure, Dendritic Cells (DCs) steadily detect pathogens and exert their antigen presentation function to induce adaptive T cell response. Respiratory Syncytial Virus (RSV) is an important respiratory pathogen in infants and young children worldwide. Here we show that RSV exposure polarizes DC maturation to a tolerogenic state. RSV exposed DCs (RSV-DCs) are unable to prime allogeneic CD4+ T cell proliferation and cytokine production. Strikingly, RSV exposed DCs are able to efficiently inhibit on-going Mixed Leukocyte Reaction (MLR) *in trans*. Phenotypic characterization of RSV-DCs indicates that they express a variety of surface inhibitory molecules and secrete high amount of the cytokine IL-10. Autocrine IL-10 receptor signaling is required for tolerogenic conversion. A direct comparison with pharmacologically generated tolerogenic DCs indicates RSV-DCs are much more potent at inhibiting CD4+ T cell alloproliferation. Furthermore, we find that RSV-DCs propagate their tolerogenic signal through expansion of regulatory T cells (Tregs). RSV-

DCs induce the selective expansion of CD4+/CD25+/FoxP3+/CTLA+/GITR+ Tregs in the bulk T cell population. These Tregs are able to inhibit on-going MLR *in trans,* indicating their functional potency. An analysis of the non-proliferating target CD4+ T cells indicates that they are in a state of phenotypic and functional anergy. These T cells express anergy markers and are unresponsive to secondary anti-CD3/CD28 restimulation. Interaction of B7 negative co-stimualtor PD-L1 and its receptor PD-1 are required for Treg expansion and function as blockade led to a reversal of anergy induction in the target population. These *in vitro* observations led us to investigate the impact of RSV-DCs on immune tolerance *in vivo*. We did preliminary studies demonstrating RSV exposure induces BALB/c bone marrow derived DCs (BM-DCs) tolerogenic characterized by inhibition of C57BL/6 CD4 T cell alloproliferation and upregulation of mouse PD-L1 molecule. Similarly to what we observed in human cells, RSV-mouse DCs are able to expand a population of CD4+CD25+FoxP3+ regulatory T cells. These cells with immune suppressive function can then be adoptively transferred into murine models of autoimmune diseases and organ transplantion to suppress antigen-specific immune activation *in vivo*.

Respiratory Syncytial Virus Triggers Immune Tolerance through Induction of Tolerogenic Dendritic Cells and Expansion of Regulatory T Cells

by

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A Dissertation

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DEDICATION

In memory of my beloved grandparents,

J.Guo (Professor of Chemistry) and S.Wang,

who inspired my dream to become a scientist.

CHAPTER ONE

Introduction and Background

Dendritic Cells and Immune Response

Dendritic Cells

Dendritic cells, first characterized by Zanvil A.Cohn and Ralph M. Steinman in 1973 (1) were given their name because of their unusual branched morphology (Figure 1.1). They play a central role in linking the innate immune recognition of invading pathogens to the initiation of adaptive immune responses (2). Dendritic cells are present in nearly every tissue of the body. These cells exist at high densities near potential points of microbial entry, such as the skin, respiratory, urogenital and gastrointestinal mucosa, as well as in the blood (3). Since their initial characterization, an extensive body of work has demonstrated that DCs are uniquely capable of priming naïve T and B cell response to invading pathogens. Aside from their critical function in inducing adaptive immune responses, DCs have been recently found to play a central role in the induction and maintenance of immune tolerance (4).

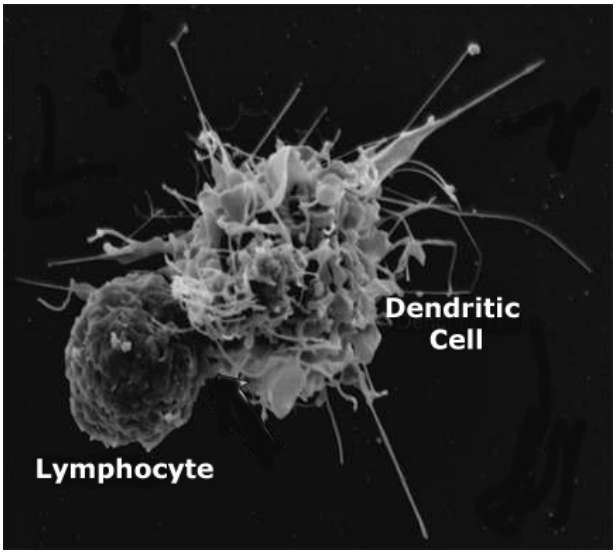


Figure 1.1: Dendritic cell. Dendritic cell get its name from its dendrite-like shape, it plays an important role in linking innate immune recognition of invading pathogens and initiation of adaptive immune response. Antigen-loading mature dendritic cells interact and activate both T and B lymphocytes in the lymph nodes.

The Origin and Development of Dendritic Cells

As shown in Figure 1.2, dendritic cells are derived from hematopoietic stem cells within the bone marrow. These CD34+ stem cells differentiate into common myeloid precursors (CMP) and common lymphoid precursors (CLP). Myeloid precursors differentiate mainly into 3 populations of cells, CLA+ cells, CLA- cells and monocytes (5). CLA+ cells subsequently differentiate into CD11c+CD1a+ cells, these cells migrate into the skin epidermis and become Langerhans cell. CLA- cells differentiate into CD11c+CD1a- cells, which migrate into the skin dermis and other tissues in the body to become interstitial DCs (6). In their peripheral locations, both Langerhans cells and Interstitial DCs serve as antigen-sampling sentinels, they can be further activated by exogenous stimulus such as invading microbial products. In addition to CLA+ and CLA- cells, myeloid cells also give rise to another DC precursor, the monocytes. In the steady

state, monocytes are present in the periphery as pre-DCs, in the context of inflammation, monocytes differentiate into immature DCs in response to soluble inflammatory factors such as GM-CSF, IFN-alpha and IL-4. These DCs resemble interstitial DCs and can be further activated through direct recognition of microbial products or self-associated inflammatory molecules such as CD40L or TNF-alpha. In the early studies, these DCs are considered to have myeloid origin simply because they can be differentiated from bone marrow myeloid precursors by adding myeloid growth factors such as GM-CSF *in vitro*. However, recent studies indicate that CMPs are not the only precursors to generate conventional DCs (7, 8).

Another cell type derived from hematopoietic stem cells is the common lymphoid precursor cells (CLPs). These cells are able to further differentiate into plasmacytoid DCs (pDCs), which are characterized by secretion of high amount of type I interferon (9). pDCs are lineage-CD4+CD123+ cells which become activated in response to viral challenge or CD40L stimulation. The notion that pDCs are of lymphoid origin was supported by the initial findings that gene transcripts of pre-T cell receptor as well as RAG mediated IgH D-J gene rearrangements are found in pDCs but not in mDCs (10, 11). However, recent evidence has indicated that RAG as a marker for lymphoid lineage may not be as specific as it was initially assumed (7, 8).

Recent studies suggest that there seems to have no distinct lineage boundaries for all DC subsets, either CLPs or CMPs are capable of generating both myeloid DCs and plasmacytoid DCs. Transfer of either CLP or CMP from mouse bone marrow to into irradiated recipients is sufficient to generate all DC subtypes (7, 8). These reflect a remarkable developmental flexibility in DC generation. However, both *in vitro* and *in*

vivo experiments indicated that the CMP or CLP cells that are able to develop into both myeloid DCs and plasmacytoid DCs are restricted to only a small proportion of Flt3+ cells in the bone marrow precursor populations (12, 13). Only 2.0-2.5% of total mouse bone marrow cells (about 1 out 92 CLPs and 1 out of 18 CMPs) are flt3+ cells, there cells are able to differentiate into both conventional DCs and pDCs (7, 12). Flt3- cells in both CMPs and CLPs are poor producers of DCs. Flt3- CMPs differentiate into monocytes, granulocytes and flt3- CLPs differentiate into T cell, B cell and NK cells respectively (14).

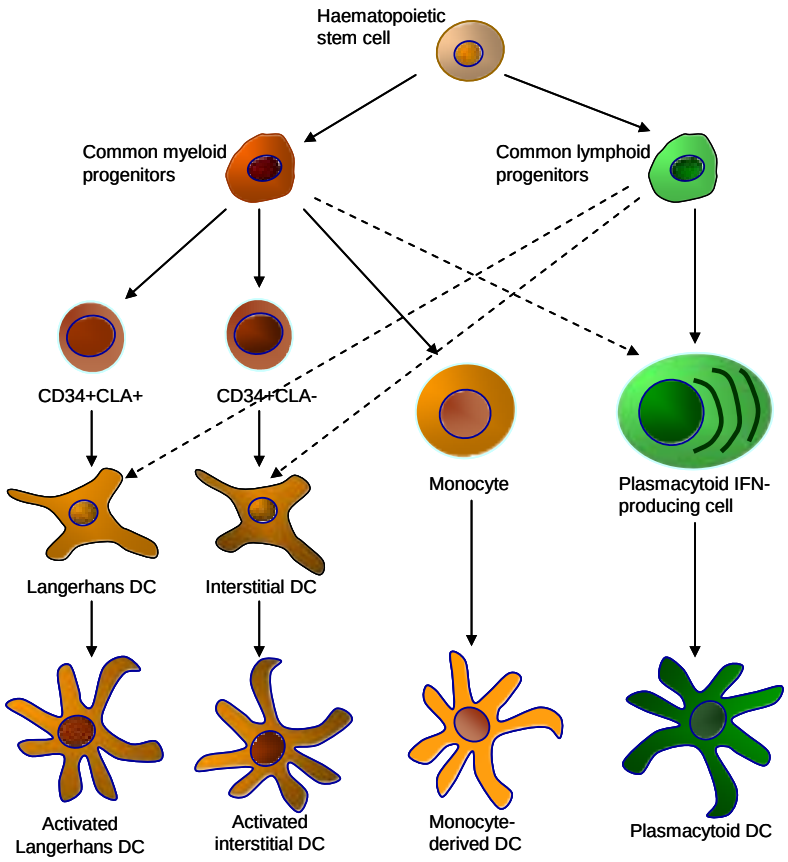


Figure 1.2: Pathways of DC development. Hematopoietic stems cells differentiate into Langerhans cells, interstitial DCs, mDCs of myeloid origin and pDCs of plasmacytoid origin. Under certain conditions, all DCs can be generated by either myeloid or lymphoid progenitors alone.

Dendritic Cell Subsets

DC subsets from heterogeneous origins can be classified based on their lineage, distribution and immune function.

As illustrated in the last section, DCs can be classified based on their lineages as myeloid DCs and plasmacytoid DCs. Besides this, DCs can also be classified based on their anatomical distributions. According to their location inside or outside the lymphoid organs, DCs can be classified as migratory DCs and resident DCs. Langerhans cells and interstitial DCs are migratory DCs, they are widely distributed in the peripheral non-lymphoid tissues and migrate to the local lymphoid organs to present captured antigens. Splenic DCs and thymic DCs are examples of lymphoid organ resident DCs, they do not migrate through the lymph and they only collect and present antigens in the lymphoid organ itself.

DCs can also be classified based on their immune function. A central function of DCs is to capture antigens and present them to naïve T cells to initiate and polarize specific T cell response. Thus DCs can be classified by the ability to prime Th1, Th2, Th17 or Treg responses based on the nature of the pathogen and the environmental context in which the antigen is encountered. DCs secrete IL-12 in response to bacterial and other intracellular pathogens, and induce Th1 response (15). In response to extracellular pathogens, DCs express OX40 ligand and triggers Th2 response (16). Under certain conditions, DCs are able to secrete IL-6, IL-23 and TGF-beta, thus priming TH17 response (17). Furthermore, under tolerogenic conditions, DCs are able to produce IL-10, TGF-beta or retinoic acid, and promote the development of regulatory T cell response (18, 19).

To summarize, DCs are a highly heterogeneous population of cells whose functions vary depending on the microenvironment in which they reside and the pathogens they encounter.

Immature Dendritic Cells

All dendritic cells are derived from hemopoietic bone marrow progenitor cells and traffic to peripheral locations in an immature state. In this immature state (before encountering pathogens), DCs serve as sentinels of the immune system. Characterized by high endocytic activity and low surface MHC expression (Figure 1.3), immature DCs constantly sample the surrounding environment and scan for the presence of pathogens. This scanning is performed through the use of pattern recognition receptors (PRR), which identify specific invariant aspects of microbial pathogens, also called pathogen associated molecule pattern (PAMP) (20, 21). These DC PRR includes Toll-like receptor (TLR) and C-type lectins, which recognize specific chemical signatures found on subsets of pathogens, such as lipopolysaccharide (LPS), peptidoglycans, single stranded RNAs and unmethylated DNA sequences (22-24). TLRs fall into two classes, those which are expressed on the surface such as TLR1, TLR2, TLR4, TLR5, TLR6, TLR11 and those which exist in intracellular endocytic compartments such as TLR3, TLR7, TLR8, TLR9 (25). Each of these molecules binds to a PAMP specific for a particular invading pathogen (Table 1.1). Stimulation of DCs through one or more often a combination of these receptors induces maturation. Following pathogen recognition, they are taken up by receptor-mediated endocytosis, phagocytosis or pinocytosis, processed and subsequently presented in the context of surface MHC molecules.

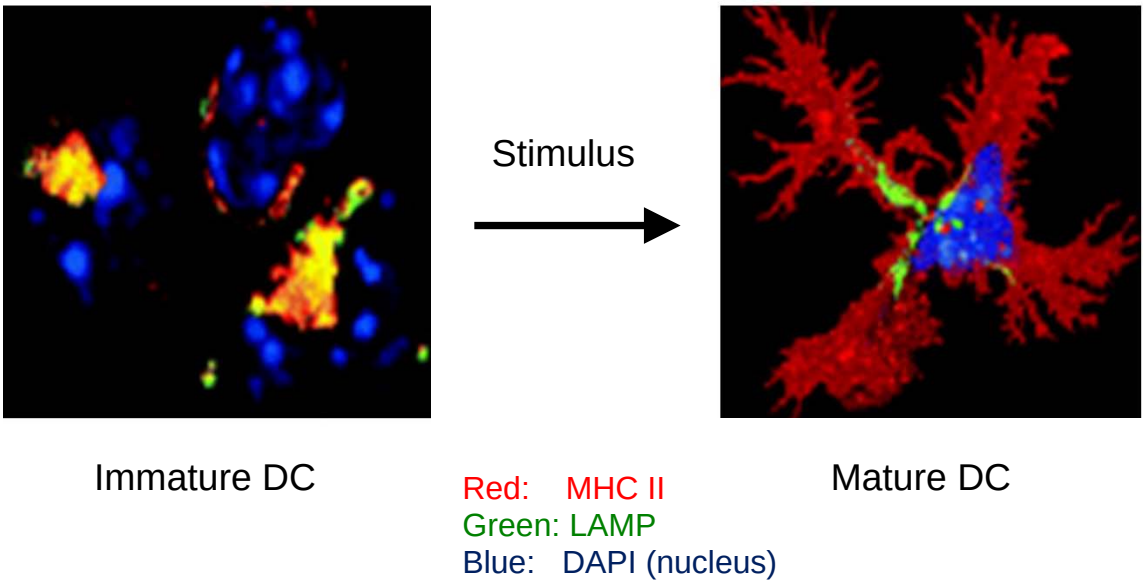


Figure 1.3: DC maturation. DC maturation is characterized by its morphological change (extension of dendrites) and translocation of intracellular MHC II molecule to the surface. Surface MHC II is loaded with antigen peptides and is to be recognized by T cell receptor. (Picture adopted from Villadangos et al, 2005, *Immunol Rev*.)

Dendritic Cell Maturation and Activation

In their mature state, DCs serve as "professional" stimulators of T cell response. Upon detection of pathogens by toll-like receptors, TLR signaling initiates a complex stepwise genetic and cell biological program broadly referred to as maturation. TLRs function as monodimers or heterodimers and have different ligand specificity. For example, TLR4 is the receptor for LPS, LPS binds to LPS-binding protein (LBP) and CD14. The ligand-bound CD14 interacts with TLR4, this interaction recruits MyD88 to the cytoplasmic tail and signals through a series of kinases such as IRAK, TRAF-6, TAK1 and IKK. IKK liberates NFkB from its inhibitor IkB so that it can translocate to the nucleus to turn on a variety of genes required for maturation-induced physiological changes (26).

Table 1.1: Classification of toll like receptors in human

Location	Receptors	Ligands	Pathogen types
Cell surface	TLR1	Triacyl lipopeptides	Bacteria
	TLR2	Glycolipids, lipopeptides, lipoproteins, lipoteichoic acid	Bacteria
		Heat shock protein	Host cells
		Zymosan	Fungi
	TLR4	Lipopolysaccharide	Gram-neg bacteria
		Viral envelop proteins	Viruses
		Heat shock proteins, fibrinogen	Host cells
	TLR5	Flagellin	Bacteria
	TLR6	Diacyl lipopeptides	Mycoplasma
	TLR11	Profilin	Toxoplasma gondii
Endosomal membrane	TLR3	Double-stranded RNA, poly I:C	Viruses
	TLR7	Single stranded RNA, imidazoquinoline, loxoribine	Viruses, small synthetic compounds
	TLR8	Single-stranded RNA, small synthetic compounds	Viruses, small synthetic compounds
	TLR9	Unmethylated CpG DNA	Bacteria

Another outcome of TLR signaling is the activation of Lysosome Associated Membrane Protein 1 (LAMP1), which is located on the surface of MHC class II compartment (MIIC) (27). LAMP1 acidifies the endosomal compartments and results in the degradation of endocytosed or phagocytosed extracellular pathogens into antigenic

peptide fragments. The resultant peptide fragments replace class II-associated invariant-chain peptide (CLIP) that has been pre-loaded to the MHC-II molecule within the MIIC (28, 29), and finally, antigen-bearing MHC-II is transported to the cell surface for antigen presentation for CD4+ T cells (30, 31). As with all nucleated cells, DCs are also able to present intracellular antigens via MHC-I pathway, cytoplasmic viral or bacterial proteins are degraded in the proteasomes and the resultant peptides are transported via transporter-associated-with-antigen-processing (TAP) in to the ER. In the ER, antigenic peptide is loaded to the newly synthesized MHC-I molecule with the help of several catalytic chaperons such as tapasin and calreticulin. The peptide-bearing MHC-I is then translocated to the cell surface for antigen presentation to CD8+ T cells (32-34). Unlike other antigen-presenting cells, DCs are uniquely capable of cross-presenting extracelluar antigens through MHC-I pathway to CD8+ T cells. This cross-presentation capability is crucial for combating intracellular pathogens that do not infect DCs (35, 36). There are 3 competing hypothesis as to the mechanism of antigen cross-presentation. The first hypothesis proposes a phagosome-to-cytosol pathway, endocytosed or phagocytosed pathogens are transported to the cytoplasm, processed into peptides, transported by TAP into ER, and then loaded onto MHC-I molecule to be translocated to the cell surface (37, 38). In the vesicular pathway, pathogens are degraded into peptides in the intracellular endosomes and loaded directly onto MHC-I molecules that have trafficked into the vesicle from plasma membrane or ER (39). The third hypothesis is the phagosome-to-ER pathway, in which proteins are retrograde-transported into cytoplasm through SEC61 and get degraded in the proteasome, a process called ER Associated Degradation (ERAD). The resultant peptides are transported back to the same phagosome through TAP for

loading to the MHC-I molecules (40, 41). Following a period of rapid, activation induced pinocytosis, fully matured DCs are no longer able to engulf new pathogens; they instead express high levels of stable surface antigenic-peptide-loaded MHC molecules.

Another feature of mature DCs is the expression of B7 family co-stimulatory molecules CD80 and CD86, these molecules are responsible for delivering a secondary stimulatory signal to activate T cells. Ligation of co-stimulatory molecules to their receptor CD28 on T cells initiate signaling transduction inside T cells responsible for IL-2 transcription, which is crucial for T cell survival and proliferation (42, 43). Along with the maturation process, TLR signaling also results in the upregulation of chemokine receptors on DC surface, which instructs the migration of DCs to the local lymphoid organs (Figure.1.4). Additionally, mature DCs also express high levels of adhesion molecules such as C-type lectin DC-SIGN, ICAM1 and secrete chemokine CCL18, which help to attract and interact with naïve T cells (44, 45). By the time they enter peripheral lymphoid organs, DCs have already transformed to a phenotypical and functional mature state. As DCs enter the T cell zone, T cells scan the peptide-MHC complex displayed on DC surface for their binding specificities, which results in binding of peptide-MHC to the antigen-specific TCR (46). At the same time, CD40 molecule on DCs interacts with CD40 ligand on T cells, which delivers a signal to upregulate co-stimulatory molecules CD80 and CD86 on DC surface over and above the levels initialized by TLR signaling (47). Costimulatory molecules CD80 and CD86 bind to CD28 receptor on T cell surface, which transduces a secondary signaling for T cell activation (48). The completion of both MHC-TCR binding and CD80/CD86-CD28 binding triggers signal transduction through ZAP70 inside the T cell (49), which induces

the activation of the transcription factors NFAT, AP-1 and NFkB (50, 51). The direct result of this signaling transduction is the synthesis and secretion of IL-2 which triggers T cell clonal expansion (Figure 1.5).

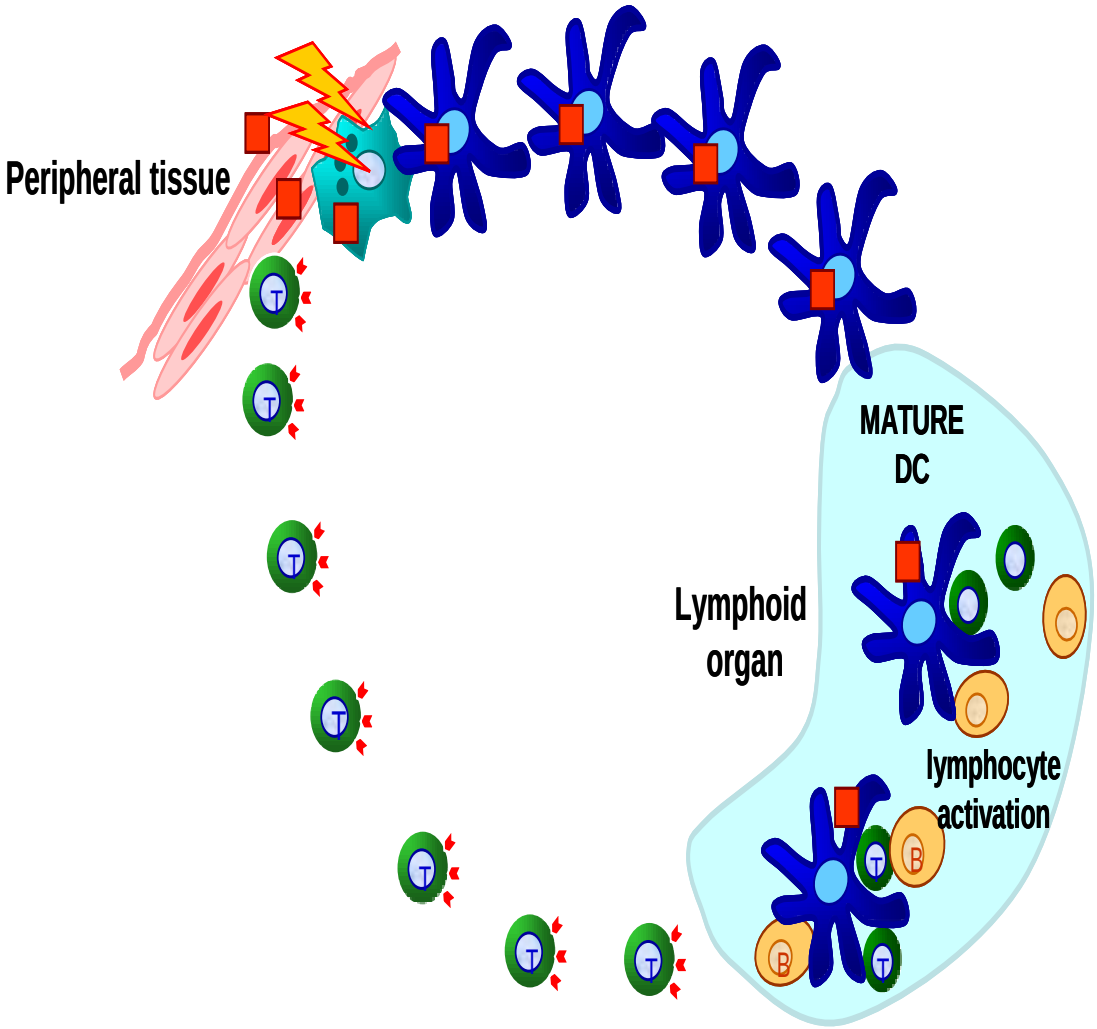


Figure 1.4: DC activation and migration. DCs in the peripheral tissues detect and capture invading pathogens. Internalization and processing of pathogen lead to DC maturation characterized by surface MHC translocation and costimulatory molecule expression. When activated DCs migrate to the local lymph nodes, they activate naïve T cell and B cells, the former then migrate to the site of infection and facilitate the killing of the pathogen.

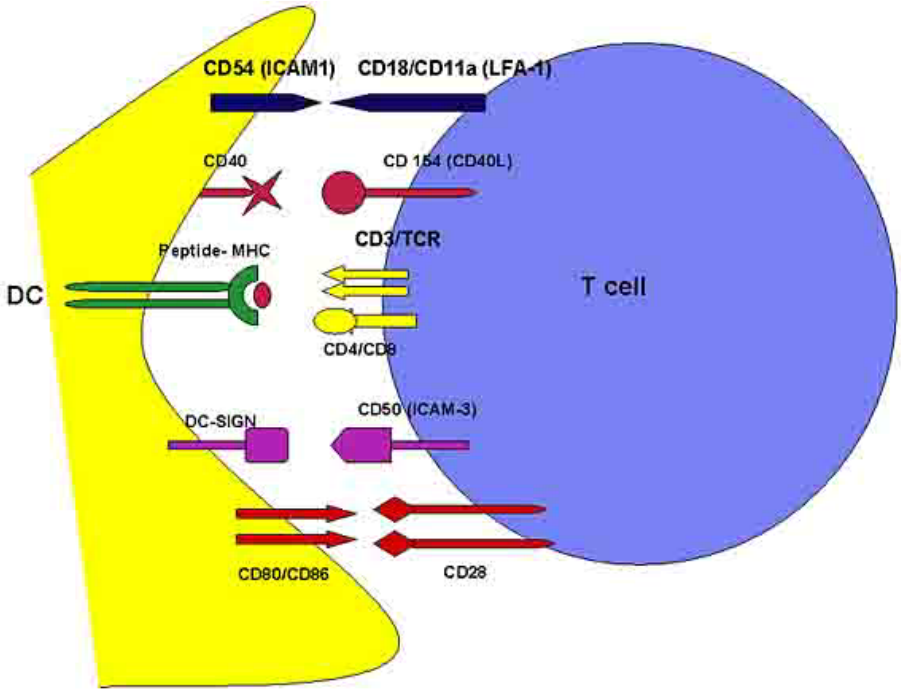


Figure 1.5: DC and T cell interaction. The interaction between mature DCs and T cells involves binding of peptide-MHC to TCR, as well as binding of co-stimulatory molecules CD40 to CD40L and CD80/CD86 to CD28, both of which are required for T cell activation. The bindings of adhesion molecules such as DC-SIGN and ICAM-3 are important for cell attraction and interaction.

Viral Strategies to Subvert DC Function

Viruses coevolve with their host immune system so that they can exploit ways to survive in the host. DCs are ubiquitous antigen presentation cells in the adaptive immune system, thus viruses have evolved strategies to subvert DC function for modulation of T cell immunity (Figure 1.6).

One mechanism viruses have evolved is to evade the detection and uptake by DCs. For example, HIV Tat protein inhibits phagocytic uptake by DCs thereby limiting downstream antigen availability (52). Internalization of HIV through DC-SIGN leads to sequestration of the virus within an endosomal compartment where it is protected from antigen presentation (53). Without the ability to uptake viruses, intracellular DC TLRs

may not have the ability to sense viral PAMPs, such as ssRNA or CpG DNA. Additionally, viral antigens will not traffic to intracellular DC processing compartments. Evading uptake therefore represents an effective method of blunting T cell anti-viral immunity.

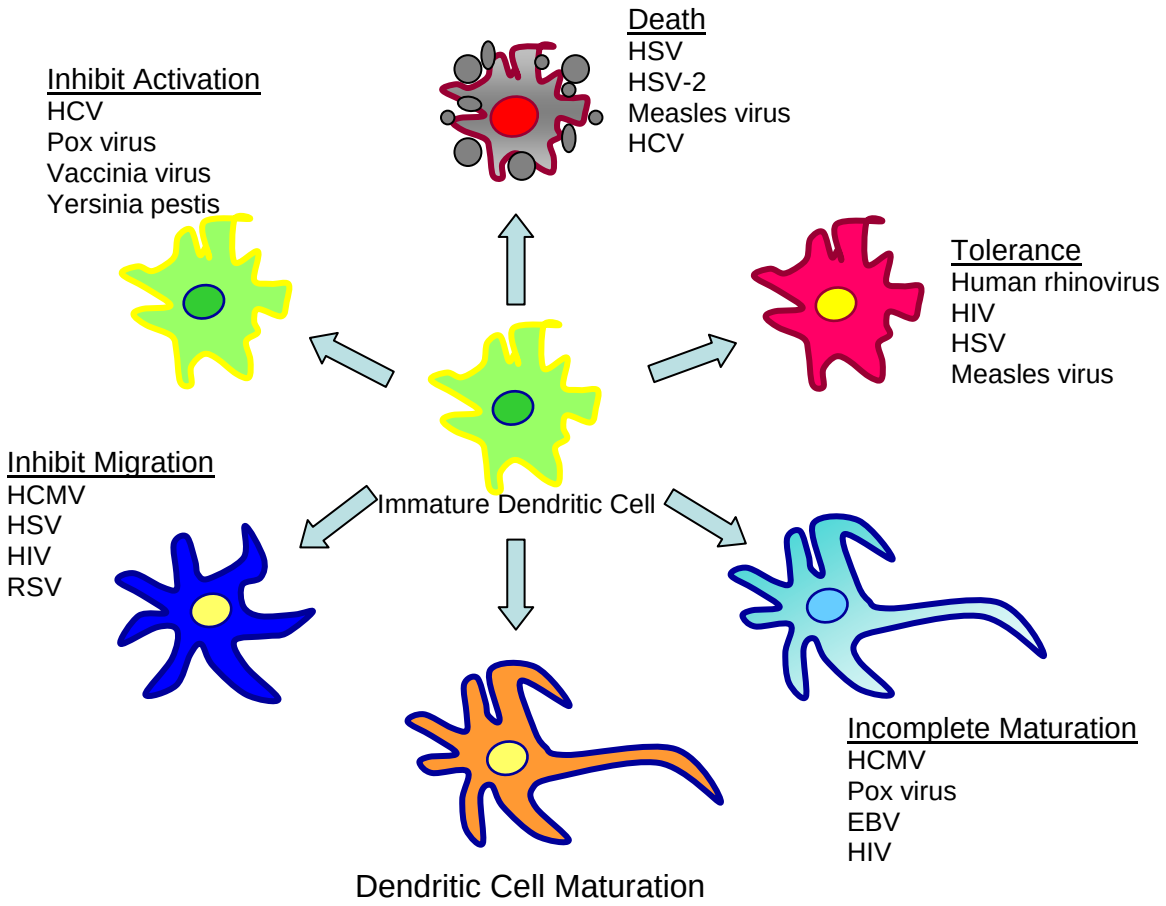


Figure 1.6: Viruses subvert DC function. Viruses develop different strategies to induce DC death, inhibit their activation, migration or maturation, or induce DC mediated immune tolerance. In this way, DC mediated induction of adaptive immune response is subverted, which favors the viruses to survive.

Viruses can also inhibit DC maturation. For example, HCMV infection does not induce DC maturation, instead, it effectively downregulates the expression of surface MHC-class I, CD40 and CD80 molecules, as well as effector cytokine secretion. These

HCMV-infected DCs are unable to generate virus-specific cytotoxic T cell response, as immature DCs can not efficiently prime new responses (54).

Viruses are also able to block the migration of DCs to the lymph nodes. For example, HCMV blocks the migration of infected DCs in response to lymph node chemotactic chemokines CCL19 and CCL21. Expression of CCR7 is markedly downregulated in infected cells in response to subsequent stimulation by TLR agonists (55). As the lymph node architecture provides an environment for efficient naïve T cell antigen scanning, the presentation of peripheral antigen to this population is effectively eliminated.

Perhaps the most direct route to subverting DC function is to kill DCs. HSV infection of immature DCs leads to their rapid apoptosis in a manner dependent on tumor necrosis factor–related apoptosis-inducing ligand (TRAIL). Infection leads to a downregulation of the anti-apoptotic protein c-FLIP making DCs susceptible to TRAIL killing (56). The use of apoptotic machinery in the elimination of dendritic cells may suppress local inflammation and prevent further recruitment of iDCs to the site of infection.

Inhibition of MHC processing and presentation is another effective way to block DC function. HCMV utilizes multiple strategies to downregulate MHC class I and upregulate non-classical MHC molecule HLA-G (57, 58). Both the gB protein of herpes virus and Vpx protein of HIV-2 disrupt class II processing by binding directly to the invariant chain leading to its degradation (59-61). The inability to present viral antigens on the surface of the DCs blocks T cell priming even in the context of high costimulation, as CD28 signal is delivered in the absence of TCR engagement.

Recently, emerging evidence indicate that viruses are able to skew DC maturation towards a tolerogenic state, these tolerized DCs secrete inhibitory cytokine IL-10, upregulate surface inhibitory molecules and are unable to drive T cell activation. At face value, this strategy represents a potent mechanism for blocking anti-viral T cell expansion. Tolerogenic DCs will be reviewed later.

Immune Tolerance and Tolerogenic DCs

Immune Tolerance

The stochastic nature of immunoglobulin receptor rearrangement necessitates a process for removal of self reactive lymphocytes. Immune tolerance is the active process by which the magnitude or duration of an immune response is inhibited in order to limit collateral tissue damage. In mammals this tolerance is established and maintained by two mechanisms: central tolerance and peripheral tolerance (Figure 1.7).

Central tolerance is an integral part of T cell development. In the thymus, T cells first undergo positive selection to ensure they are able to recognize MHC molecules (62, 63). These selected T cells then undergo a process of negative selection in which cells that react strongly with self-peptide-MHC complex are killed and thereby deleted from the overall T cell repertoire (64, 65). Although a critical fail-safe to prevent autoimmune disease, this process of central tolerance is incomplete. Many moderately-self-reactive T cells escape clonal deletion and migrate to the periphery.

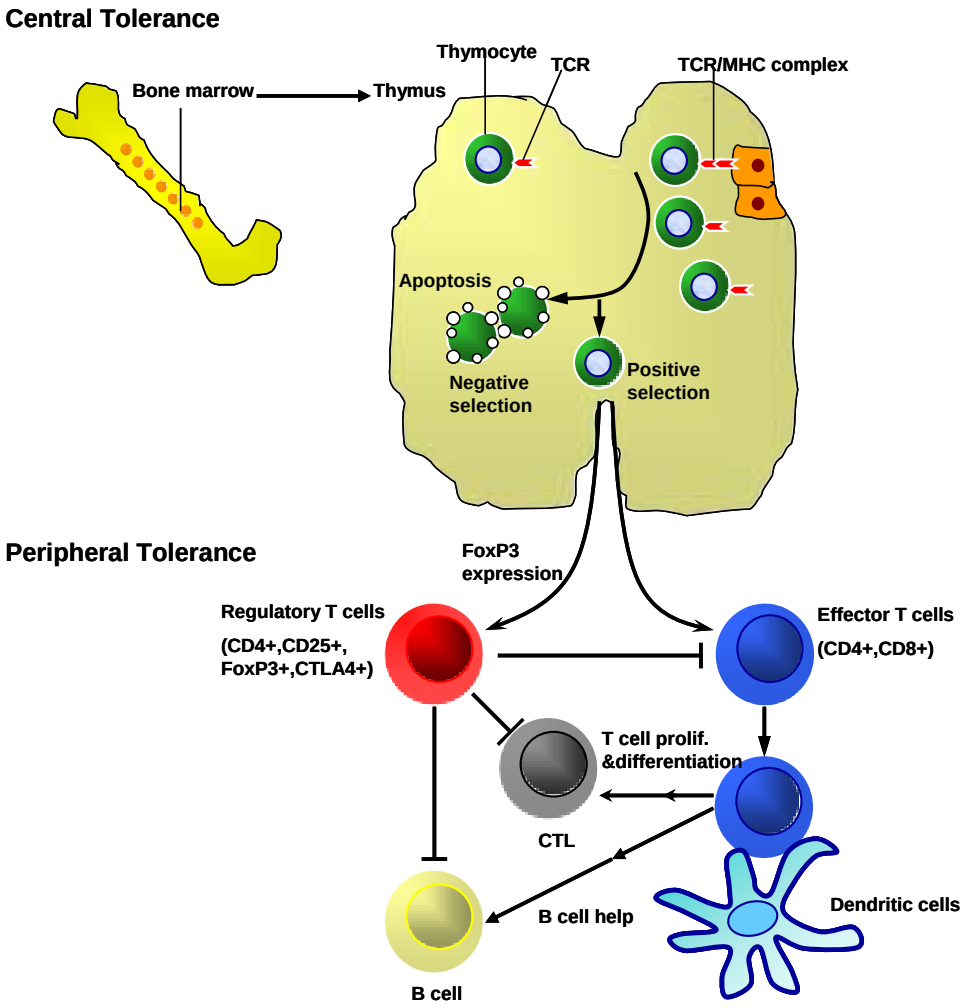


Figure 1.7: Central and peripheral tolerance. Central tolerance happens when thymocytes which recognize self-MHC are positively selected followed by a negative selection in which thymocytes that react too strongly with MHC-self peptide complex are deleted from the T cell repertoire. A random proportion of thymocytes that moderately react with MHC-self peptide complex are signaled to express FoxP3/CTLA-4 and become Tregs. Immature T cells that react minimally to self-peptide migrate to the peripheral as effector T cells while Tregs migrate to the peripheral to shut off immune activation to self-antigen or innocuous antigen, which is known as peripheral tolerance. (Figure adapted from Gregersen & Behrens, 2006, *Nat.Rev.Genet.*)

Once in the periphery, the destructive capacity of these self-reactive T cells is kept in check by a variety of mechanisms collectively referred to as peripheral tolerance. An important mechanism is the induction of T cell anergy. In the absence of costimulatory signals, even though self-reactive T cells encounter cognate self-peptide

presented by MHC, they do not divide, but rather become refractory to both primary and secondary stimulation (66, 67). Self-reactive T cells may also be suppressed by regulatory T cells (Tregs). Tregs are derived from the thymus. Upon recognition of their specific antigen, they can broadly suppress nearby effector T cells in a non-specific manner (68).

In the steady state therefore, the final pool of self reactive cells has been culled by the gauntlet of central tolerance to a population of moderate and low affinity cells kept in check by ever vigilant peripheral tolerance mechanisms.

DCs and Immune Tolerance

During central tolerance, thymic DCs and thymic medullary epithelial cells are responsible for shaping the T cell repertoire. DCs are located in thymic medulla, where they capture and present self-antigens. T cells which recognize self-peptides presented by these DCs are deleted from the repertoire. This important role of DCs in shaping the T cell repertoire was demonstrated by pulsing thymic DCs with a model antigen. Addition of these loaded DCs to a thymic culture system resulted in the selective deletion of antigen-specific thymocytes (65, 69). Although effective at removing high affinity cells, central tolerance is at best incomplete. Many self-reactive T cells which escape clonal deletion migrate into the peripheral population. Additional tolerance mechanisms are required to limit the impact of these T cells in the periphery.

In the periphery, DCs are believed to be the pivotal mediators of immune tolerance to self-antigens. Constitutive ablation of DCs in mice resulted in the development of spontaneous fatal autoimmunity under steady state conditions (70). Initially, it was thought that phenotypically immature DCs which take up and present

self-proteins derived from normal tissue turnover are responsible for the induction of peripheral tolerance. In 2002, Bonifaz et al showed that antigen delivery by anti-DEC-205 antibodies to immature DC induced CD8+ T cell tolerance (71). Simultaneously Mahnke et al. demonstrated that anti-DEC-205 antibodies targeted to immature DC *in vivo* expanded CD25+/CTLA-4+ Tregs (72). These observations led to the belief that immature DCs in the steady state induce immune tolerance while mature DCs induce immunegencity. Over time, considerable evidence has mounted against this dichotomous model of DC function. In 2008, researchers from the same group demonstrated that anti-DEC-205 actually targeted mature DC *in vivo*, and these phenotypically mature cells are potent inducers of Foxp3+ Tregs (19).

Tolerogenic DCs

Tolerogenic DCs, like mature immunogenic DCs, are defined by their functional and phenotypic characteristics.

Whereas immunogenic DCs are often functionally defined as strong stimulators of alloproliferation, tolerogenic DCs have the ability to inhibit on-going alloproliferation *in trans* in a dose dependent manner. A second, intimately related *in vitro* property of tolerogenic DCs is their ability to drive Foxp3+ Treg expansion (Figure 1.8). These unique properties of tolerogenic DCs lead to their profound potency *in vivo*, namely the ability to induce durable tolerance to transplanted allogeneic grafts and suppress the development of autoimmue diseases. A review of current literature demonstrates that tolerogenic DCs which satisfy these functional criteria share a variety of common phenotypic characteristics, namely the expression of ITIM containing receptors and

ligands. The phenotypic expression of these molecules points to a central role for IL-10 receptor signaling during the DC maturation process (Figure 1.9).

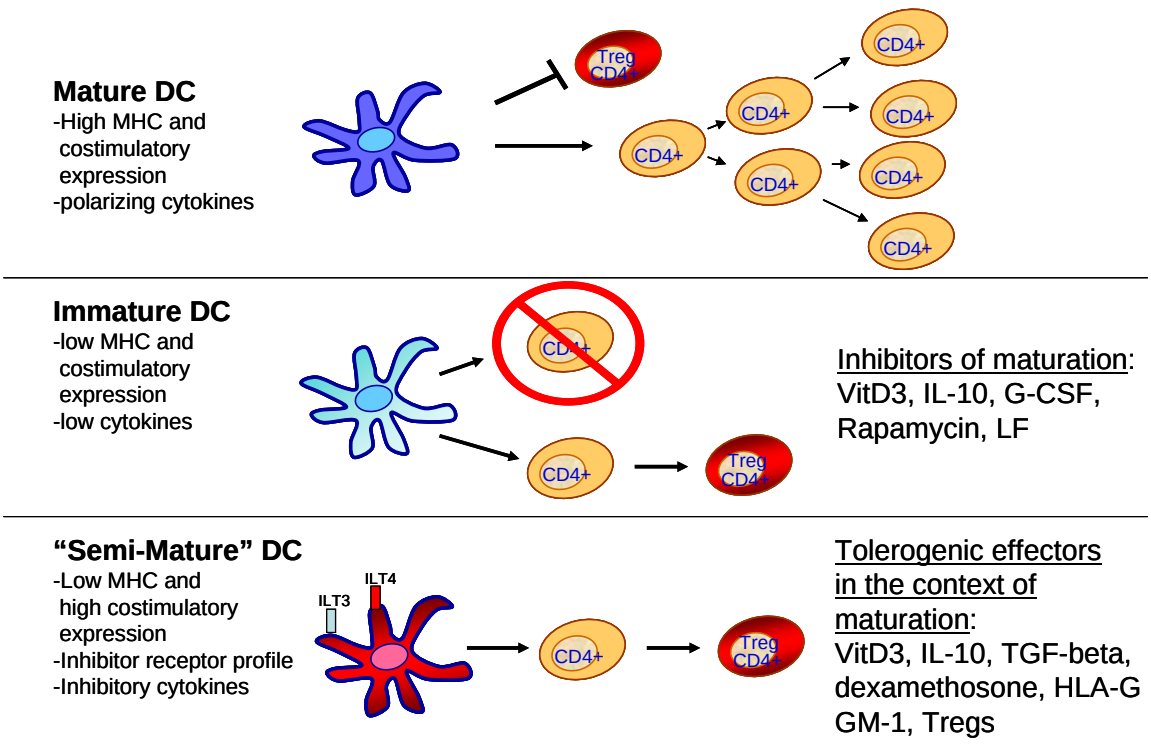


Figure 1.8: A comparison of immunogenic and tolerogenic DCs. Immunogenic DCs express high levels of MHC and costimulatory molecules, they are able to stimulate the proliferation of CD4+ effector T cells. Tolerogenic DCs express low levels of MHC and costimulatory molecules, but produce inhibitory receptors and cytokines. They are able to inhibit CD4+ effector T cell proliferation and promote FoxP3+ Treg expansion.

Phenotypic characterization of tolerogenic DCs includes the expression of surface ITIM containing receptors and their ligands, and the secretion of inhibitory cytokines (e.g. TGF-beta, IL-10), both of which contribute to the inhibition of T cell response. ILT family members ILT3 and ILT4 belong to a family of Ig-like inhibitory receptors which display a long cytoplasmic tail containing immunoreceptor tyrosine-based inhibitory motifs (ITIMs) (73). Transduction of ILT3 and ILT4 in DCs results in blocked proliferation of allogeneic T cells and generation of Foxp3+ Tregs (74). Microarray and

qPCR data indicate that IL-10 regulates the expression of ILTs in DCs and monocytes (75, 76). Treatment of myeloid DCs with extrogenous IL-10 display high levels of ILT3 and ILT4 on their surface (77). PD-L1 is the ligand of programmed death 1(PD-1), PD-1 contains an ITIM motif responsible for the recruitment of SHP phosphatase (78). Ligation of PD-1/PD-L1 plays an important role in inhibiting T cell response (79). Blocking PD-L1/PD-1 signaling pathway augmented HIV-specific CD4 and CD8 T cell function in chronic HIV infection (80). Both our and other's laboratories have observed a role of IL-10 in regulating PD-L1 expression on tolerogenic DCs. Neutralizing IL-10 by antibody reversed upregulated PD-L1 expression in DCs from tumor patients, while extrogenous addition of IL-10 in DC culture from normal donor upregulated PD-L1 surface expression (81). Paired immunoglobulin-like receptors (PILRs) are orthologues of ILTs which also contains an Immunoreceptor Tyrosine-based Inhibitory Motifs (ITIM) (82). They play a role in regulating CTL triggering by blocking the access of CD8 molecules to MHC-I, PIR-B-deficient DCs evoked CTLs more efficiently, leading to accelerated graft and tumor rejection (83). PILR-alpha has been demonstrated as IL-10-inducible by microarray (75). The expression of Indoleamine 2, 3-dioxygenase (IDO), an enzyme of tryptophan catabolism, has been associated with tolerogenic phenotype. Transduction of IDO gene into DCs suppressed allogeneic T cell proliferation *in vitro* (84). IL-10 has been shown to regulate the expression of IDO in DCs. The presence of IL-10 during DC maturation prevented IFN-γ-induced down-regulation of IDO, resulting in sustained expression of functional IDO even in mature, IFN-γ-activated DCs (85). IL-10 also regulates the expression of other tolerogenic DC markers, such as HLA-G, Inhibin Beta A (INHBA), Aquaporin 9 (AQP9), Signaling Lymphocytic Activation

Molecule (SLAM), etc. (75), which are believed to be expressed on all tolerogenic DCs. Functions as they relate to tolerance induction are unclear at this point.

It is now clear that tolerogenic DCs present in the gut (86), liver (87) and lung (88) or pharmacologically generated tolerogenic Vit-D3-DCs and Dex-DCs display a mature phenotype (89, 90). IL-10 is believed to play a pivotal role in regulating the expression of both immunoinhibitory receptors and cytokines during tolerogenic DC maturation process. Inhibitory cytokine TGF-beta seems not to be involved in tolerogenic DC induction. Elegant work using dominant negative form of TGF-beta receptor II under the control of CD11c promoter (CD11c-dnTGFbetaRII) showed that DC homeostasis is independent on TGF-beta signaling (91).

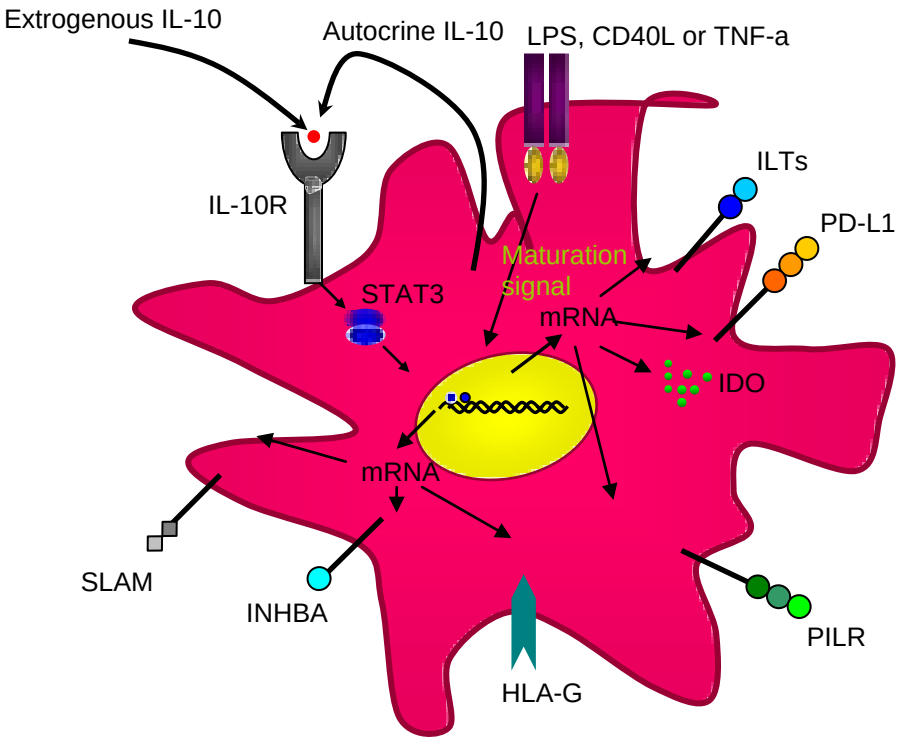


Figure 1.9: IL-10 signaling dominates the generation of tolerogenic DCs. The expression of inhibitory molecules ILTs, PD-L1, PILR, HLA-G, INHBA, SLAM and IDO are regulated by IL-10 signaling pathway.

Tolerogenic DCs, in a consensus definition, are subsets of mature-polarized DCs that function in inhibition of immune activation. IL-10 regulates the generation of inhibitory molecules in tolerogenic DCs, leading to the functional competency of immune regulation (Figure 1.9).

Virus-induced Tolerogenic DCs

As discussed above, IL-10 receptor signaling during maturation plays a pivotal role in the induction of tolerogenic DCs. Human cytomegalovirus (hCMV) expresses no less than 7 IL-10 isoforms during the initial phase of infection (92). CMV-IL-10 expression occurs in the immediate early phase and is therefore coincident with on-going DC maturation (93, 94). Raftery et al demonstrated that exposure of DCs to CMV-IL-10 polarized these cells toward a tolerogenic phenotype characterized by high IDO expression (95). This tolerogenic polarization occurred only when CMV-IL-10 was delivered in the context of on-going maturation. Besides expressing IDO, CMV induced tolerogenic DCs upregulate inhibitory ligand PD-L1, a common feature of tolerogenic DCs. Blocking the interaction of PD-L1 and its receptor PD-1 restored antigen-specific T cell function (96). Inhibitory molecule ILT-2 and ILT-4 are also reported to be upregulated on DCs upon CMV exposure (97). These findings suggest a role for tolerogenic DCs in the pathophysiology of CMV infection.

Epstein-Barr virus (EBV) is another herpesvirus that is able to produce viral encoded IL-10 which suppresses immune function (98). EBV-IL-10 has 84% amino acids identical to human IL-10. Addition of EBV-IL-10 into DC culture skewed them to a tolerogenic state characterized by reduced ability to stimulate T cell proliferation and

induction of alloantigen-specific hyporesponsiveness (99). Tolerogenic DCs may contribute to the long-term persistence of these herpesviruses in the human population even in the context of an active antiviral T cell response.

Besides herpesviruses, other viral families have also independently devised strategies to induce tolerogenic DCs. HIV is a lentivirus belonging to the family of retroviridae. Exposure to HIV induced IL-10 production in DCs, which in turn were capable of inhibiting allogeneic T cell proliferation *in trans* in an IL-10 dependent manner. IL-10 secretion not only induced tolerogenic DCs but also induced CCR5 upregulation, leading to more efficient HIV infection (100). PD-L1 on DCs and PD-1 on T cells were selectively upregulated upon HIV infection (80, 101), blocking their interaction augmented HIV-specific CD4 and CD8 T cell function (80). Antiviral therapy reduced IL-10 production, resulting in downregulation of PD-L1, in agreement with the central role of IL-10 in controlling tolerogenic molecule expression (101). HIV infection also induced high levels of surface HLA-G expression, which played a role in HIV persistence (102). Other reports also indicated Foxp3+ Treg expansion by HIV that correlated with the viral load (103, 104). Expansion of Tregs played a major role in the maintenance of a tolerogenic microenvironment that favored viral infection and replication. Successful antiviral therapy resulted in depletion of Treg cells in PBMC or mucosal tissue (104).

Hepatitis C virus (HCV) belongs to the family of Flaviviridae. HCV-infected DCs produce elevated IL-10 and decreased IL-12 (105), indicating a skew of immune response from Th1-inducing state to tolerance-inducing state. Blocking IL-10 restored impaired T cell proliferation (105). PD-L1 upregulation of HCV-DCs was responsible

for impaired T cell function as indicated by antibody blockade of PD-L1 and PD-1 interaction (106). Boettler et al demonstrated a role of HCV-DC expanded CD4+CD25+ Tregs in the suppression of virus-specific CD8+ T cell response (107). Treg expansion contributed to viral persistence in chronically HCV-infected patients. Much like CMV, these immune modulatory mechanisms suggest a role for tolerogenic DCs in persistent HCV infection even in the face of high avidity CTL responses.

Measles virus (MV) is a Paramyxovirus that induces immune tolerance though targeting and altering DCs. MV infection produced high amount of IL-10 that was responsible for tolerogenic DC induction (108). MV selectively upregulated and targeted inhibitory receptor SLAM on tolerogenic DC surface to suppress both antigen-specific and allogeneic T cell proliferation (109). Beside these, CD4+CD25+ Tregs were also induced upon MV infection which contributed to the immuosuppression (108).

Unlike the herpes-, flavi- and lenti-viral family members, measles does not establish chronic infections in their hosts. The exploitation of tolerogenic DC maturation by these viruses may indicate a selective advantage, not only in the establishment of long term tolerance but also during the initial phases of viral expansion.

In summary, a variety of viruses have convergently evolved mechanisms to induce tolerogenic DCs which favors the subversion of host immune activation. Reported viral strategies to induce tolerogenic DCs are summarized in Table 1.2.

Table 1.2: Viruses from different families convergently evolved strategies to induce tolerogenic DCs

Family	Virus	Induce IL-10	Inhibit T cell prolif.	Express PD-L1	Express HLA-G	Express ILTs	Express SLAM	Expand / induce Treg	Others
Herpes-virus	CMV	CMV-IL-10 (93, 94)	Yes (95)	Yes, blockade restores prolif. (96)	Yes (110)	ILT-2 and ILT-4 (97)			Express IDO (95)
	EBV	EBV-IL-10 (98)	Yes (99)			ILT-3 and ILT-4 (111)			Induce Anergy (99)
Lenti-virus	HIV	Yes (100)	Yes (80, 100)	Yes, blockade restores prolif. (80)	Yes (102)	Yes (112)	Yes (113)	Yes (103, 104)	
Flavi-virus	HCV	Yes, blockade restores prolif. (105)	Yes (105, 106)	Yes, blockade restores prolif. (106)				Yes (107)	
Paramyxo-virus	RSV	Yes (unpub. data)	Yes ((114) and unpub. data)	Yes, blockade restores prolif. (unpub. data)	Yes (unpub. data)	Yes (unpub. data)	Yes (unpub. data)	Yes (unpub. data)	Induce anergy (unpub. data)
	Measles Vrirus	Yes (108)	Yes (108, 109)				Yes, suppression is SLAM depen-dent (109)	Yes (108)	
Picona-virus	Rhino-virus	Yes (115, 116)	Yes (117)	Yes, blockade restores prolif. (117)					Express Sialoadhesin. Induce anergy (117)

Regulatory T Cells

Tregs

Regulatory T cells (Tregs) were initially described by Gershon *et al*. in the early 1970s and were called suppressive T cells (118). Regulatory T cells can be defined as a T-cell population that functionally suppresses an immune response by influencing the activity of another cell type.

Regulatory T cells come in many forms, some express CD8 transmembrane glycoprotein (CD8+CD28- T cells), some express CD4, CD25, Foxp3 (CD4+CD25+Foxp3+ Tregs) and some do not express these markers but secrete inhibitory cytokine IL-10 (Tr cells), while others secrete TGF-beta (Th3 cells). CD4+CD25+Foxp3+ T cells are referred to as naturally occurring Tregs and have been intensively studied (119).

The Development of Naturally Occurring Tregs

Naturally occurring Tregs derive from the thymus. In the thymus, all T cells are initially CD4-CD8-TCR-, they combine their TCR genes to form a functional molecule and test with cells in thymic cortex for an adequate level of interaction with self-MHC, a process known as positive selection (62, 63). If they passed this selection, they begin to express both CD4 and CD8 on their surface and move to the thymic medulla, where they are negatively selected for their binding affinity with self-peptides presented by thymic DCs (64, 65). The negative selection is a “Goldilocks” process, T cells that react too strongly with self-peptide-MHC are signaled to undergo apoptosis thus deleted from the T cell repertoire; cells that have minimal interaction with self-peptide-MHC are selected as effector T cells and release to the periphery; cells that react intermediately are either

delected, anergized or a random proportion of them are selected as Tregs. These selected Tregs begin the transcription of genes such as FoxP3 and produce anti-apoptotic molecules to protect them from being deleted (120, 121). These cells later become functional Tregs and are release to the periphery for non-antigen-specific suppression of T cell activation. Tregs have a larger TCR diversity than effector T cells, biased towards self-peptides. In periphery, Tregs can be further expanded in response to tolerogenic signal, their expansion is dependent on IL-2 (122) (Figure 1.10).

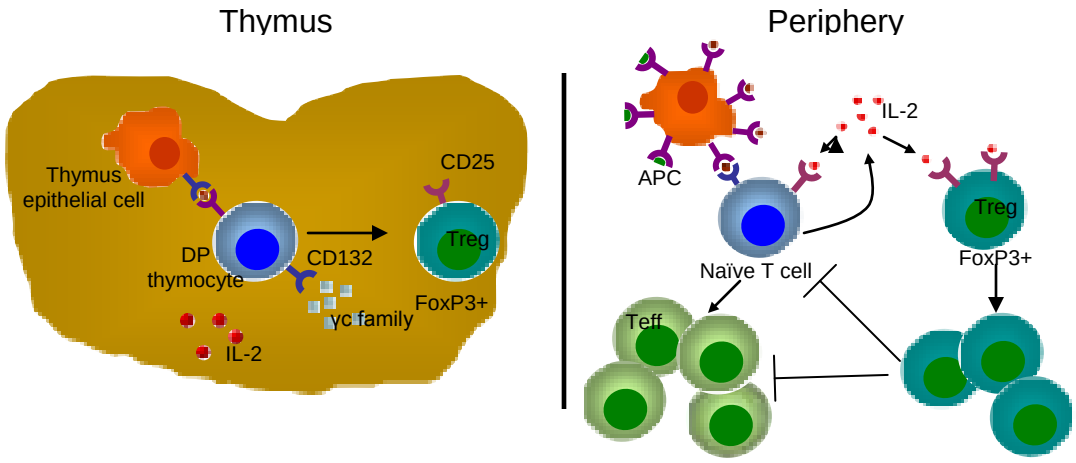


Figure 1.10: Treg development. Tregs are developed in the thymus by positive selection. Selected cells begin to express CD25 and FoxP3. In the periphery these cells can be further expanded in response to tolerogenic signaling.

Treg Phenotype and Function

In steady condition, Tregs suppress the immune activation to self-antigen or innocuous antigens, which if not controlled properly, induce the development of autoimmune diseases. The critical role regulatory T cells play within the immune system is evidenced by the severe autoimmune syndrome that results from a genetic deficiency in regulatory T cells (123).

Other than expressing Foxp3, Tregs are also demonstrated to specifically express CTLA-4 and GITR. The production of anti-inflammatory cytokines, such as TGF-beta and IL-10, has been shown to also contribute to natural Treg suppressive activity *in vivo* (124). CTLA4-expressing natural Tregs induce the expression by APCs of the enzyme indoleamine 2, 3-dioxygenase (IDO), which degrades tryptophan, and lack of this essential amino acid has been shown to inhibit T-cell activation and promote T-cell apoptosis (125)

Viruses Favor the Treg Generation and Function

Because Tregs generate favorable conditions for the persistence of viruses, it is conceivable that the induction, maintenance and function of Tregs could also be manipulated by viruses. During HBV infection, CD4+CD25+Foxp3+ Tregs increased dramatically in both PBMC and liver-infiltrating lymphocytes (126), serum TGF-beta was responsible for the conversion of peripheral CD4+CD25- T cell to CD4+CD25+ Tregs (127). Foxp3+ Treg expansion by HIV is correlated with the viral load (103, 104). Expansion of Tregs played a major role in the maintenance of a tolerogenic microenvironment that favored viral infection and replication. Successful antiviral therapy resulted in depletion of Treg cells in PBMC or mucosal tissue (104). During HCV infection, Boettler et al demonstrated a role of HCV-DC expanded CD4+CD25+ Tregs in the suppression of virus-specific CD8+ T cell response (107).

Respiratory Syncytial Virus and Viral Immunity

Respiratory Syncytial Virus (RSV) is an important respiratory pathogen in infants and young children world wide. RSV infection causes hospitalization of nearly 125,000

children and 1800 deaths in the United States annually. Worldwide, the hospitalization rate is even higher with a mortality rate of nearly 5%. Re-infection is common throughout life, even when exposed to the same viral strain (128, 129). Up to date, clinically there is no effective vaccine against this virus, initial attempt of vaccination using formalin-inactivated virus eventually led to exacerbation of the disease upon exposure to the natural stains (130, 131). Thus, virological and immunological studies on RSV pathogenesis are active research area; the ultimate goal is to develop effective vaccine against this virus.

RSV is a member of the genus *Pneumovirus* of the family *Paramyxoviridae*. It is an enveloped virus with a single stranded negative sense genome. The viral genome consists of 10 genes, and these are translated into 11 proteins (Figure 1.11). The fusion protein (F protein) facilitates the fusion of the viral and host membranes, and is demonstrated to be able to induce helper T cell type 1 immune response (132). Attachment G protein, associated with enhanced disease, induces a Th2-type immune response (133). Phosphoprotein P is an essential subunit of the viral RNA-dependent RNA polymerase; siRNAs targeting P protein strongly inhibit RSV replication *in vivo* (134). Non-structural protein NS1 and NS2 suppress type I IFN production in infected cells through the inactivation of IRF-3 (135). Also, the viral genome transcribes nucleocapsid protein N, small hydrophobic protein SH, polymerase L and matrix protein M and M2/22k.

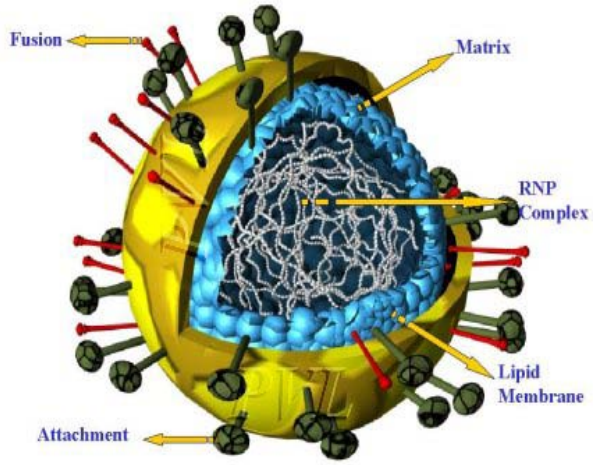


Figure 1.11: RSV structure. RSV is an enveloped virus with single stranded negative sense genome. Viral genome consists of 10 genes encoding 11 proteins. Among them, Fusion protein (F protein) and Attachment protein (G protein) are crucial for the infection.

Previous studies indicate that RSV infection causes a significant increase in the number of mature DCs in mouse lungs (136, 137) and has the capacity to infect and replicate in these cells (138). It is believed that RSV induces immune dysfunction in both T cell effector function and development of CD8+ T cell memory. Chang and colleagues indicated that RSV suppressed CD8+ T cell effector activity in the lung and this suppression can be reversed by *ex vivo* treatment of exogenous IL-2 (139, 140). CD8+ effector T cell expansion in these studies did not induce durable memory responses, reflecting a defect in CD4+ T cell help. The development of antigen specific CD8+ T cells may arise from cross-presentation of cell debris from dead virus (141, 142). RSV infected DCs also inhibit CD4+ T cell response and render them inefficient at proliferation and IFN-gamma secretion (114, 143). Inhibition of T cell activity has been suggested to be mediated by soluble inhibitory molecules secreted by RSV-DCs, which reduce their capacity to induce secretion of IFN-gamma by T cells (144). Although

inhibition of T cell response is an acknowledged phenomenon induced by RSV-infection, the detailed mechanism underlying it is not clear.

Rationale

DCs are ubiquitous antigen presenting cells that are crucial for the initiation of antiviral adaptive immune response. Several virulent microorganisms have developed molecular mechanisms to impair DC function and prevent clearance by adaptive immunity (145-147). Previous studies have indicated that RSV exposure inhibits dendritic cells' function in priming T cell activation, proliferation and cytokine production (114, 148). However, detailed mechanism is still to be elucidated. Thus, our first aim is to explore both phenotypical and functional characteristics of RSV-exposed DCs. Regulatory T cells function to inhibit T cell activation. Treg expansion has been observed during the course of infection with several viral species, such as HBV (126), HIV (103, 104) and HCV (107). Our second aim therefore is to determine whether RSV infection does the same job to expand Tregs and whether RSV-exposed mouse DCs are functionally similar to human DCs. By accomplishing these aims, we will be able to have a more detailed understanding of how dominant immune tolerance is induced by RSV and our study will serve as a preliminary study for the utilization of RSV-DCs to induce antigen-specific immune tolerance in murine models of autoimmune diseases and organ transplantion.

Hypothesis

The overall hypothesis of this dissertation is that RSV infection skews DC maturation to a tolerogenic state. These tolerogenic DCs propagate their tolerogenic signals through

generation of regulatory T cells. This hypothesis will be addressed through the following subaims.

1. Determine whether RSV exposure polarizes DC maturation to a tolerogenic state. We will characterize DC phenotypical and functional properties after exposure to RSV.
2. Determine whether RSV-DCs propagate their tolerogenic signals through the generation of regulatory T cells. We will analyze RSV-DC primed CD4+ T cells and examine the presence and function of Tregs while elucidating the mechanisms of the induction of these Tregs by RSV-DC.
3. Determine whether RSV-exposed mouse DCs have the same immune suppressive property as RSV-exposed human DCs in their ability to expand regulatory T cells.

Dissertation Overview

This dissertation was dedicated to understand how RSV induces tolerance in the immune system. Chapter 2 describes findings that RSV infection skews DC maturation to a tolerogenic state. Chapter 3 shows that RSV-DCs propagate their tolerogenic signals through generation of regulatory T cells and demonstrates that RSV-exposed mouse DCs have the similar property as RSV-exposed human DCs. Finally, Chapter 4 summarizes the dissertation and preview implications and possible applications based on existing results.

CHAPTER TWO

RSV Exposure Polarizes DC Maturation toward a Tolerogenic State

Introduction

Respiratory Syncycial Virus (RSV), a negative-stranded RNA paramyxovirus, is the leading respiratory pathogen in infants and young children worldwide. Reinfection is common throughout life. Children can get re-infected with the same strain of virus (149) and immunocompetent adults experience recurrent RSV infections (150-152). The mouse model of RSV infection has yielded a numbers of important conclusions. It is thought that a critical mechanism that causes illness in primary RSV infection is an aberrant CD8+ T cell response with inefficient CTL function (139, 153). RSV-specific CD4+T cells appear to be skewed towards the secretion of IL-4 which is responsible for enhanced weight loss and illness as well (114, 154). Given the wide spectrum of alterations of the T lymphocyte functions observed in RSV infected mice, we hypothesized that they might indeed reflect alterations at the dendritic cell level. Dendritic cells (DCs) are the primary antigen presenting cells (APCs) that guide the development and polarization of an adaptive immune response (2, 155). DCs have the unique ability to induce primary immune responses and control immune tolerance through the induction of both T-cell anergy and the generation of regulatory T-cells (156). In periphery, tolerogenic DCs are capable of secreting immune inhibitory cytokines and inhibit T cell response. Previous study indicated that RSV-exposed mouse DCs are poor stimulators of T cell proliferation (114), this led us to investigate whether RSV infection induce blood mDC maturation to a tolerogenic state. To address this

question, we investigated both the phenotypic and functional tolerogenic characteristics of RSV-DCs. We comprehensively analyzed the cytokine secretion profile of RSV-DCs and expression of surface immune inhibitory molecules. After that, we assessed the ability of RSV-DCs in driving CD4 T cell proliferation in a mixed leukocyte reaction. Finally we compared RSV-DCs to pharmacologically generated tolerogenic DCs to evaluate their tolerogenic potency.

Material and Methods

RSV

RSV was propagated by infecting Hela cells for 5-6 days at 37°C. Viral titer was assessed by tissue culture infection dose (TCID50) calculation. TCID50 is defined as the dilution of assay sample at which 50% of a susceptible HeLa cell culture inoculated becomes infected. Briefly, TCID50 value: -m = log10 starting dilution - [p-0.5] x d. The equation is defined where m is the log10 TCID50 (per unit volume inoculated per replicate culture), d is the log10 dilution factor, and p is the proportion of wells positive for viral infection. After 5-6 days infection period, culture supernatant was collected and cells were processed through a few freeze-and-thaw cycles to release the viruses followed by centrifugation to remove the cell debris. The supernatant was combined and centrifuged in a 10% sucrose gradient at 4000rpm for 30min to collect the virus-enriched layer. For all subsequent experiments, multiplicity of infection (MOI) equals to 1 was used.

Blood mDC

Leukocyte-enriched blood samples were obtained from local blood bank (Carter Blood Care). Peripheral Blood Mononuclear Cells (PBMCs) were separated by centrifuging blood in a Ficoll gradient (density centrifugation). After centrifugation, the buffy layers on top of red blood cells were collected (Figure 2.1). PBMCs were then incubated with magnetic microbeads conjugated to anti-CD3, anti-CD14, anti-CD16, anti-CD19, and anti-CD56 and then passed over a magnetic column to eliminate major cells types such as B cells, T cells, etc. (Figure 2.2). The negative fraction was collected and stained for DC specific surface markers LINEAGE-FITC, CD123-PE, HLA-DR-PerCP, and CD11c-APC. The stained cells were then sorted on a FACS ARIA cell sorter. mDCs were defined as LINEAGEneg, HLA-DR+, CD11c+ cells (Figure 2.3) . Purity of the isolated mDCs averaged 97%.

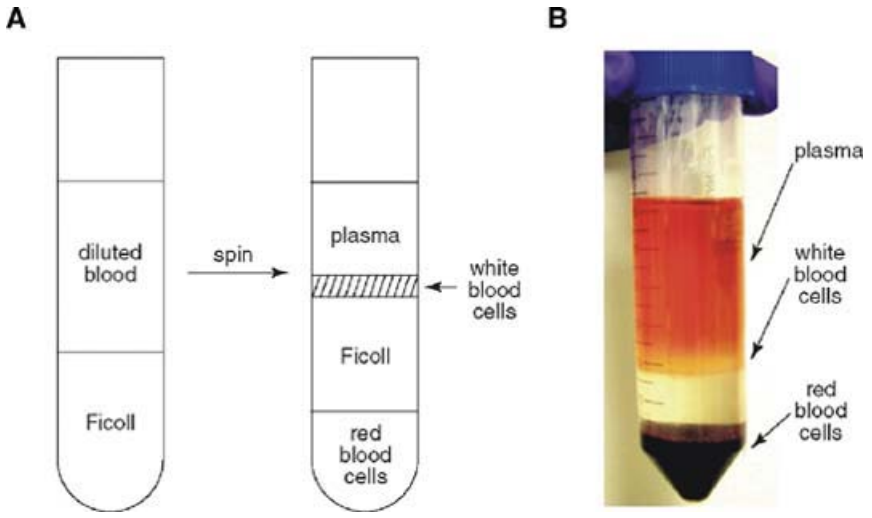


Figure 2.1: Isolation of white blood cells (PBMCs) from human blood (gradient centrifugation). Diluted blood was laid on top of Ficoll solution followed by high speed centrifugation. Plama, white blood cells and red blood cells were separated in different layers and can be enriched.

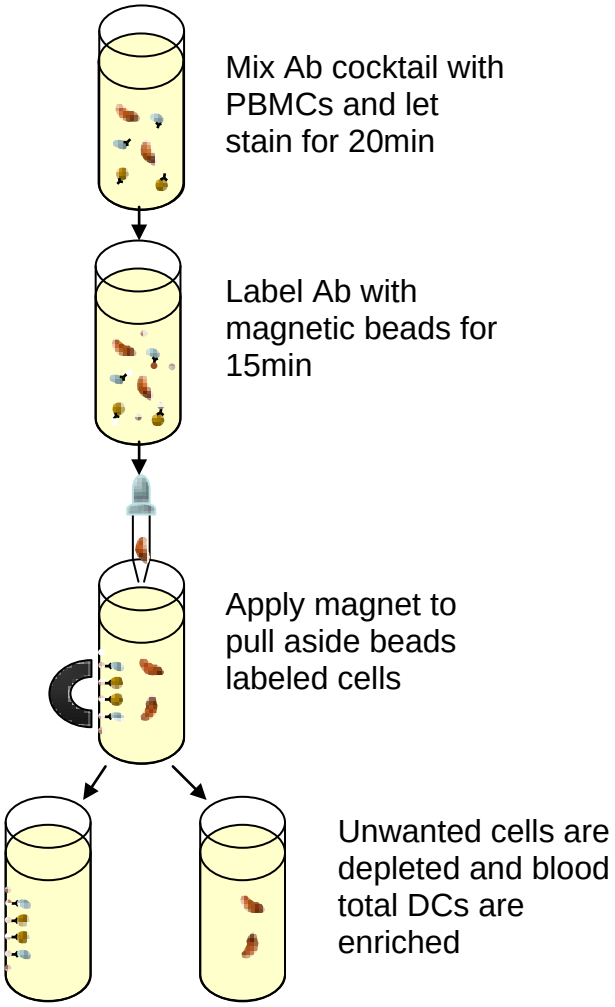


Figure 2.2: Enrichment of total blood DCs from PBMCs. Antibody cocktail that labels all cell types except for DCs was mixed with PBMCs for 20min followed by wash-off of excess antibody. These antibodies were then conjugated with magnetic beads for 15min. Then, magnetic field was applied to the cells to pull aside antibody labeled unwanted cells. Blood total DCs were left untouched.

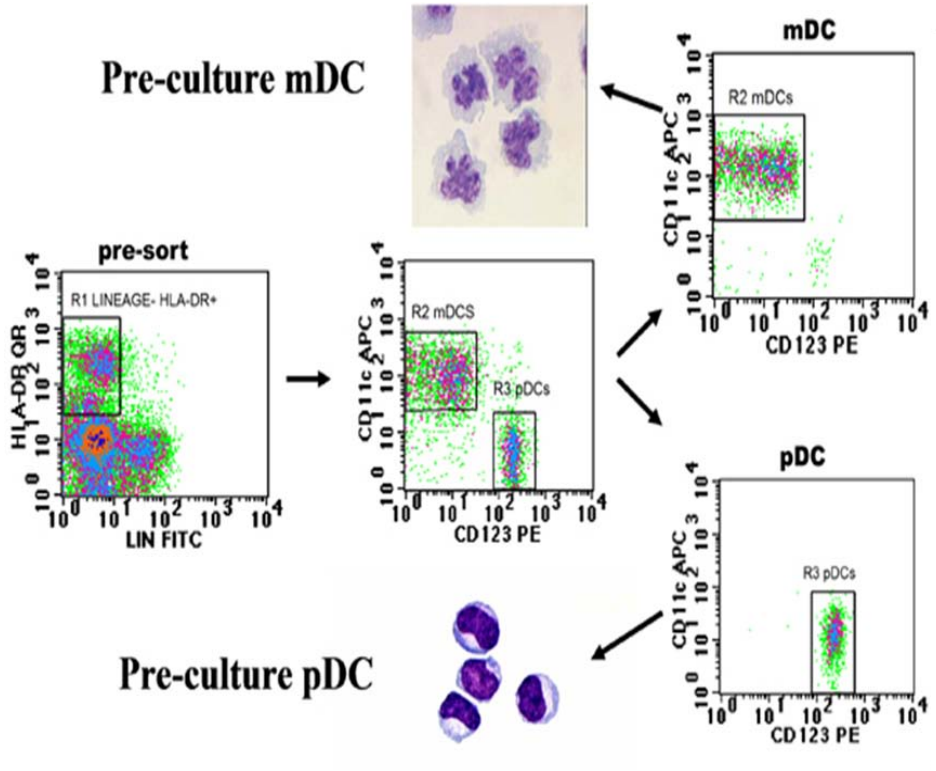


Figure 2.3: Further separation of mDCs and pDCs. Total DCs from beads enrichment were stained for LINEAGE-FITC, CD123-PE, HLA-DR-Quantum Red and CD11c-APC. The stained cells were sorted on FACS-Aria, and LINEAGEneg, HLA-DR+, CD11c+ mDCs were collected for subsequent experiments.

Detection of IL-10 Production

DCs were unexposed or exposed to either Flu or RSV for 18h, and then DC culture supernatants were collected for analysis. IL-10 concentration in the culture was measured by Luminex multiplex technology. Cytokine concentrations were measured with Beadlyte cytokine assay kit (Upstate, NY) using a Bio-Plex Luminex 100 XYP instrument and calculated using Bio-Plex manager 4.0 software with a 5-parameter curve-fitting algorithm applied for standard curve calculations.

Microarray Assay

Total RNA was isolated using the RNeasy kit (Qiagen) according to the manufacturer's instructions, and RNA integrity was assessed by using an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA).

Affymetrix GeneChips. Double-stranded cDNA was generated from 2 to 5 ug total RNA, followed by single-round *in vitro* transcription with biotin-labeled nucleotides, using the Affymetrix RNA transcript labeling kits (Affymetrix, Santa Clara, CA). Biotinylated cRNA targets were purified using the Sample Cleanup Module (Affymetrix), and subsequently hybridized, according to the manufacturer's standard protocols, to AffymetrixHGU133A GeneChips (which contain 22283 probe sets). Arrays were scanned using an Affymetrix confocal laser scanner.

Microarray Data Analysis. Microarray Suite, version 5.0 (MAS 5.0; Affymetrix) software was used to assess fluorescent hybridization signals, to normalize signals, and to evaluate signal detection calls. Raw signal intensity values for each probe set were analyzed by algorithms in MAS 5.0. A maximum of 8 samples was assigned randomly for hybridization and staining each run day to minimize technical variability.

Normalization of signal values per chip was achieved using the MAS 5.0 global method of scaling to the target intensity value of 500 per GeneChip. Analysis was restricted to probe sets for which a present (P) call was obtained in at least 75% of GeneChips in at least one patient class evaluated (quality control probes). A gene expression analysis software program, GeneSpring, version 7.1 (Agilent), was used to perform statistical analysis, hierarchical clustering, and classification of samples. Nonparametric univariate tests (Mann-Whitney *U* or Fisher exact test) were used to rank

genes on the basis of their ability to discriminate between predefined groups of patients. The ability of the top ranked (ie, classifier) genes to discriminate the predefined class of pathogen was determined by the K-Nearest Neighbors (kNN) method.

Real-time RT-PCR Analysis of Gene Expression

Total cellular mRNA was extracted from virally exposed or unexposed DCs at 6 and 18 hours using Qiagen RNeasy® Micro Kit according to the manufacturer's instruction, then reverse transcribed to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). PD-L1, SOCS2, INHIBA, PTX3, AQP9, SLAMF1, ILT4, ILT5, ILT6, LAIR1, and PTPN2 expression in DCs were assessed by Real-time RT-PCR using primers designed by Applied Biosystems. Gene expression levels were compared to GAPDH as endogenous control and normalized as fold increase over internal control for comparison.

Antibodies, Flow Cytometric Staining and Chemicals

For examination of DC surface immunoinhibitory molecule expression. Flu or RSV exposed DCs were stained with fluorochrome-conjugated antibodies for SIGLEC1, PD-L1, ILT-4, HLA-G, SLAM and LAIR (BD Biosciences) for 30 minutes at 4°C, rinsed with PBS, centrifuged at 1200 rpm for 5 minutes and resuspended in 1% paraformaldehyde for fixation. Samples were then acquired on FACS-Calibur for detection of fluorescent intensity. Resulting data were analyzed by FlowJo software (Tree Star Inc., Ashland, OR). For antibody blockade experiments, RSV F protein antibody Synagis (5ug/ml), IL-10 antibody (10ug/ml, R&D Systems), IL-10 receptor antibody (10ug/ml, Clone 37607.11, Sigma Aldrich), PD-L1 antibody (10ug/ml, Clone

MIH1, eBioscience), and PD-1 antibody (10ug/ml, Clone J116, eBioscience) are used. For STAT3 signaling blockade experiments, STAT3 inhibitors Cucurbitacin I and Stattic purchased from CalBiochem are used at 1uM and 10uM respectively.

CFSE Staining and MLR

Freshly isolated CD4 T cells were incubated at a concentration of $5X10^6$/ml in 1.25uM CFSE for 10 minutes followed by washing twice to get rid of excess dye (Figure 2.4). For MLR, unexposed or either Flu or RSV exposed DCs were cultured with CFSE-labeled CD4 T cells (DC: T cell=1:40) for 5 days. At day 5, cells were harvested and stained with CD4-APC (BD Biosciences) followed by flow cytometric analysis. For in-trans MLR, viral exposed DCs were added to a co-culture where unexposed DCs were used as MLR stimulator and CFSE labeled allogeneic CD4 T cells were used as responders. An increased ratio of viral exposed DCs and unexposed DC were used to examine the inhibitory potency of tolerogenic DCs, briefly, the ratio of viral exposed DCs and unexposed DCs were set as 0:1250, 50:1250, 100:1250, 250:1250, 500:1250 and 1250:1250, together with 50,000 CD4 T cells. After 5 days, the percentage of CD4 T cell alloproliferation was plotted for comparison (Figure 2.5). For the antibody blockade experiments, F protein antibody, IL-10/IL-10 receptor antibody and PD-L1/PD-1 antibody were added at specific stages of MLR.

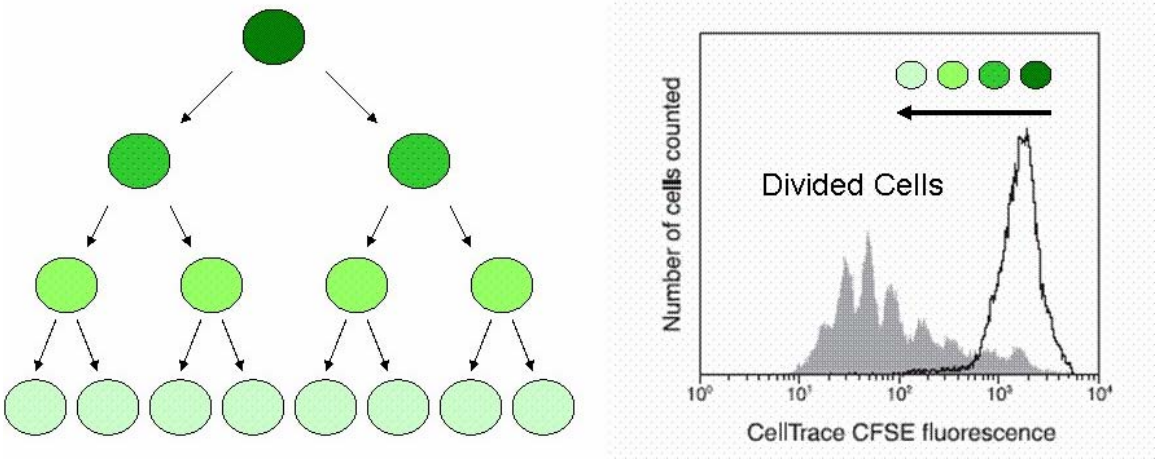


Figure 2.4: CFSE cell tracer for T cell proliferation. CFSE is a cellular dye that covalently couples via its succinimidyl group to intracellular molecules. When the cells divide, the dye is equally distributed to the daughter cells, resulting in a dilution of fluorescent intensity in the next generation. Here, CFSE is used to trace T cell proliferation, T cells express less and less fluorescence as they divide and this can be detected by flow cytometry.

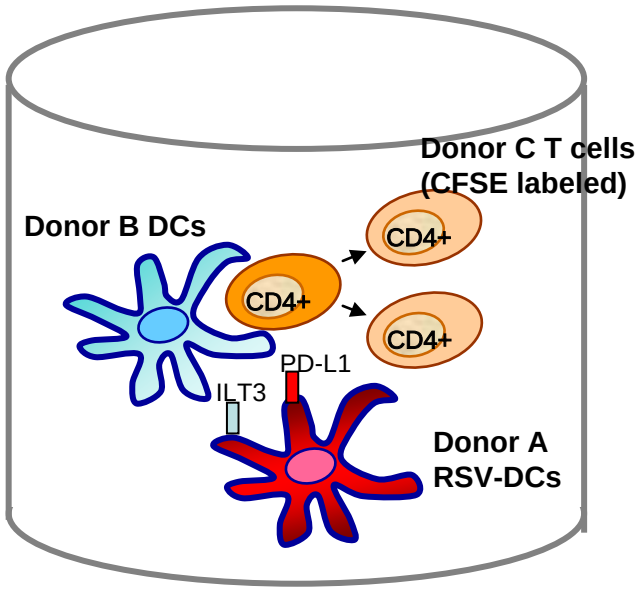


Figure 2.5: Mixed leukocyte reaction. To test the ability of RSV infected DCs to inhibit alloreaction. Infected DCs from donor A were co-cultured with normal DCs from donor B and CFSE labeled T cells from donor C. The ability of donor A RSV-DCs to inhibit donor B DC induced T cell allproliferation was indicated by CFSE dilution.

Preparation of Pharmacologically Generated Tolerogenic DCs

PBMC were purified from human peripheral blood by Ficoll-Hypaque centrifugation. Then monocytes were purified by adherence and differentiated into moDC after 6 days in the presence of GM-CSF (100ng/ml, BUMC Pharmacy) and IL-4 (50ng/ml, R&D Systems) (GM+IL-4 DC) or GM-CSF and IL-10 (100ng/ml, R&D Systems) (GM+IL-10 DC) or GM-CSF and vitamin D3 (100nM, Calbiochem) (GM+VitD3 DC). At day 6, DC are washed and recultured for 2 days in the presence of GM-CSF or GM-CSF and Dexamethasone (10nM, Sigma-Aldrich) or GM-CSF and vitamin D3 (10nM). DCs were washed twice and 2500 DC were cultured with 10^5 allogeneic T lymphocytes in 96-well U-bottom in 5% AB medium for 5 days (triplicate). 1 uCi [^{3}H]-thymidine was added for the last 18h of culture. Plates were harvested on a Tomtec Harvester 96 and proliferation detected on a Wallac microbeta trilux-u-scintillant counter (PerkinElmer, Wellesly, MA.).

Statistical Analysis

To determine statistical difference, P value was calculated for paired T test by using GraphPad Prism software and significant differences were concluded if $p<0.05$. Single asterisk indicates $p<0.05$, double asterisks indicate $p<0.01$.

Results

RSV-DCs Secrete High Amount of IL-10 and Display Upregulated Surface Immune Inhibitory Molecules

Immunomodulatory effects of IL-10 on APCs were described soon after the discovery of this cytokine in the late 1980s (157, 158). It down-regulates the expression

of Th1 cytokines, MHC class II antigens, and costimulatory molecules on DCs (159). Thus, expression of IL-10 has been indicated as a general feature of tolerogenic DCs (160). We therefore assessed the secretion of IL-10 by RSV-DCs and compared it to control or Flu-DCs. As shown in Figure 2.6, RSV-DCs secreted significantly higher amount of IL-10 than control or Flu-DCs.

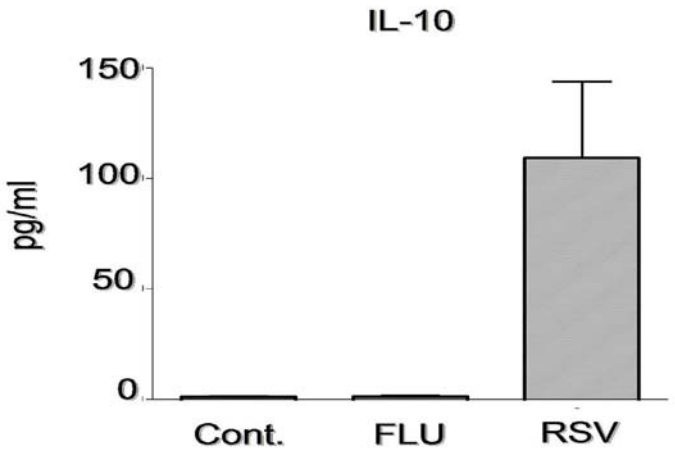


Figure 2.6: RSV infection induces DCs to secrete IL-10. mDCs were exposed to 1 MOI of either Flu or RSV for 18h. Cell culture supernatants were analyzed for the expression of IL-10 by Luminex Multiplex.

To further understand the mechanism of RSV mediated DC tolerogenic conversion, we focused on the expression of surface immune inhibitory molecules. First of all, RNA transcript profiles of RSV-DCs were analyzed. As shown in figure 2.7, RSV-DCs upregulated a variety of surface inhibitory molecules. These molecules fell into two main classes, ITIM containing inhibitory receptors and down stream transducing molecules. The inhibitory class-I immunoreceptors ILT4 (LILRB2), ILT5 (LILRB3) and ILT6 (LILRA3), have been associated with tolerogenic function in DCs (77, 161). LAIR1 and LAIR2 have been shown to inhibit DC differentiation (162). SLAMF1 is

upregulated in tolerogenic DCs and IL-10 treated monocytes, as are GAS-6, aquaporin-9, SPP1 and pentraxin-3 (75, 76). CD30 (TNFRSF8) has been described to contribute to regulatory T cell function (163, 164). SOCS2, for suppressor of cytokine signaling, is a negative regulator of DC inflammatory cytokine production (165). STAT3 is a mediator of IL-10 receptor signaling, whose activation is necessary for many of its immunosuppressive properties (166). Similarly, the protein tyrosine phosphatase PTPN2 is a critical negative regulator of inflammatory signaling (167). The TGF-beta family member inhibin-beta-1 (INHBA) mediates its suppressive function through the activation of SHIP phosphatase (168).

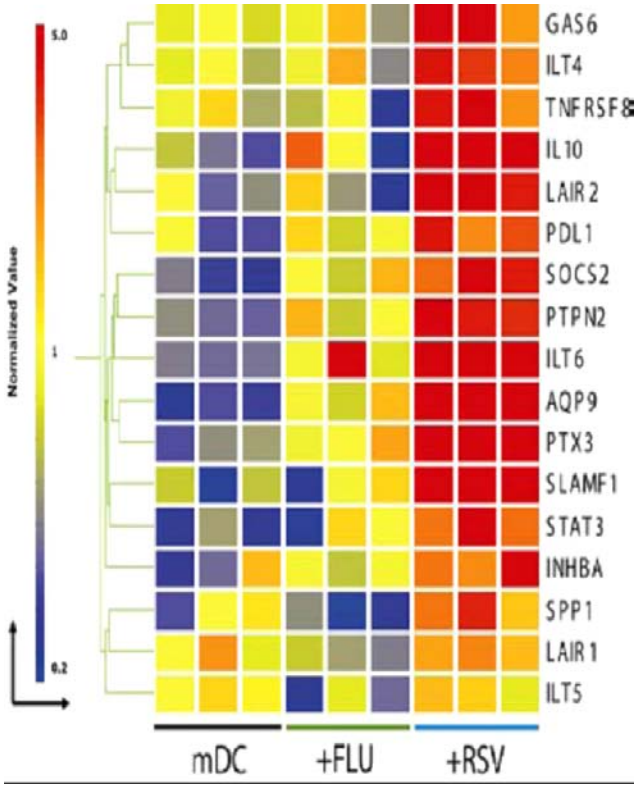


Figure 2.7: Microarray analysis of tolerogenic gene expression. mDCs (3 donors) were unexposed or exposed to either Flu or RSV for 18h. RNA was extracted, labeled and hybridized to the U133 2 plus chip (Affymetrix). Differential analysis was performed using Gene Spring 6.2 software package.

To confirm the microarray data, we did Real-time RT-PCR analysis of these surface inhibitory molecules. To monitor the expression kinetics, we untreated or treated mDCs with Flu or RSV for 6h and 18h respectively to reflect early and late viral exposure. After the treatment, DC mRNA was collected according to the standard protocol and expression level was assessed by Real-time PCR. As shown in Figure 2.8, PD-L1, SOCS2, INHBA, PTX3, AQP9 and SLAMF1 were upregulated dramatically in early infection phase (6 hour), while ILT-4, ILT-5, ILT-6, LAIR1 and PTPN2 were upregulated in the late infection phase (18 hour). Furthermore, we assessed the expression of these surface markers in protein level using flow cytometry (Figure 2.9).

Thus far, we have demonstrated that RSV infection skew DC maturation to a phenotype similar to what is defined as tolerogenic DCs, which are characterized by large amount of secretion of IL-10 and upregulation of various immunoinhibitory molecules on DC surface. As a next step, we will move on to investigate the functional properties of these tolerogenic DCs.

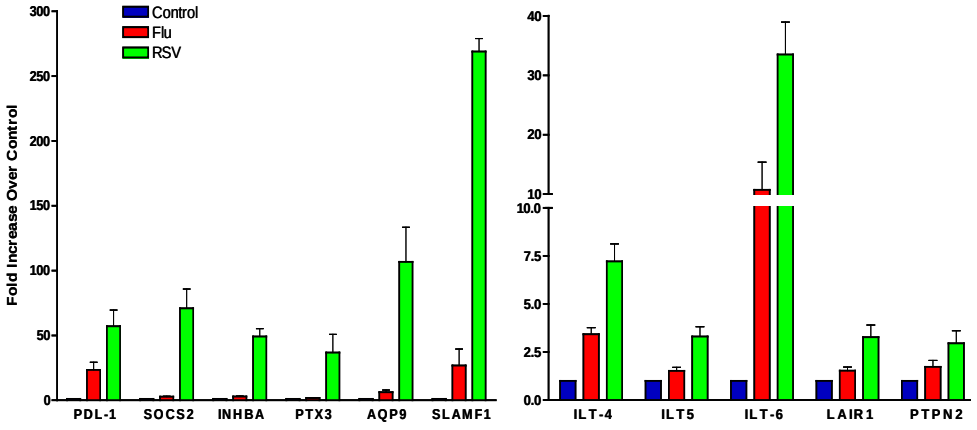


Figure 2.8: Surface tolerogenic marker expression in early and late time points. mDCs (5 donors) were unexposed or exposed to either Flu or RSV for 6 and 18 hours respectively. After that, RNA was extracted followed by Real-time RT-PCR analysis of gene expression. The left panel denotes genes that are upregulated in early infection, and the right panel denotes genes upregulated in late infection.

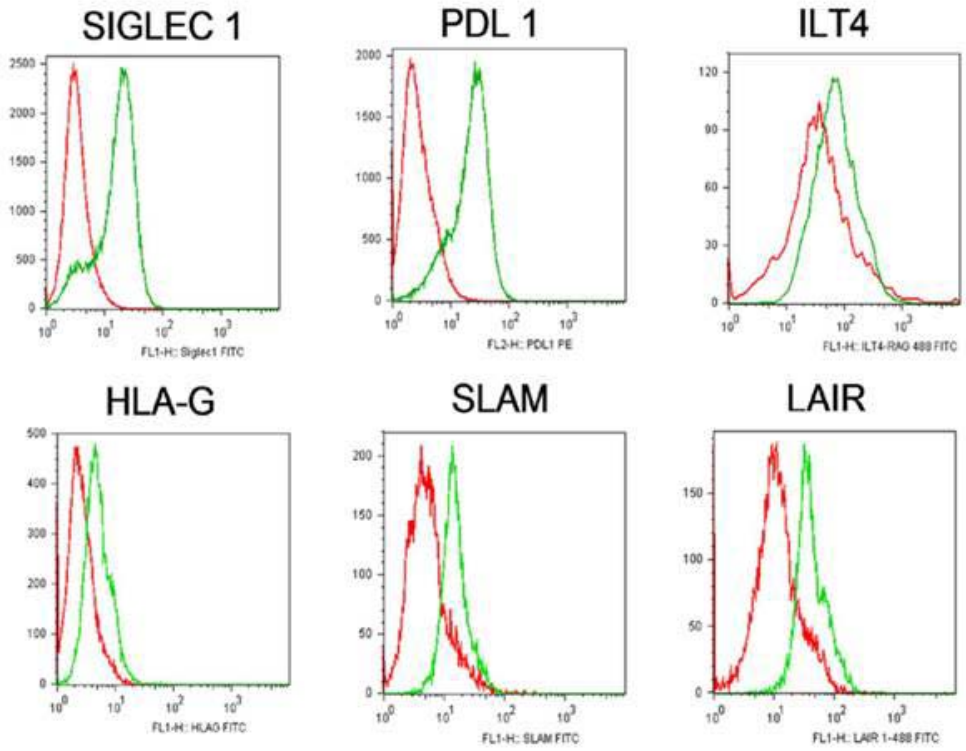


Figure 2.9: Surface expression of DC immunoinhibitory molecules. After either exposed to Flu or RSV for 18h, DCs were stained with fluochrome-conjugated antibody against SIGLEC1, PD-L1, ILT-4, HLA-G, SLAM and LAIR. FACS data reflect the expression intensity of these surface markers.

RSV-DCs are Unable to Drive CD4 T cell Alloproliferation

One important function of DCs is to prime the activation of CD4 T cells. When MHC-class II molecule engages with T cell receptor (TCR) and costimulatory molecules CD80/CD86 interact with CD28 on T cells. T cells transduce activation signals and begin to proliferate. Tolerogenic DCs are characterized by the inability to drive T cell alloproliferation. Since we have observed that RSV exposed DCs display a tolerogenic phenotype, we further investigated the functions of tolerogenic DCs to prime CD4 T cells. After unexposed or exposed to either Flu or RSV for 18h, DCs were collected and added to a culture together with CFSE labeled purified CD4 T cells from another donor.

CFSE (carboxyfluorescein diacetate succinimidyl ester) is a fluorescent cell staining dye. It is not membrane permeable, so when T cells divide, the dye distributes equally to the daughter cells. Thus measuring the intensity of the fluorescence allows us to monitoring the division (proliferation) of T cells. After co-culture DCs and T cells for 5 days, cells in the culture were collected and stained with APC-labeled anti-CD4 antibody. CD4 T cell alloproliferation was assessed by monitoring CFSE dilution. As shown in Figure 2.10, RSV-DCs were unable to drive CD4 T cell alloproliferation when compared to untreated DCs or Flu-DCs. These indicated that RSV-DCs are functional potent tolerogenic DCs.

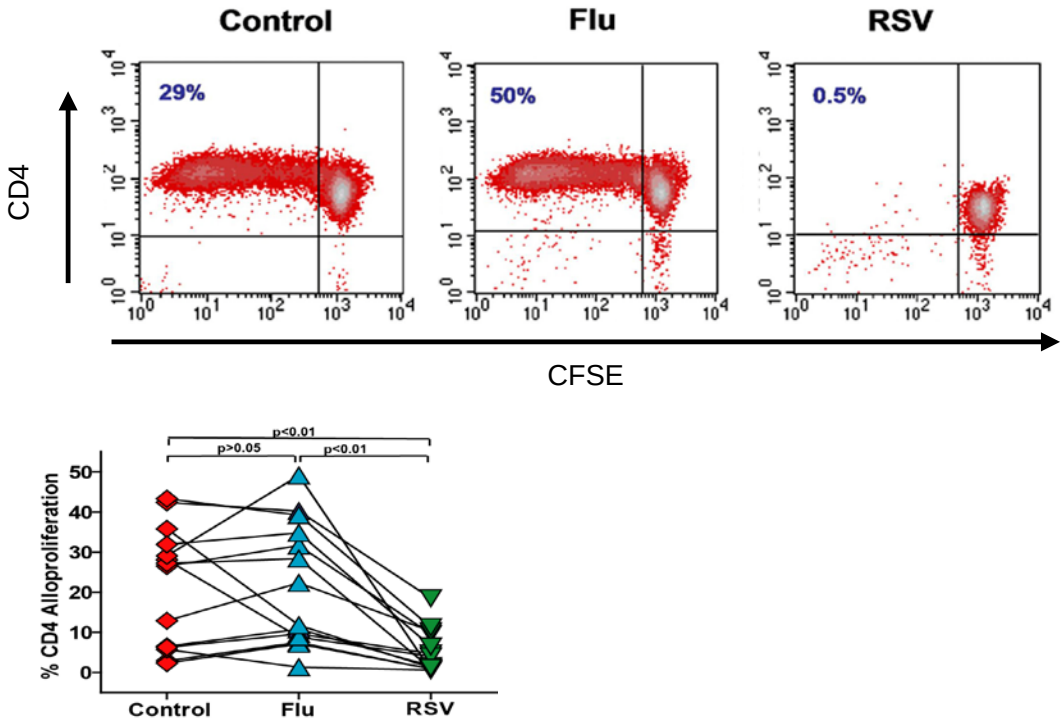
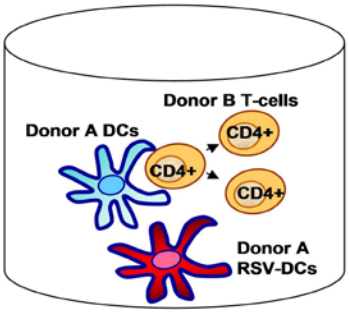


Figure 2.10: RSV-DCs are incapable of driving T cell alloproliferation. Unexposed or either Flu or RSV exposed DCs were cultured with CFSE-labeled CD4 T cells for 5 days. At Day 5, cells were stained with CD4-APC antibody, and their allogeneic proliferation was assessed as CFSE dilution by flow cytometry. The lower panel summerizes data from n=10 different donors.

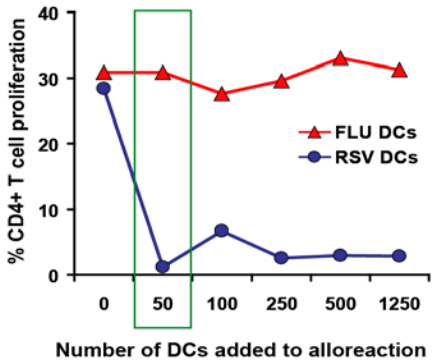
RSV DCs are Potent Suppressors of Mixed Leukocyte Reaction (MLR)

Infectious tolerance describes a phenomenon that tolerance can be transferred from one cell population to another. Tolerogenic DCs have been shown to be able to convey tolerogenic properties to other DCs (4). Thus, we next investigated whether RSV-DCs are able to suppress a DC-driven MLR. After unexposed or exposed to either Flu or RSV for 18h, DCs were collected and washed, then increasing numbers of virally exposed DCs from donor A were added to a MLR consisting of 1,250 mDCs from donor A and 100,000 CFSE-labeled CD4 T cells from donor B (Figure 2.11 A). After co-culture for 5 days, the ability of uninfected DCs to drive T cell proliferation was assessed by CFSE-dilution. As shown in Figure 2.11 B and C, as few as 25-50 RSV-DCs inhibited the ability of 1,250 un-infected DCs to drive CD4 T cell alloproliferation by more than 85%. Flu-DCs did not have the suppressive effect. The ability of RSV-DCs to suppress an on-going MLR *in trans* has been observed in at least 5 independent donors (Figure 2.11 D). Furthermore, we excluded the possibility that the inhibition of MLR by RSV-DCs was due to the carry over of the RSV in the system. As addition of RSV fusion protein antibody to neutralize live viral particles failed to offset the inhibitory capacity of RSV-DCs (Figure 2.11 E). Thus, we conclude that RSV-DCs are potent inhibitors of on-going alloreaction *in trans*.

A.



B.



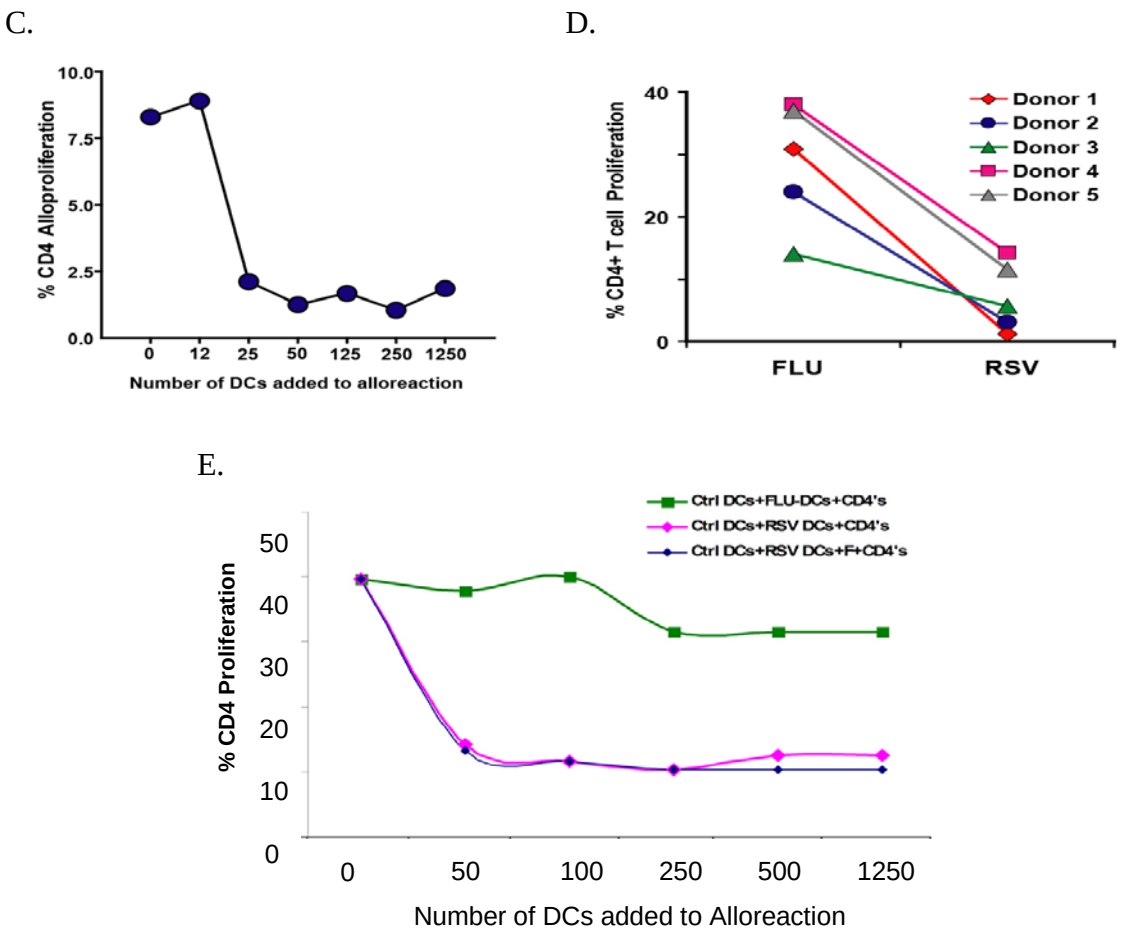


Figure 2.11: RSV-DCs confer infectious tolerance of suppress CD4 T cell alloproliferation driven by un-infected DCs. (A).Experimental design: increasing numbers of either Flu or RSV exposed DCs were added to a MLR in which unexposed DCs were used as alloreaction stimulators and CFSE-labeled allo-T cells were used as responders. After co-culturing for 5 days, the ability of viral exposed DCs to inhibit on-going MLR was assessed by T cell CFSE dilution. (B and C).The percentage of CD4 T cell proliferation at different exposed /unexposed DC ratio was plotted. Compare to Flu-DCs, as few as 25-50 RSV-DCs were able to suppress 1250 unexposed DC driven T cell proliferation by more than 85%. (D).Statistical data summarizes n=5 independent donors. (E).The in-trans allo-inhibition was not due to the carryover of viral particles from RSV-DCs, neutralizing viral particle by anti-F antibody failed to offset the inhibitory capacity of RSV-DCs.

Autocrine IL-10 is Required for Tolerogenic Conversion

IL-10 has been demonstrated to be important in inducing tolerogenic DCs. Treatment of DCs with IL-10 induces the expression of surface B7 family inhibitory molecules as well as ILT family members (75-77). IL-10 treated DCs also have a

dramatically reduced capacity to stimulate primary T cell proliferation as well as effector cytokine express (169, 170). We have observed that RSV-DCs secreted large amount of IL-10, this led us to investigate whether IL-10 was involved in RSV-mediated DC tolerogenic conversion. Blocking IL-10 signaling during the alloproliferation assay did not permit RSV-DCs to induce CD4 T cell alloproliferation (Figure 2.12, panel 4), indicating that IL-10 is not directly involved in the ability of RSV-DCs to drive a MLR. However, blocking IL-10 signaling at the time of the exposure to RSV resulted in the generation of RSV-DCs that display some allo-stimulatory capacity (Figure 2.12 panel 2). These results indicated that the autocrine IL-10 which occurs during the exposure of mDCs to RSV plays an important role in the generation of tolerogenic DCs.

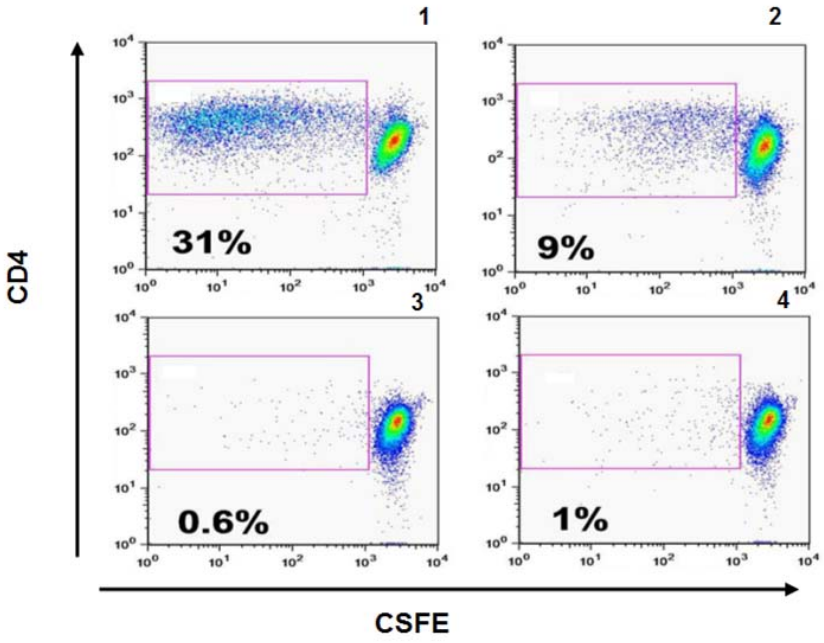


Figure 2.12: Autocrine IL-10 is required for tolerogenic DC conversion. mDCs were incubated with either isotype control (panel 1 and 3) or blocking antibodies to IL-10 and IL-10 receptor (panel 2 and 4), either 30min prior to viral exposure (panel 2) or following 18h of RSV exposure (panel 4). The ability of DCs t o induce CD4 T cell alloproliferation was assessed by CFSE dilution (n=4).

PD-L1/PD-1 Interaction is Required for Tolerogenic Function

As we have shown that RSV-DCs express various surface inhibitory molecules, the expression of these receptor and ligand systems appear to contribute to tolerogenic DC function. PD-1 is member of the extended CD28/CTLA-4 family of T cell regulators. The intracellular tail contains two phosphorylation sites located in an immunoreceptor tyrosine-based inhibitory motif and an immunoreceptor tyrosine-based switch motif, which suggests that PD-1 negatively regulates TCR signals (171). PD-1 has two ligands PD-L1 and PD-L2, studies are just beginning to elucidate the function of these ligands. It was demonstrated that Anti-CD3 mAb plus either PD-L1 or PD-L2-Ig proteins linked to beads inhibited T cell proliferation and cytokine production (172). Studies using Chinese hamster ovary (CHO) cells transfected withMHCclass II, and PD-L1 or PD-L2 in the presence or absence of B7-2 also support an inhibitory role for PD-L1 and PD-L2 (173). Since PD-L1 is rapidly upregulated at a high-level in RSV-DCs, we investigated whether the engagement of PD-L1 and its receptor PD-1 is responsible for the inhibition of T cell proliferation. RSV-DCs were added to a MLR in the presence of blocking antibodies to both PD-L1 (clone MIH1) and its receptor PD1 (clone J116) or isotype control antibodies. As shown in Figure 2.13, blocking PD1/PD-L1 engagement during MLR partially restored the allo-stimulatory capacity of RSV-DCs. These results indicate that the high level of surface expressed PD-L1 induced following RSV exposure plays a critical role in the tolerogenic function of these DCs during T cell co-culture.

A. B.

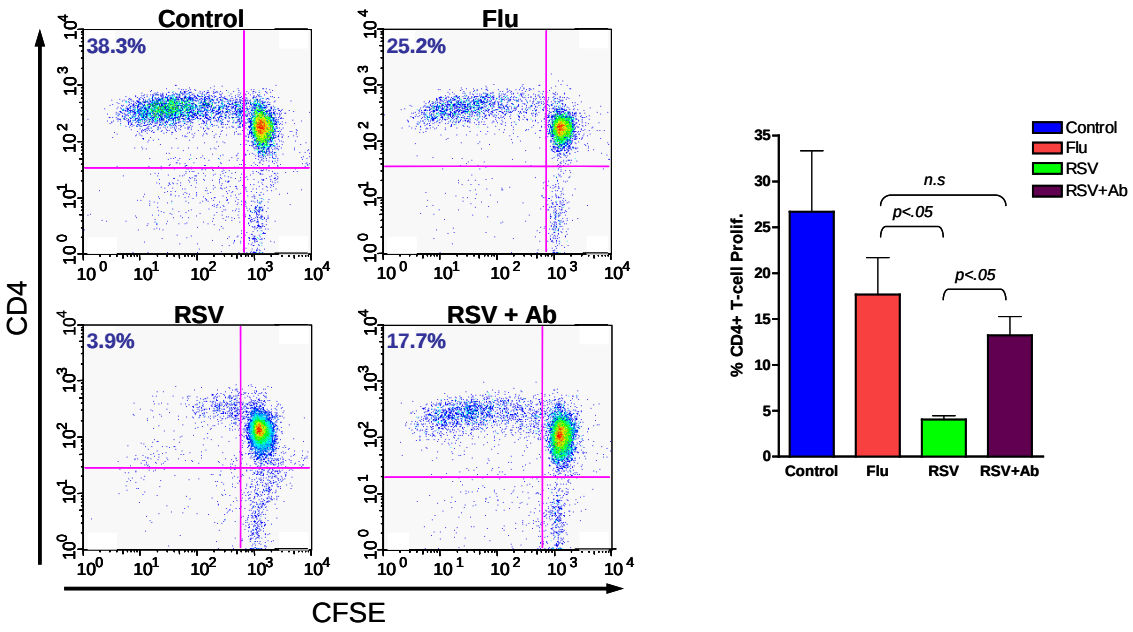


Figure 2.13: PD-L1/PD-1 signaling is required for tolerogenic DC function. A.mDCs were either unexposed (control) or exposed to 1 MOI of Flu or RSV for 18 hours. mDCs were incubated with either isotype control or blocking antibodies to PD1 and PD-L1. The ability of these cells to induce CD4+ T-cell allo proliferation was then assessed. B.Line graph summarizes n=5 independent experiments from different donors ($p<.0.05$).

IL-10 Receptor Signaling Controls PD-L1 Expression and is Required for Tolerogenic Function

As we have shown that autocrine IL-10 is important for RSV mediated DC tolerogenic conversion and PD-L1/PD-1 interaction is required for tolerogenic DC function, we next examined the relationship between IL-10 receptor signaling and the expression of PD-L1.

IL-10 signal transduction is mediated by the Janus kinase (JAK)/STAT3 pathway, STAT3 phosphorylated by JAK forms a dimer and travels to the nucleus where it turns on specific genes responsible for IL-10 mediated anti-inflammatory response (174, 175). To block the IL-10 receptor signaling, we used two STAT3 inhibitors—Cucurbitacin I

and Stattic. Cucurbitacin I is a triterpenoid compound produced from *Cucumis sativus* L. and Stattic is a synthetic vinyl-sulfone compound, both of them block STAT3 activation in a highly specific manner (176, 177). 1h before RSV exposure, DCs were treated with either STAT3 inhibitor (Cucurbitacin I or Stattic) or DMSO (solvent control). Following an 18h viral exposure, surface PD-L1 expression was examined. As shown in Figure 2.14, both Cucurbitacin I and Stattic inhibited the upregulation of PD-L1 expression following RSV exposure, which indicates that IL-10 receptor signaling controls the expression of PD-L1.

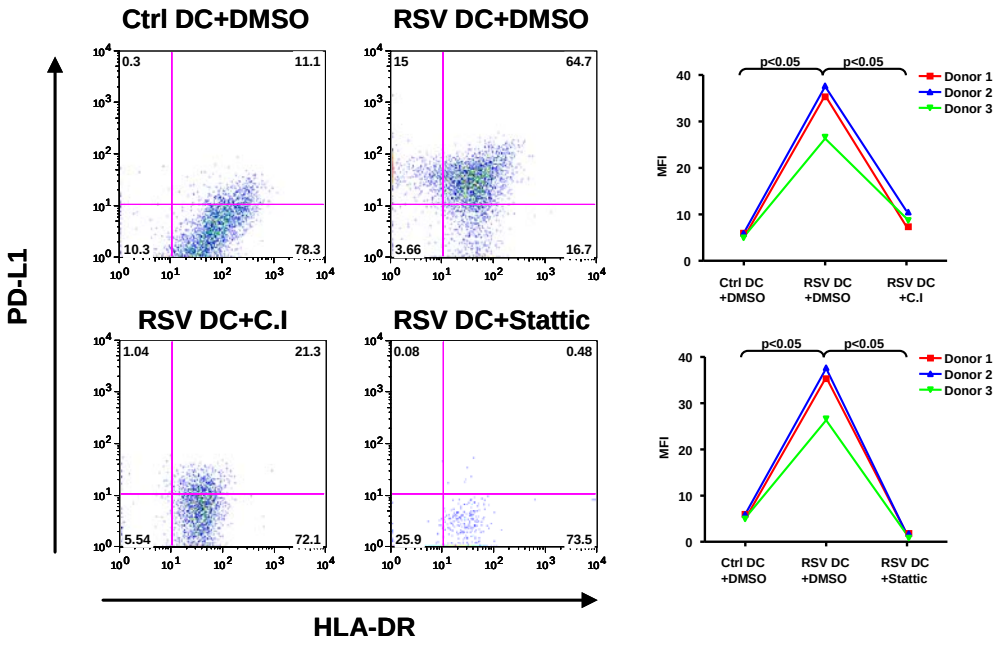


Figure 2.14: PD-L1 expression upon IL-10 signaling blockade. Blockade of IL-10 signaling by STAT3 inhibitors Cucurbitacin I or Stattic significantly inhibited the upregulation of PD-L1 induced by RSV-exposure.

Since PD-L1 is required for DC tolerogenic function, next we examined the ability of RSV-DCs to prime allogeneic T cell response upon STAT3 inhibitor treatment. Similar as described before, DCs were treated with STAT3 inhibitors 1h before RSV

exposure and after 18h viral exposure, CFSE labeled allogeneic T cells were added to co-culture for 5 days. After Day 5, T cell alloproliferation was assessed by CFSE dilution. As shown in Figure 2.15, STAT3 inhibitor treatment partially restored T cell alloproliferation capacity.

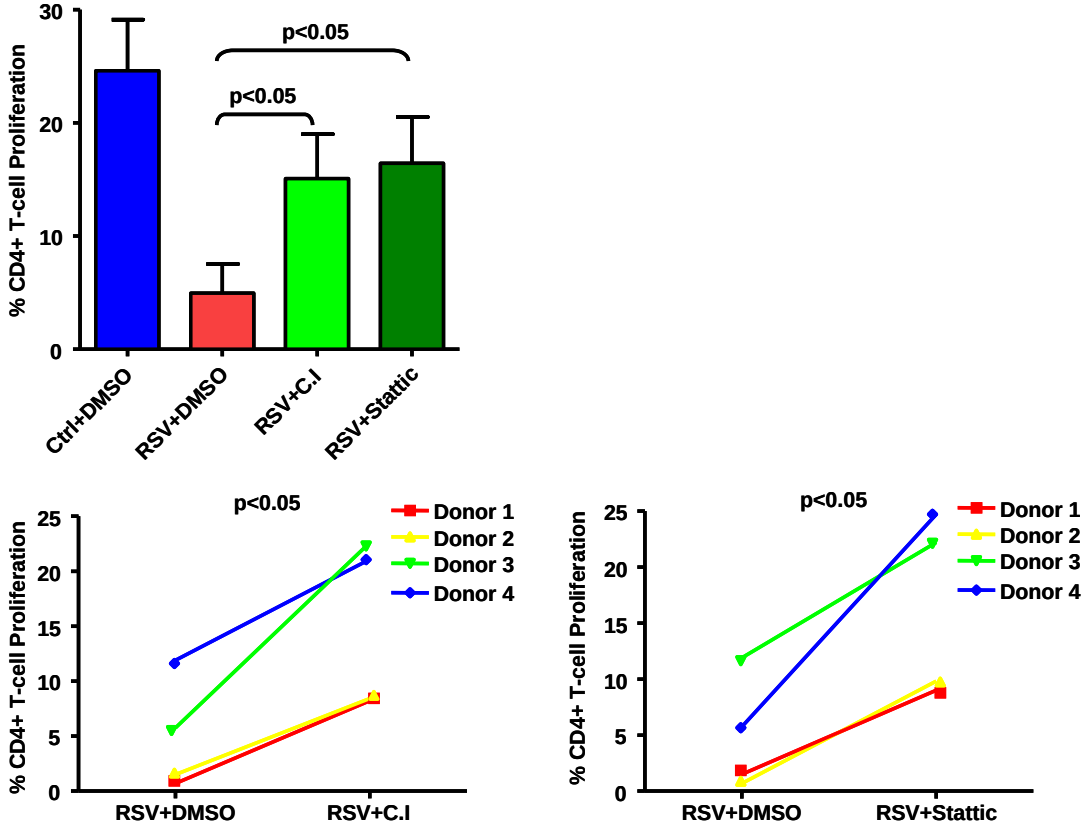


Figure 2.15: Blocking IL-10 signaling partially restores T cell alloproliferative capacity. Blocking IL-10 signaling by STAT3 inhibitors Cucurbitacin I or Stattic results in an increased T cell proliferation when compared to the DMSO solvent control.

Taken together, our findings suggest that IL-10 receptor signaling activated by RSV induce the expression PD-L1 on DC surfaces which play an important role in inhibiting allogeneic T cell activation.

RSV-DCs are More Tolerogenic than Pharmaco-DCs in Inhibiting T Cell Alloproliferation

Diverse strategies have been used to modify DCs *in vitro* such that a tolerogenic or regulatory phenotype can be generated. These include the use of immunosuppressive cytokines such as IL-10, TGF-beta, TNF-alpha, etc.(160), or the use of immunosuppressive drugs, such as Vitamin D3, Dexamethasone, Rapamycin, Cyclosporin, etc.(178). We did a side-by-side comparison of RSV-DC, IL-10-DC, VitD3-DC, DEX-DC in terms of their tolerogenic function.

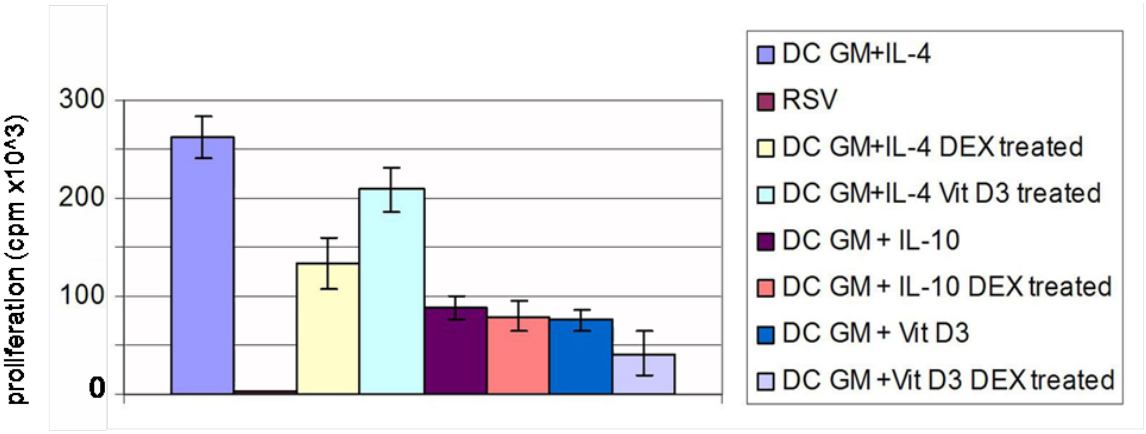


Figure 2.16: The ability of pharmaco-DCs and RSV-DCs to inhibit T cell allogeneic proliferation. Presence of immune suppressive drug during DC differentiation generates DCs that are more tolerogenic than after DC differentiation. RSV-DCs are strikingly more potent than pharmaco-DCs to inhibit T cell alloproloferation.

To compare the functional capacity of tolerogenic DCs, we tested their ability to drive CD4+ T cell alloproliferation. In this experiment, pharmaco-DCs generated in 3 different ways were used. GM+IL-4 DEX-DC and GM+IL-4 Vit D3 DC were generated by culturing monocyte 6 days in the presence of GM-CSF and IL-4, and then followed by culturing in the addition of DEX or Vit D3 for 2 more days. GM+IL-10 DC and GM+IL-10 DEX DC were generated by culturing monocyte with GM-CSF and IL-10 for 6 days,

then in GM+IL-10 DEX DC condition, DEX was added for culturing 2 more days. GM+Vit D3 DC and GM+VitD3 DEX DC were made by culturing monocyte with GM-CSF and Vit D3 for 6 days, then in GM-Vit D3 DEX DC condition, DEX was added for culturing 2 more days. All these pharmacologically generated tolerogenic DCs together with GMIL-4 DC and RSV DC are co-cultured with CD4+ T cells for 5 days. At day 5, radioactive thymidine incorporation assay was used to assess T cell proliferation.

As shown in Figure 2.16, all tolerogenic DCs showed inhibition of CD4+ T cell proliferation when compared to GMIL-4 DCs. Differentiation of monocyte to DC in the presence of IL-10 or Vit D3 generates DCs that are more tolerogenic than DEX or Vit D3 treated GMIL-4 DCs. However, all these pharmaco-DCs are much less potent than RSV-DCs to inhibit T cell alloproliferation.

Another important feature of all tolerogenic DCs is the ability to inhibit an on-going alloreaction *in trans*. To compare the ability of tolerogenic DCs to inhibit an on-going alloreaction, Pharmaco-DCs (generated by different strategies) and RSV DCs were added in an increased number to an on-going alloreaction in which untreated DCs were used as stimulators and allogeneic T cells were used as responders. T cell proliferation was assessed by radioactive thymidine incorporation assay. As shown in Figure 5.17, GM+Vit D3 and GM+Vit D3 DEX DCs are able to significantly inhibit *in trans* alloreaction when compared to pharmaco-DCs generated following other protocols. RSV-DCs are strikingly more potent to inhibit on-going alloreaction, as few as 50 RSV-DCs are able to nearly completely shut down the proliferation of 50000 T cells driven by 1250 untreated DCs.

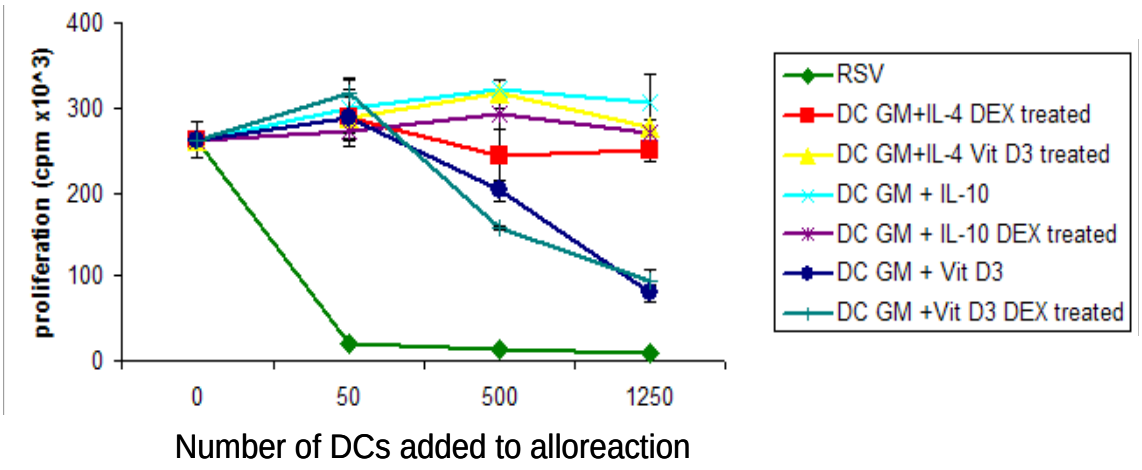


Figure 2.17: The ability of pharmaco-DCs and RSV-DCs to inhibit on-going alloreaction. GM+Vit D3 DC and GM+Vit D3 DEX DC are able to inhibit on-going alloreaction, RSV-DCs are strikingly more tolerogenic than pharmaco-DCs.

Taken together, RSV-DCs are much more potent than pharmaco-DCs to inhibit CD4+ T cell alloproliferation as well as mixed leukocyte reaction *in trans*.

Discussion

RSV infection is the leading cause of hospitalization in the first few years' life of an individual. Nearly 100% of young children have been infected at least once by the age of two. However, up till now there is no effective vaccine against this virus, early attempt of vaccination using formalin inactivated virus resulted in even enhanced disease symptoms (128, 129). Thus, understanding the mechanism of RSV-mediated immune evasion has been important for vaccine development.

In this chapter, we have demonstrated that DCs, upon RSV exposure, were skewed to a tolerogenic state. A cytokine secretion analysis indicated what RSV-DCs secrete large amount of immune inhibitory cytokine IL-10. Secretion of IL-10 appears to be a general feature of all tolerogenic DCs. Both 1alpha,25-Dihydroxyvitamin D(3) and

Dexamethasone induced tolerogenic DCs show increased secretion of IL-10 (179). These IL-10 secreting VitD3- or DEX-DCs are able to suppress T cell response *in vitro* or *in vivo* (180, 181). In our study, we found that RSV-DCs secrete IL-10 and this autocrine IL-10 was found to be important for RSV-mediated DC tolerogenic conversion. Upregulation of surface immunoinhibitory receptor and ligand systems are another feature of all tolerogenic DCs. It is demonstrated that HLA-G and ILT interaction leads to development of tolerogenic DC with the induction of anergic and immunosuppressive T cells (182). Tolerogenic DCs isolated from cancer patients express highly upregulated PD-L1, which is responsible for impaired T cell activation in these patients (81). In our study, we found that various inhibitory molecules were upregulated on RSV-DC surface. Importantly, PD-L1/PD-1 signaling was required for RSV-DCs' function in suppressing T cell proliferation.

Strikingly, we have found that RSV-DCs were able to confer infectious tolerance, as few as 25-50 RSV-DCs were able to suppress the ability of 20 times as much un-infected DCs to drive MLR. This remarkable suppressive function led us to investigate whether regulatory T cells were expanded, which is the subject of the next chapter.

To sum up Chapter two, we found that RSV exposure did not instruct DCs to elicit effective anti-viral immune response; rather, these DCs were skewed to a tolerogenic state featured by IL-10 secretion and surface inhibitory molecule expression. These cells not only are unable to drive T cell response but also are able to confer infectious tolerance to other un-infected cells. These will help to explain why adaptive immune response (especially memory response) is hard to establish following RSV infection in patients.

CHAPTER THREE

RSV-DCs Induce Regulatory T Cell Expansion and Conventional T Cell Anergy

Introduction

Respiratory Syncytial Virus (RSV) is a leading respiratory pathogen in infants and young children worldwide. RSV infection causes hospitalization of nearly 125,000 children and 1800 deaths in the United States annually (183, 184). Worldwide, the hospitalization rate is even higher with a mortality rate of nearly 5% (128). Viral exposure does not induce protective immunity and repeated infections continue to occur as children get re-exposed to the same viral strain in subsequent seasons (149). These observations indicate that mechanisms may exist for viral subversion of adaptive host response. Dendritic cells play a central role in initiation and regulation of adaptive immunity (2). In the previous chapter, we have reported that RSV subverts immune response by skewing the maturation of DCs to a tolerogenic state. RSV-DCs secrete high amount of IL-10 and express a variety of immune inhibitory molecules (ILTs, HLA-G, PD-L1, etc). Furthermore, RSV-DCs are incapable of activating naïve CD4+ T cell response *in vitro* or *ex vivo*. Strikingly, RSV-DCs are able to inhibit the allo-proliferative capacity of unexposed DCs *in trans* at very low cell numbers. The potent inhibitory capacity leads us to investigate the role of Tregs in propagating this tolerogenic signal.

Tregs play a critical role in the maintenance of immune tolerance (185). Antigen specific Tregs are induced by tolerogenic DCs in the context of both soluble and receptor mediated inhibitory signals (186). It is reported that in the presence of TGF-beta, PD-L1 signaling regulates generation of adaptive FoxP3+CD4+ Tregs both *in vitro* and *in vivo*

(187, 188). Tregs have the capacity to transfer their tolerance-inducing ability to other cell populations, a term known as “infectious tolerance” (189). The propagation of infectious tolerance by Tregs can be achieved either by inducing tolerogenic DC through a inhibitory feedback loop (190) or directly differentiating Teff cells into IL-10 or TGF-β-producing adaptive Tregs (191). In this study, we demonstrate that RSV-induced tolerogenic DCs selectively expand Tregs which in turn inhibit Teff cell proliferation and function. Furthermore, we demonstrate PD-L1/PD-1 signaling is required for Treg expansion by RSV-induced tolerogenic DCs. By inducing Treg expansion, a small number of RSV induced tolerogenic DCs may prevent the development of an effective antiviral response.

Peripheral T cell tolerance is an essential property of the immune response that is regulated by the cooperation of the intrinsic anergy of T cells with active suppression by other cells. T cell anergy is a state in which T cells fail to respond to previously encountered antigenic stimulation by functional APCs. Initially, T cell anergy was found to be induced in a $CD4^+$ T cell clone *in vitro*, when the cells were stimulated by TCR (first signal) without a costimulatory CD28 signal (second signal) (192). Because such cells lose the ability to produce IL-2 upon secondary stimulation *in vitro*, and recover from the anergic state when exposed to exogenous IL-2, T cell anergy is defined as the state in which T cells lose the ability to produce IL-2 autonomously.

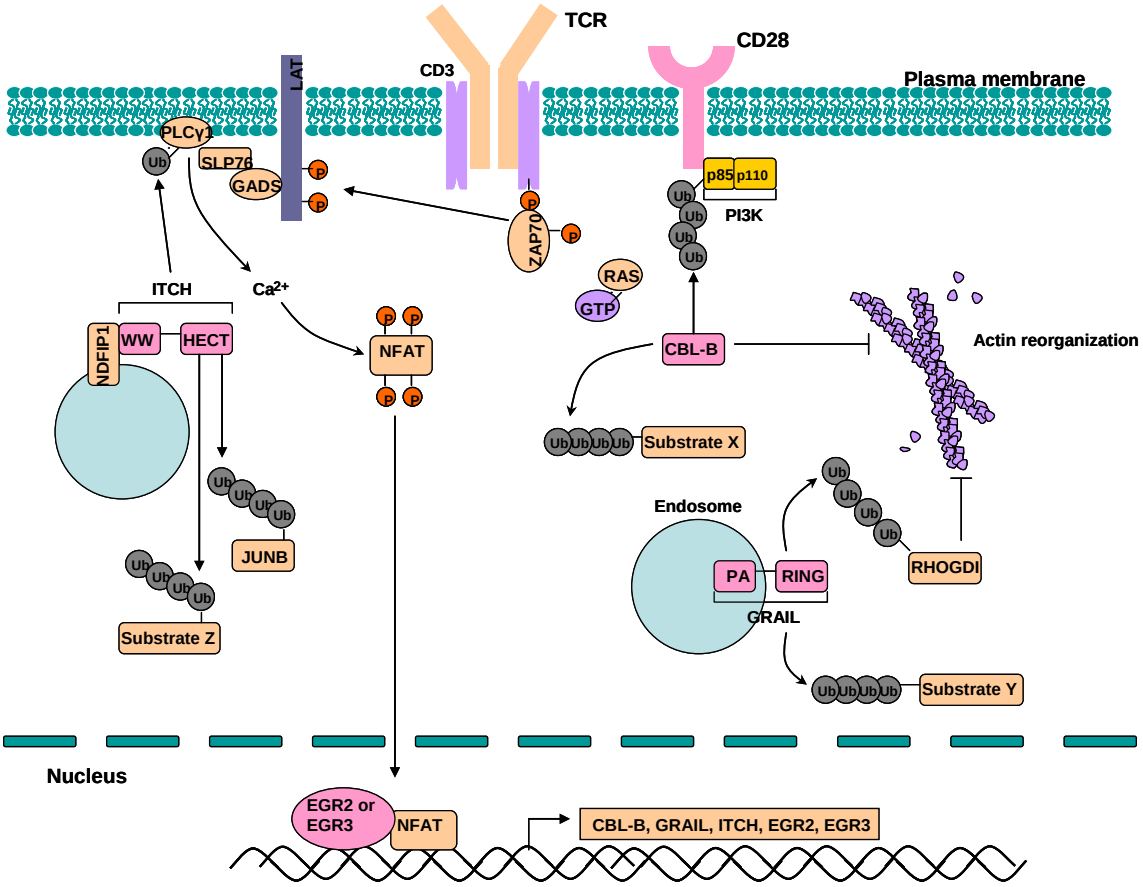


Figure 3.1: E3 ubiquitin ligases in regulating TCR signaling. TCR signaling results in translocation of NFAT (Nuclear Factor of Activated T cells) into the nucleus, without appropriate contribution from other transcription factors activated by the MAPK signalling pathway, a genetic anergic signal is imparted on the T cell. Specifically, NFAT causes the upregulation of expression of three E3 ubiquitin ligases, CBL-B (Casitas B-lineage lymphoma B), GRAIL (gene related to anergy in lymphocytes) and ITCH (itchy homologue E3 ubiquitin protein ligase), These E3 ubiquitin ligases, unrelated at the polypeptide level, target different signalling nodes that are essential for T-cell activation. ITCH (along with its related E3 ubiquitin ligase NEDD4 (neural precursor cell expressed, developmentally downregulated 4)) has been shown to monoubiquitylate both phospholipase Cγ1 (PLCγ1) and protein kinase Cθ (PKCθ) for lysosomal degradation and termination of signalling downstream of linker for activation of T cells (LAT). CBL-B polyubiquitylates the p85 subunit of PI3K (phosphoinositide 3-kinase) and prevents PI3K from associating with CD28, thus attenuating this co-stimulatory signalling cascade. CBL-B expression also leads to deficient T-cell synapse formation by inhibiting actin reorganization downstream of the RHO-family small GTPase VAV1. GRAIL has also been shown to modulate the activation of RHO-family members by polyubiquitylating and stabilizing the RHO inhibitor RHOGDI (RHO guanine-dissociation inhibitor). Thus, the combined effects of ITCH, CBL-B and GRAIL serve as a potent inhibitory signal to prevent T-cell activation after stimulation through the T-cell receptor (TCR), as could occur during strong binding to self-protein–MHC complexes in the periphery. (Figure adapted from Fathman & Lineberry, 2007, *Nat.Rev.Immunol.*)

Anergized T cells display defect in intracellular signaling transduction. This defect is thought to involve the function of certain E3 ubiquitin ligases. These E3 ligases translocate to the T cell membrane (probably to immunological synapses) and colocalize with target substrates such as phospholipase Cγ1 (PLCγ1) and protein kinase Cθ (PKCθ). This colocalization initiates the mono-ubiquitination and possible lysosomal degradation of PLCγ1 and PKCθ, leading to diminished TCR signaling and resulting in compromised immunological synapse function and T cell responses. Among these E3 ubiquitin ligases, Cblb, GRAIL and Itch are best characterized T cell anergy markers (193-195). In this chapter, we analyzed RSV-DC primed T cells and found that they were rendered phenotypical and functional anergy.

Material and Methods

Preparation of RSV and mDCs

A2 RSV was prepared as previously described; briefly, HEp-2 cells were infected at a Multiplicity Of Infection (MOI) of 1.0. After incubation at 37°C for 4-5 days, cells were lysed and centrifuged in a sucrose gradient; supernatant containing enriched viruses was collected. Viral titer was determined by tissue culture infections dose (TCID50) calculation, for all subsequent experiments, MOI of 1.0 was used.

mDCs were isolated from human blood PBMC as described previously, briefly, PBMCs were obtained from centrifuging blood in a Ficoll gradient (GE Healthcare). DCs were enriched by incubating PBMCs with magnetic microbeads conjugated to anti-CD3, anti-CD14, anti-CD16, anti-CD19, and anti-CD56 antibodies and then passed over a magnetic column. After enrichment, mDCs and pDCs were further separated and

purified by labeling cells with LINEAGE-FITC, CD123-PE, HLA-DR-PerCP, and CD11c-APC antibodies followed by FACS sorting. The purity of isolated mDCs averaged 97%.

Mixed Leukocyte Reaction and T Cell Sorting

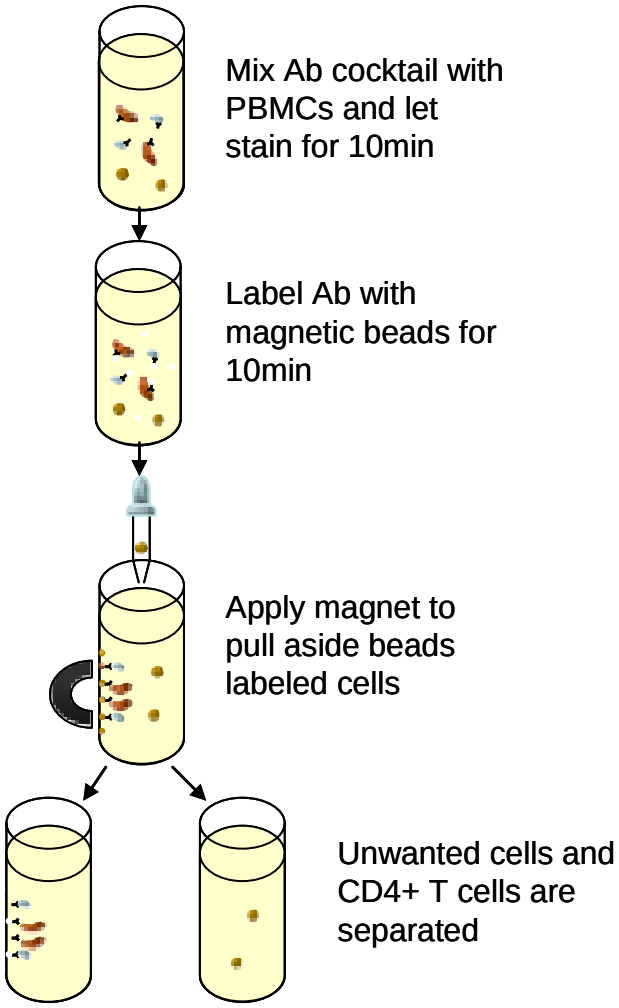


Figure 3.2: Strategies to purify CD4+ T cells from PBMCs. Antibody cocktail that labels all cell types except for CD4+ T cells are incubated with PMBCs for 10min followed by wash-off of excess antibodies. Then antibodies are conjugated with magnetic beads for 10min. Magnetic field is applied to the cell mixture, beads labeled unwanted cells are pulled aside. CD4+ T cells are free in the medium and can be collected.

Untreated, Flu or RSV treated mDCs were cultured at 37°C for 18h. Allogeneic CD4+ T cells were freshly isolated from blood PBMCs by negative selection using EasySep® Human CD4+ T cell Isolation Kit (Stemcell Technologies, Figure 3.2) and labeled with Carboxyfluorescein succinimidyl ester (CFSE) at a concentration of 1uM for 10min followed by washing.

Untreated, Flu or RSV treated mDCs respectively were mixed with allogeneic T cells at a ratio of 1:40 and cultured at 10^6 cell/ml at 37°C for 5 days. At day 5, CD4+ T cell alloproliferation was assessed by flow cytometry in a CFSE dilution assay. T cells which proliferated once (designated G1) were sorted by FACS-Aria for Treg analysis. Non-proliferating T cells (designated G0) were sorted for T cell anergy analysis. In the Treg suppression assay, FACS-sorted G1 population was added to an on-going MLR using untreated mDC from a second donor as stimulators and CFSE labeled CD4+ T cell from a third donor as responders, G1:DC:T=1:1:20, and co-culture for 5 days followed by the FACS analysis.

Antibodies, Flow Cytometric Staining and Analysis

Analysis of Tregs markers was performed by surface staining of CD25-APC (BC96, eBioscience) and GITR-PE (eBioAITR, eBioscience), and intracellular staining of FoxP3-FITC (hFOXY, eBioscience) and CTLA-4-PE-Cy5 (BNI3, BD Pharmingen). Cells were first stained for surface molecules for 30min, and then permeabilized with Cytofix/Cytoperm (BD Biosciences) for 20min followed by intracellular molecule staining (196). Cells were fixed with 4% paraformaldehyde before FACS analysis. mDC surface PD-L1 and PD-L2 expression was assayed using PE-conjugated PD-L1 (MIH1, eBioscience) or PD-L2 antibody (MIH18, eBioscience). Blocking antibodies against PD-

L1 (10ug/ml, MIH1) and PD-1 (10ug/ml, J116) were purchased from eBioscience. All flow cytometric data were acquired by FACS-Calibur or FACS-Aria (BD Biosciences, San Jose, CA) and analyzed by FloJo software (Tree Star Inc., Ashland, OR).

Real-time RT-PCR Analysis of Gene Expression

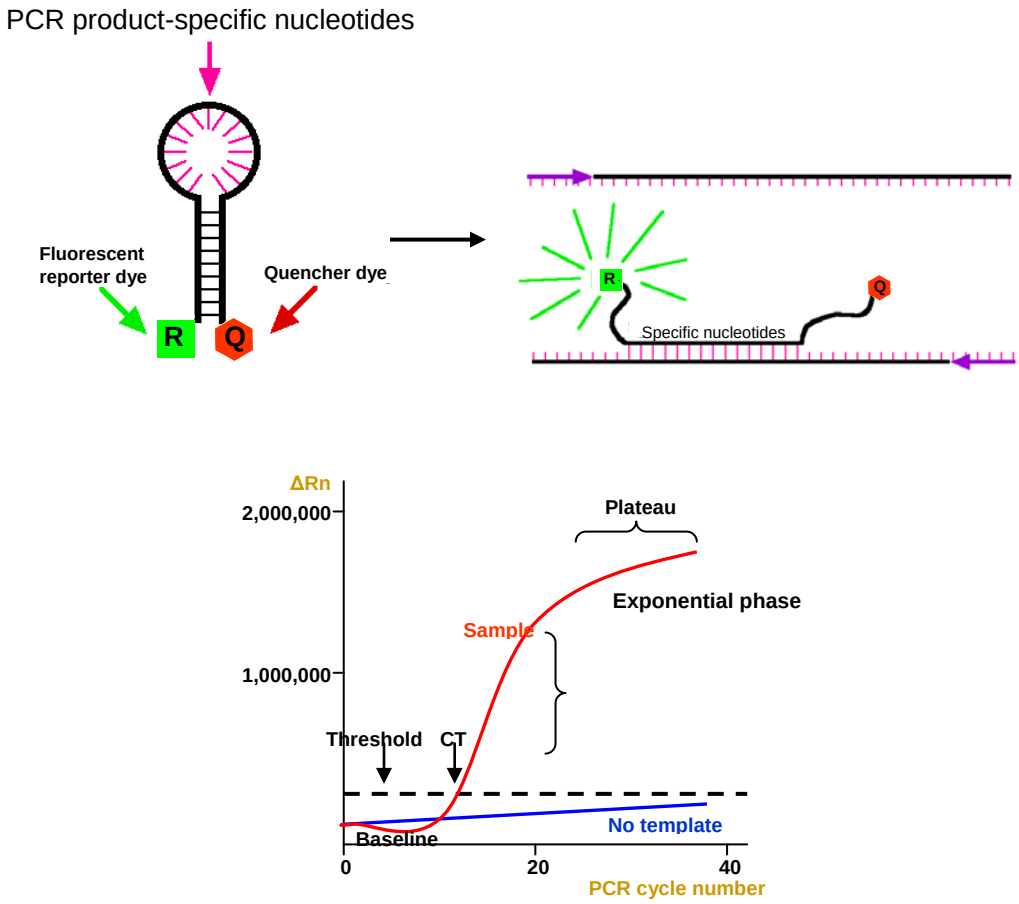


Figure 3.3: Real-time PCR analysis of gene expression. A molecular beacon is a fluorescent reporter dye and a quencher dye labeled oligo-nucleotides that binds specific to the gene sequence. When a new strand is synthesized by PCR, molecular beacon binds to it and emits fluorescence excited by appropriate wavelength of light. The amount of fluorescence is directly proportional to the amount of PCR product amplified. The read-out of the real-time PCR reaction is a curve with the X axis showing the PCR cycle number and the Y axis showing the arbitrary copy number of the product. The higher the DNA concentration is, the less cycle number is needed to reach the plateau phase.

Total cellular mRNA was extracted from sorted cell using Qiagen RNeasy® Micro Kit according to the manufacturer's instruction, then reverse transcribed to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). CD25, CTLA-4, FoxP3, PD-1, Ebi-3 and IL-12a expression in Tregs and PD-L1, PD-L2 expression in RSV-DCs and Cblb, GRAIL and Itch expression in anergized T cells were assessed by Real-time RT-PCR using primers designed by Applied Biosystems. Gene expression levels were compared to GAPDH as endogenous control and normalized as fold increase over internal control for comparison (Figure 3.3).

Detection of Cytokine Production

Control-, Flu- or RSV-G0 T cells were stimulated with anti-CD3/CD28 Dynabeads (Invitrogen) for 48h and the supernatants were collected for analysis. IL-2 concentration in the culture was measured by Luminex multiplex technology. Cytokine concentrations were measured with Beadlyte cytokine assay kit (Upstate, NY) using a Bio-Plex Luminex 100 XYP instrument and calculated using Bio-Plex manager 4.0 software with a 5-parameter curve-fitting algorithm applied for standard curve calculations.

Mouse Experiments

BALB/c mice were sacrificed; femurs and tibias were removed and washed in 70% ethanol and ice-cold RPMI-1640. Bone marrow was flushed out and red blood cells were lysed by resuspending cells in 3-10ml ammonium chloride solution for 3 min followed by wash. Bone marrow cells were then culture in RPMI-10% FCS complete medium for 7 days in the presence of 50ng/ml mouse recombinant GM-CSF and IL-4.

Same amount of GM-CSF and IL-4 were added at Day 3 to re-boost the cells. To characterize mouse DCs, FITC anti-mouse MHC class II (I-A/I-E) (eBiosciences, clone M5/114.15.2), PE anti-mouse PD-L1 (eBiosciences, clone MIH5), PE-Cy5 anti-mouse CD80 (eBiosciences, clone 16-10A1) and APC anti-mouse CD11c (eBiosicences, clone N418) were used to stain DC surface molecules. At Day 7, about 60-70% cells had differentiated into DCs as indicated by flow cytometric analysis. Harvested DCs were either untreated or treated with Flu or RSV for 18h.

C57BL/6 mice were sacrificed, spleens were removed and washed in ice-cold RPMI-1640. Splenocytes were release by physical disruption and subjected to filtration for a rough purification. CD4+ T cells were then purified from splenocytes by negative selection using Mouse CD4+ T Cell Isolation Kit (Miltenyi Biotec). Briefly, splenocytes were labeled by Biotin conjugated antibody cocktail followed by wash to remove excess antibodies. Antibody labeled cells were then incubated with anti-biotin microbeads. Magnetic field was applied to pull aside antibody-beads labeled unwanted cells, leaving only untouched CD4+ T cell in the medium. For the analysis of CD4+ T cell proliferation, CD4+ T cells were further labeled with 1.25-2uM of CFSE for 10min followed by addition of 20% FCS to stop the reaction.

BM-DCs from BALB/c mice were either untreated or treated with Flu or RSV for 18h to obtain control-, Flu- or RSV-DCs. CD4+ T cells (either unlabeled or CFSE-labeled) from C57BL/6 mice were co-cultured at 37°C with BALB/c BM-DCs for 6 days. For the analysis of CD4+ T cell alloproliferation, CFSE-labeled T cells were stained with APC anti-mouse CD4 antibody (BD Pharmingen, Clone RM-4-5) followed by flow cytometric analysis. For the assessment of Treg expansion, CD4+ T cells, following DC

co-culture, were analyzed using a Mouse Regulatory T cell Staining Kit (eBiosciences). Briefly, cells were stained with FITC anti-mouse CD4 (clone: RM4-5) and APC anti-mouse CD25 (clone: PC61.5). Then cells were fixed and permeabilized followed by intraceullar staining with PE anti-mouse/rat FoxP3 antibody (clone FJK-16s) for flow cytometric analysis.

Statistical Analysis

To determine statistical difference, P value was calculated for paired T test by using GraphPad Prism software and significant differences were concluded if $p<0.05$. Single asterisk indicates $p<0.05$, double asterisks indicate $p<0.01$.

Results

RSV-DCs Expands a Population of CD25^{hi}/FoxP3^{hi}/CTLA-4^{hi}/GITR+ T Cells

As we have previously demonstrated in Chapter 2, RSV-DCs inhibit allogeneic CD4+ T cell proliferation when compared to untreated DCs or Flu exposed DCs. More strikingly, RSV-DCs are able to confer infectious tolerance to inhibit MLR driven by unexposed DCs. These led us to investigate the role of Tregs in the culture, since it is demonstrated that an immunoregulatory feed back loop exist between tolerogenic DCs and Tregs (190, 197). Tolerogenic DCs induce the expansion Tregs, Tregs then induce tolerogenic maturation of immature DCs (Figure 3.4).

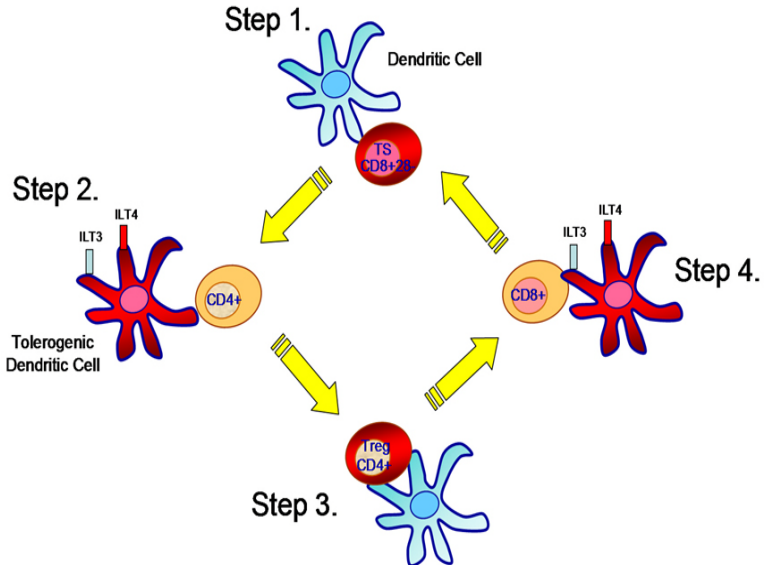


Figure 3.4: Cycle of infectious tolerance. Immunoregulatory feed back loop exist between tolerogenic DCs and Tregs. Step1, regulatory CD8+/CD28- T cells encounter immature DCs and induce tolerogenic maturation. Step2, newly converted tolerogenic DCs skews naïve CD4 T cells to Tregs. Step3, Tregs induce tolerogenic maturation of immature DCs. Step4, tolerogenic DCs polarizes naïve CD8 T cells to regulatory CD8+/CD28- cells.

Tregs are potent inhibitors of adaptive T cell response. As it has been shown that Tregs are enriched in lungs of RSV-infected mice (198), we tested whether the RSV-DCs selectively induce the expansion of Tregs *in vitro*. Following co-culture with control, Flu or RSV exposed DCs for 5 days, allogeneic T cells were stained with Treg markers CD25, FoxP3 (Forkhead box P3, a transcription factor modulating regulatory gene expression (199)), CTLA-4 (Cytotoxic T-Lymphocyte Antigen 4, a member of CD28 costimulatory family which deliver inhibitory signals to T cells(200)) and GITR (Glucocorticoid-Induced Tumor necrosis factor Receptor (201)) to analyze by flow cytometry. As shown in Figure 3.5, T cells co-cultured with RSV-DCs displayed an increased population of $CD25^{hi}/FoxP3^{hi}/CTLA\text{-}4^{hi}/GITR+$ T cells compared to their untreated or Flu treated counterpart, indicating the expansion of Tregs. To further characterize these regulatory cells, we investigated their function to inhibit MLR.

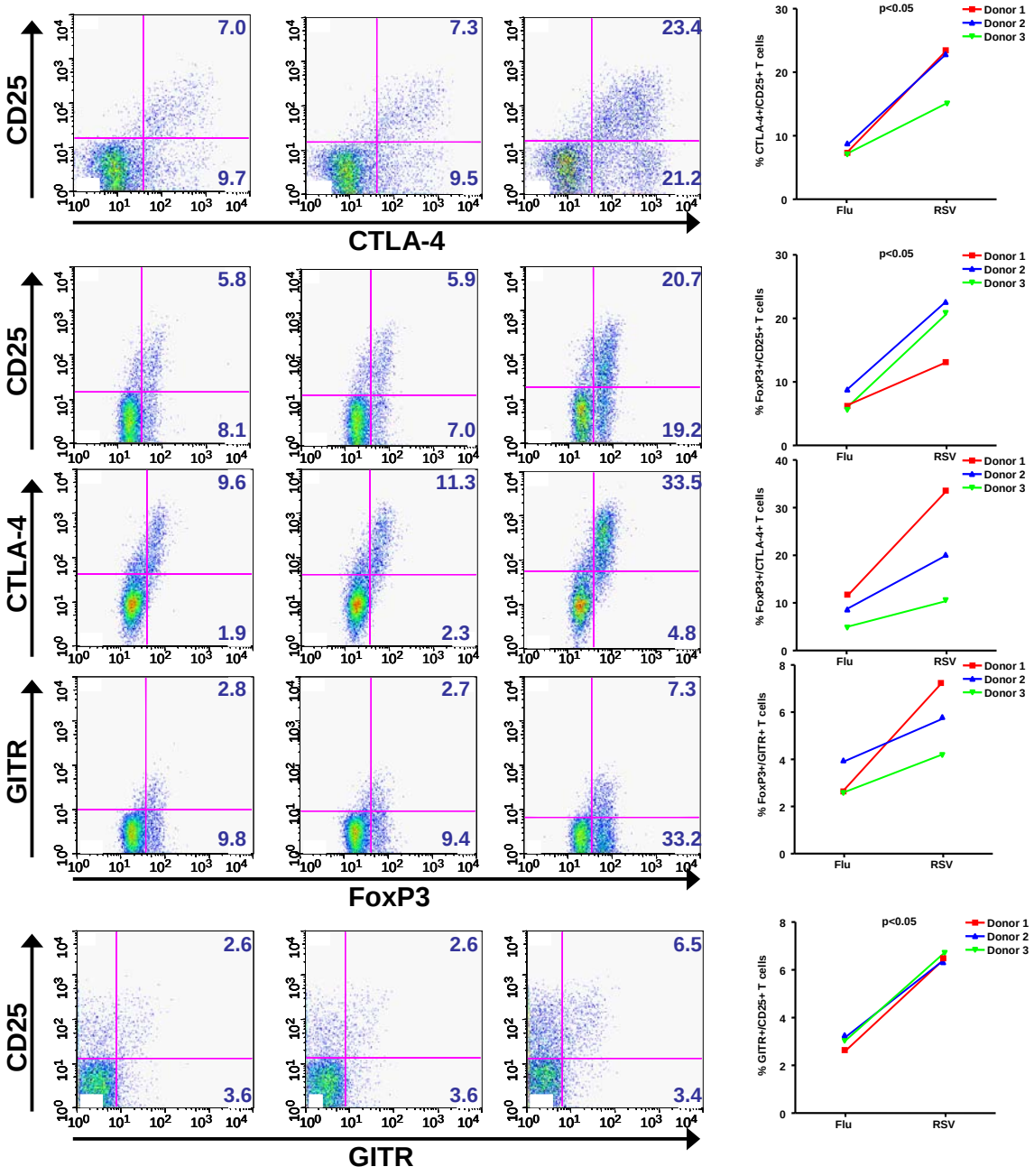


Figure 3.5: RSV-DCs expand a population of CD25+FoxP3+CTLA-4+GITR+ T cells. After untreated or Flu- or RSV- infected for 18h, mDCs were cultured with CD4+ T cells for 5 days. Then Treg markers CD25, FoxP3, CTLA-4 and GITR expressions were analyzed by flow cytometry. Figure shown is a representative of n=3 independent experiments.

RSV-DC Primed G1 T Cells are Able to Suppress On-going MLR

Since Tregs proliferate poorly when compared to effector T cells (202), we sorted by flow cytometry the first generation of proliferating cells (G1) (Figure 3.6) and determined whether they were able to suppress T cell proliferation in an MLR (203). The sorted G1 population from control, flu or RSV conditions respectively was added to an MLR where mDCs from a second donor were used as stimulator and CFSE-labeled CD4+ T cells from a third donor were used as responders (Fig.1B, G1:DC:T=1:1:20). After 5 days' co-culture, the alloproliferation of respondent CD4+ T cells was assessed by flow cytometry as of CFSE dilution.

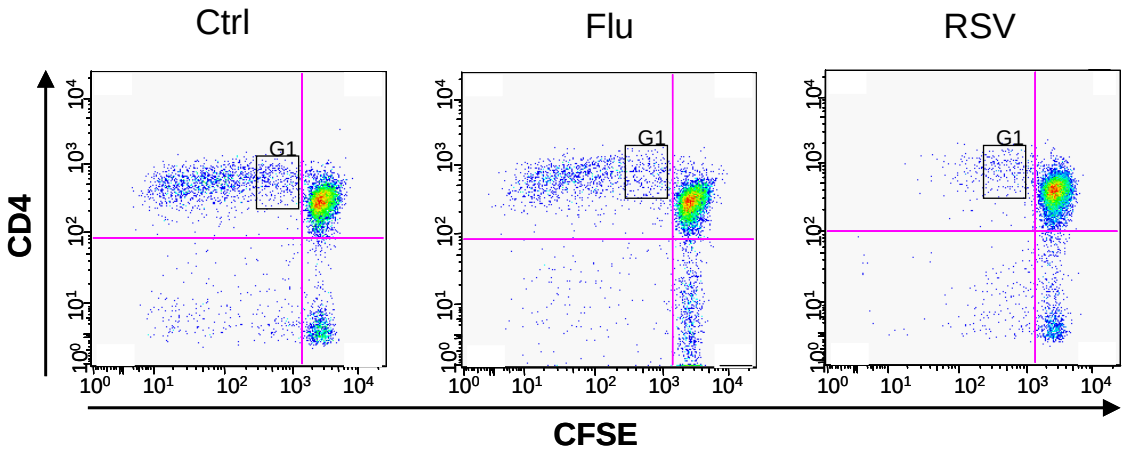


Figure 3.6: Sorting of Treg enriched G1 population. After co-culturing control, Flu or RSV DCs with allogeneic CFSE-labeled CD4 T cells for 5 days, cells were stained with CD4-APC. The first generation of proliferation (G1) was sorted by FACS Aria, gating strategy shown.

As shown in Figure 3.7, RSV-G1 T cells were capable of significantly suppressing CD4 T cell alloproliferation when compared to untreated-G1 or Flu-G1 T cells ($n=3$, $p<0.05$). The functional *in trans* allo-inhibition data suggested the existence of Tregs in RSV-G1 T cell population.

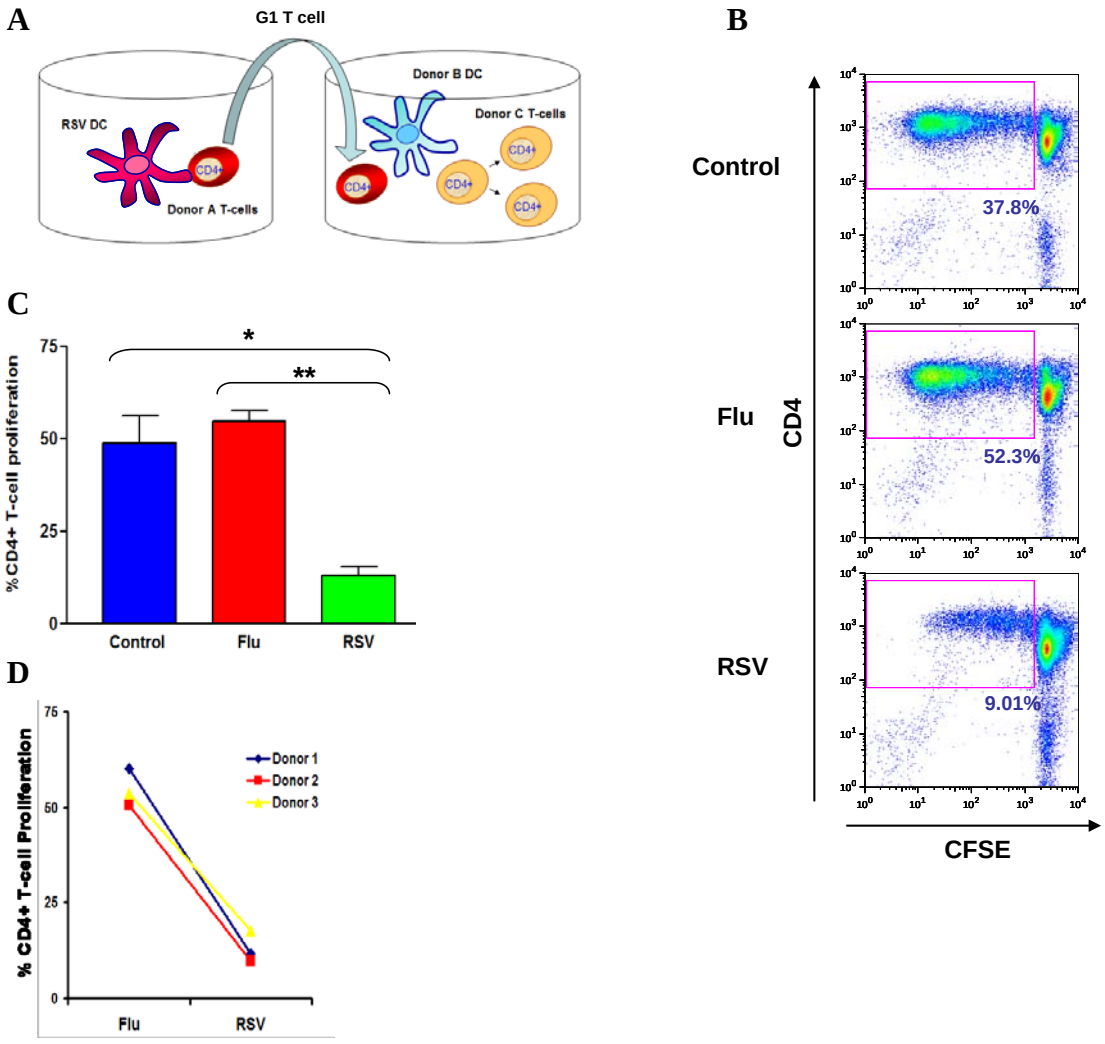


Figure 3.7: G1 T cells have immunosuppressive function. (A).Sorted G1 T cells from control, Flu or RSV conditions were added to a on-going MLR in which uninfected DC from a second donor was used as stimulators and CFSE labeled-CD4+ T cells from a third donor as responders. (B).Inhibition of allo-proliferation was indicated as CFSE-dilution of CD4+ T cells in the on-going MLR. RSV-G1 T cells significantly inhibited MLR. (C and D).Bar graph summarizes the percentage of CD4+ T cell proliferation in control, Flu or RSV-G1 cell added on-going MLR (Ctrl-RSV $p<0.05$, Flu-RSV $p<0.01$, n=3).

Phenotypical Characterization of G1 T Cells

To confirm the observation that Tregs exist in RSV-G1 T cells, we did phenotypical analysis of G1 T cells. After sorted by FACS-Aria, G1 T cells from control,

Flu or RSV conditions were permeablized for intracellular staining of FoxP3 followed by flow cytometric analysis. As shown in Figure 3.8, RSV-G1 cells contained more than 20% FoxP3^{hi} Tregs, which is significantly higher than control- or Flu-G1 T cells ($p<0.05$).

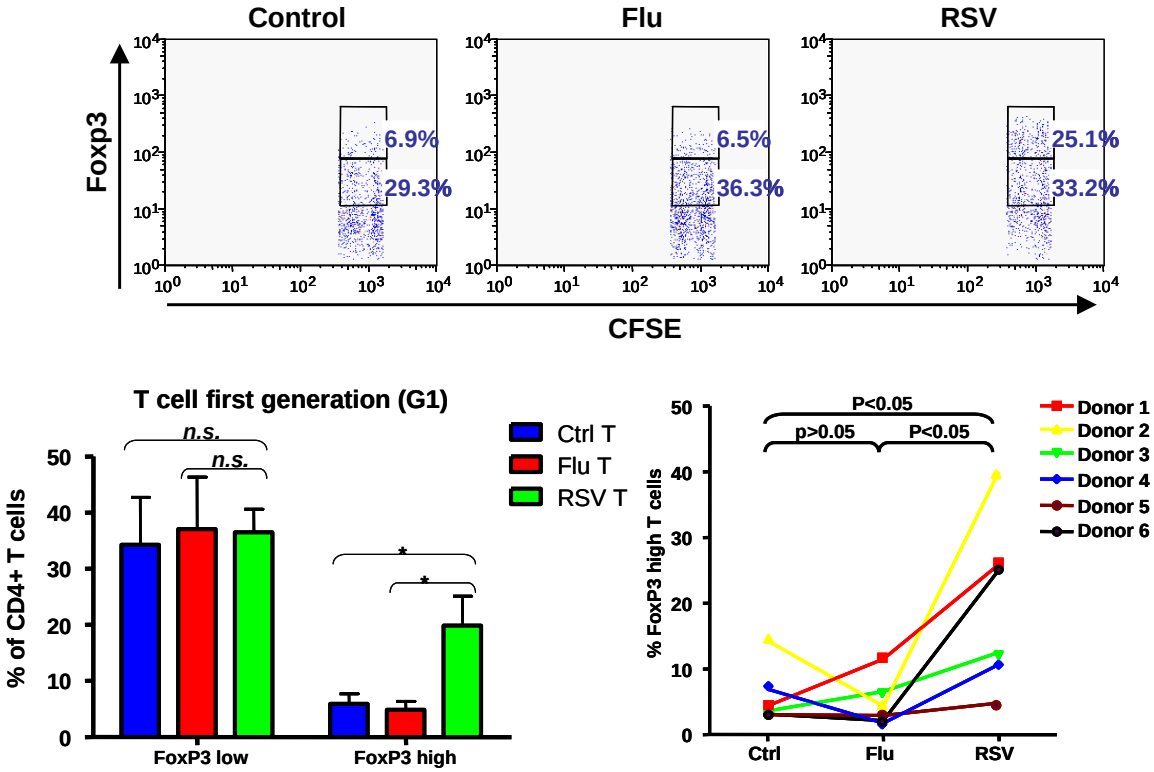


Figure 3.8: Flow cytometric analysis of FoxP3 expression in G1 T cells. CFSE-labeled CD4+ T cells were cultured with control, Flu or RSV-DCs for 5 days, first generation of proliferation G1 T cells were isolated for examination of FoxP3 expression. Flow gates indicate FoxP3^{hi} and FoxP3^{lo} population respectively. Bar graph is a summarization of percentage of FoxP3^{hi} and FoxP3^{lo} population in control, Flu and RSV conditions. Data represent n=6 independent experiments (Foxp3 high: Ctrl-RSV $p<0.05$, Flu-RSV $p<0.05$).

The limited number of G1 T cells after cell sorting makes it difficult to perform flow cytometric analysis of other Treg markers. As an alternative to further analyze the expression of Treg-associated phenotypical markers in G1 cells, we performed Real-time RT-PCR to assess CD25, FoxP3, CTLA-4 and PD-1 mRNA level. G1 T cells from

control, Flu or RSV were lysed to extract RNA. RNA was then reverse transcribed to cDNA. Equal concentration of cDNA from 3 conditions was used to perform Real-time PCR analysis using primers specific for those Treg markers. Consistent with the flow cytometry data for the bulk population, RSV-G1 T cells expressed increased levels of CD25, FoxP3, CTLA-4 and PD-1 mRNAs compared to control-or Flu-G1 T cells (Figure 3.9).

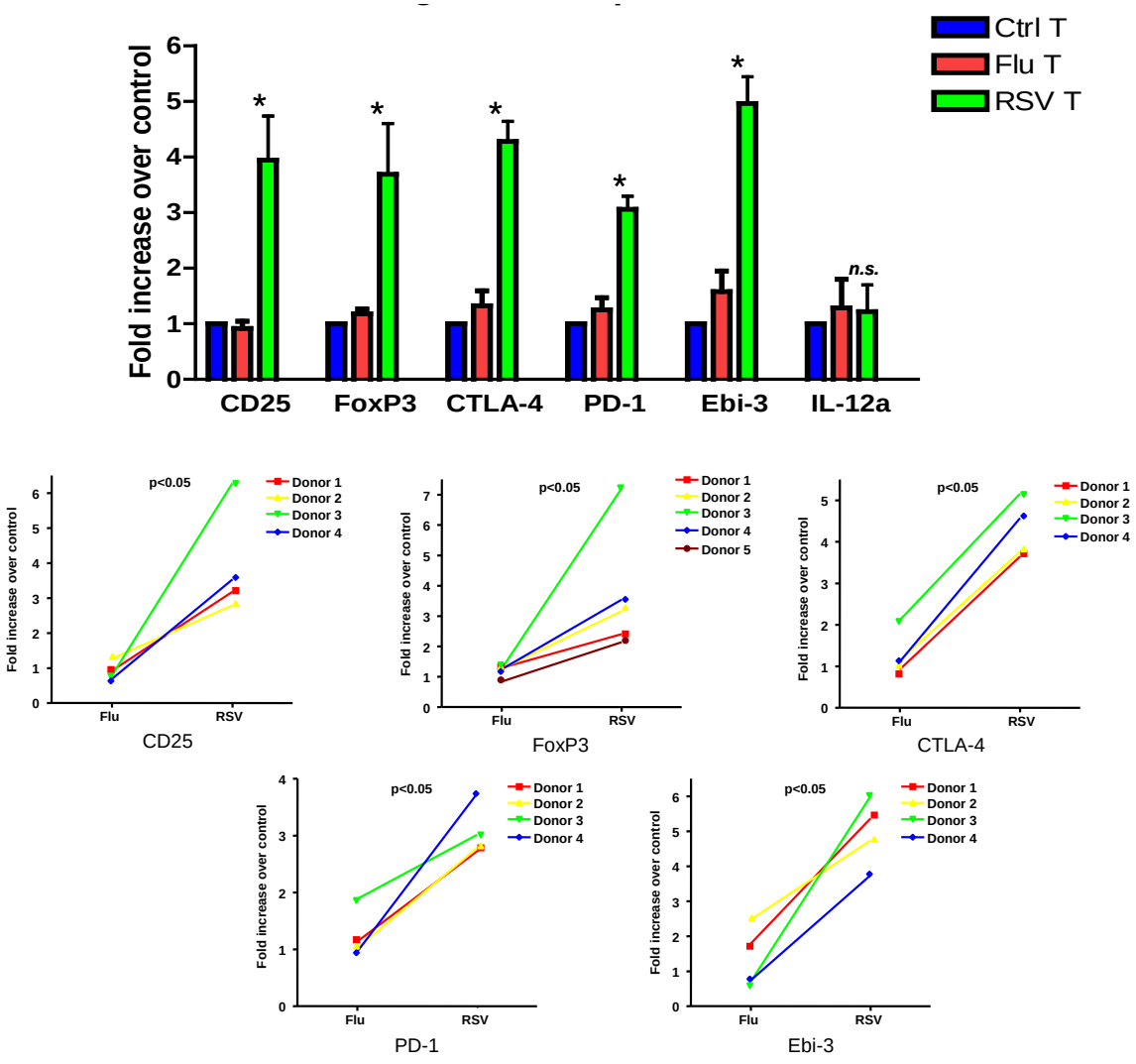


Figure 3.9: Real-time RT-PCR analysis of Treg markers CD25, FoxP3, CTLA-4, PD-1 expression and Treg inhibitory cytokine IL-35 subunit Ebi-3 and IL-12a expression. Statistical line graph summarizes data from 4 or 5 independent donors ($p<0.05$).

Recently, it has been reported that Treg function is dependent on the expression of IL-35, a heterotrimer of Epstein-Barr-virus-induced gene3 (Ebi-3) and interleukin-12 alpha (IL-12a/p35) (204, 205). To determine whether RSV-DC expanded Tregs produce IL-35, we monitored the expression of IL-35 subunits Ebi-3 and IL-12a by Real-time RT-PCR. As shown in Figure 3.9, RSV G1 T cells showed increased Ebi-3 expression ($p<0.05$) compared to untreated DCs or Flu G1 T cells, while the expression levels of IL-12a were similar among the three conditions. The analysis of protein and mRNA expression from RSV-G1 T cells indicates RSV-DCs induce the expansion of regulatory T cells.

Taken together, these data indicate that RSV-DCs selectively induce the expansion of Tregs, these Tregs exist primarily in G1 population.

RSV-DC Expanded Tregs Induce Conventional CD4+ T cell Anergy

Anergy is a tolerance mechanism, in which T cells are functionally inactivated following the antigen encounter. Anergic cells remain alive for an extended period of time in a hyporesponsive state characterized by the impairment of T cell proliferation and cytokine production upon exposure to pleiotropic stimuli (206, 207). One well characterized mechanism by which Tregs function is through the induction of anergy in targeted effector T cell population. We have observed that a large proportion of CD4+ T cells did not proliferate upon stimulation with RSV-DCs, we asked then whether these non-proliferating T cells were anergic.

As described above, mDCs either untreated or treated with Flu or RSV respectively for 18h were co-cultured with allogeneic CD4+ T cell (DC:T=1:40). After 5 days, $CFSE^{high}$ non-proliferative T cells (designated G0) were isolated by cell sorting

(Figure 3.10). To test whether these non-proliferating cells were in a state of anergy, G0 cells were stimulated for 2 days with anti-CD3/CD28 microbeads (5ul beads/10^6 T cells). In the presence of T cell receptor signal and CD28 co-stimulatory signal, control G0 T cells and Flu G0 T cells demonstrated dramatic expansion (30% and 23.8% respectively). However, RSV G0 T cell did not proliferate well (9.3%) even in response to strong anti-CD3/CD28 stimulation (Figure 3.11). This hypo-responsiveness is characteristic of T cell anergy.

As anergized T cells fail to produce IL-2 in response to TCR signaling (208), so next we determined whether RSV-G0 T cells produced effector cytokine upon stimulation. In the presence of anti-CD3/CD28 microbeads, control-, Flu- and RSV-G0 T cells were cultured for 2 days and supernatants were collected for assessment of IL-2 production. As shown in Figure 3.12, RSV-G0 T cells secreted significantly lower levels of IL-2 when compared to control- or Flu-G0 T cells after anti-CD3/CD28 stimulation ($n=6$, $p<0.05$). This indicated that CD4 effector T cells from RSV-DC co-culture are functionally anergized.

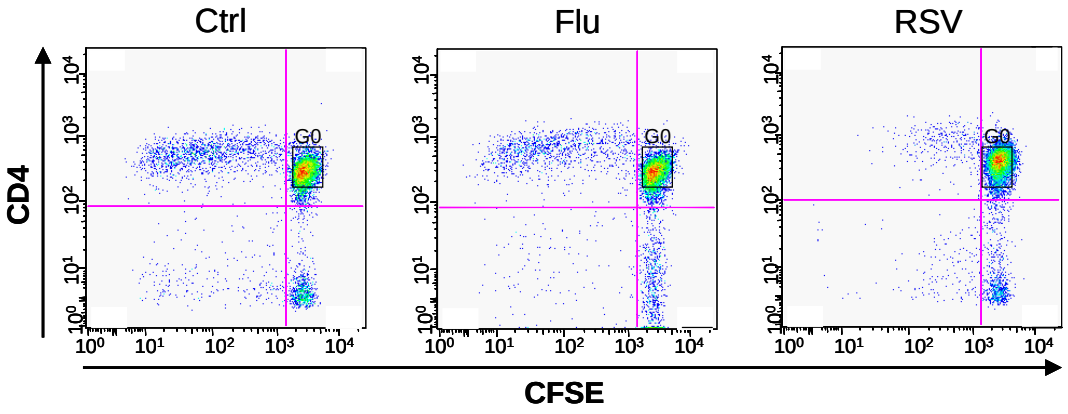


Figure 3.10: Sorting strategy of Nonproliferating G0 population. After co-cultured with control, Flu or RSV DC, Nonproliferativing (G0) portion of CD4+ T cells were sorted by FACS-Aria in all three conditions, gating strategy shown.

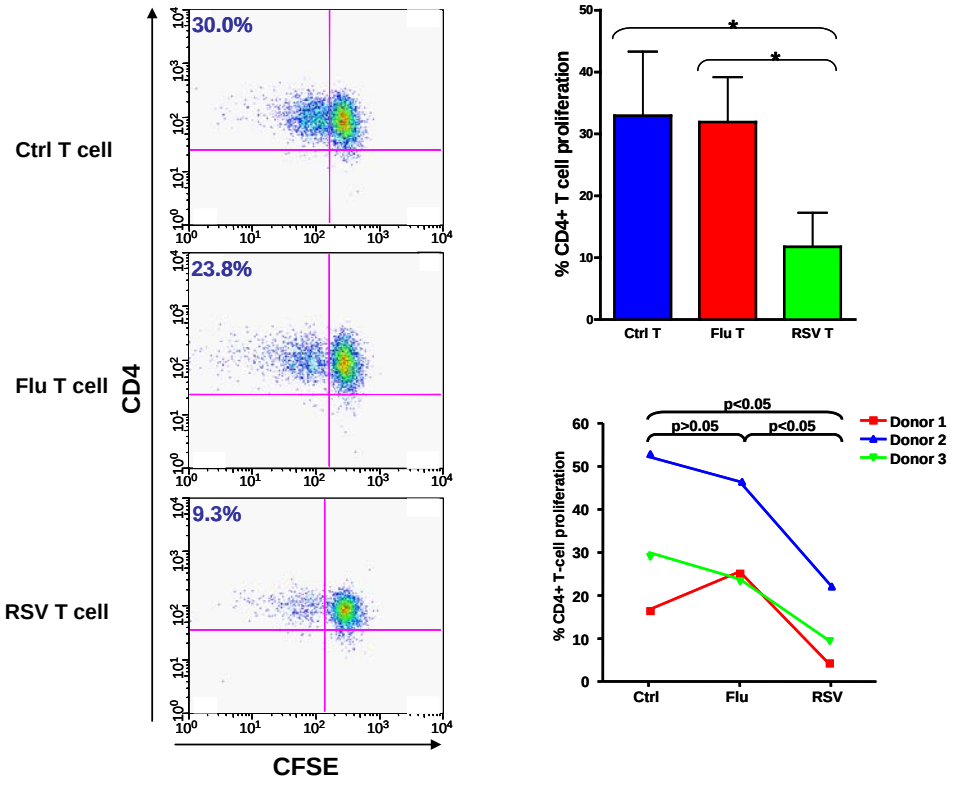


Figure 3.11: The restimulation of the G0 T cells. Non-proliferating CD4+ T cells sorted from control, Flu or RSV conditions were stimulated with anti-CD3/CD28 microbeads for 2 days. CD4+ T cell proliferation was assessed by CFSE dilution. RSV-G0 T cells showed significant less proliferation upon restimulation when compared to control or Flu G0 T cells. Bar graph summarized the percentage of proliferation in n=3 different donors (Ctrl-RSV $p<0.05$, Flu-RSV $p<0.05$).

E3-ubiquitin ligases Cblb, GRAIL and Itch are negative regulators of TCR signaling and intrinsic mediators of T cell anergy. The expression of these proteins has been used to identify anergic T cell populations (Figure 3.1) (209). We examined whether the non-proliferating G0 T cell population expressed these markers. mRNA from control-, Flu- or RSV-G0 T cell was isolated and the expression of anergy markers was determined by Real-time PCR. As shown in Figure 3.13, both Cblb and Itch in RNA levels were upregulated in RSV-G0 T cells when compared to control- or Flu-G0 T cells

(n=3, $p<0.05$). The anergy marker GRAIL demonstrated similar expression in all 3 populations.

Collectively, these data indicate that RSV-DCs, in addition to expanding Tregs, induce phenotypical and functional anergy in T effector cell populations.

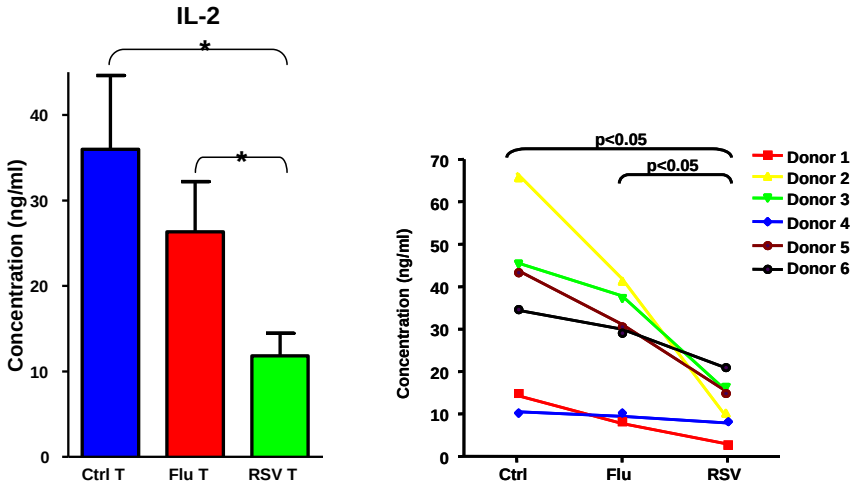


Figure 3.12: The production of T effector cytokine IL-2 in control-, Flu- or RSV-G0 T cells upon anti-CD3/CD28 re-stimulation. RSV G0 T cells secrete significantly less effetor T cell cytokine IL-2 compare to control or Flu G0 T cells. Bar graph summarizes data from n=3 independent experiments.

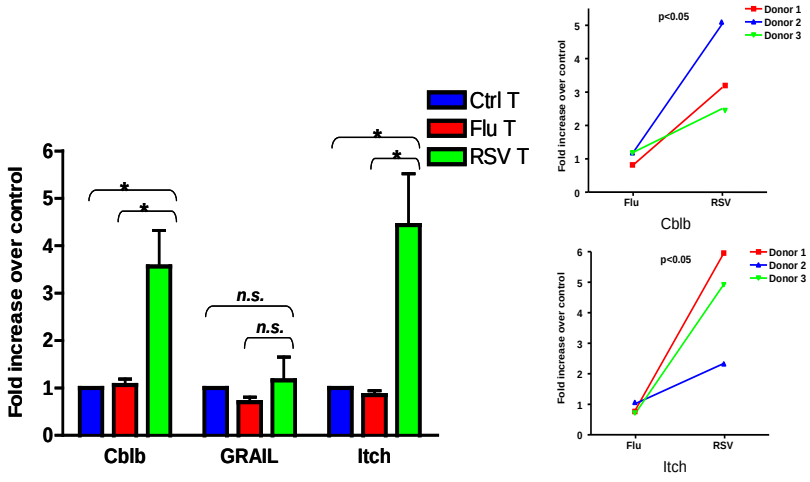


Figure 3.13: RSV-G0 T cells express specific anergy markers. RSV-G0 T cells express significantly higher amount of E3 Ubiquitin ligases Cblb and Itch, which are specific anergy markers.

PD-L1/PD-1 Signaling is Required for RSV-DCs Mediated Treg Expansion and Anergy Induction

We next investigated the mechanism through which RSV-DC induced Treg expansion. As we have demonstrated in Chapter 2, RSV exposed DCs express a variety of immune inhibitory molecules including Programmed Death Ligand 1 (PD-L1). PD-L1 is a B7 family negative co-stimulator, its receptor PD-1 contains a cytoplasmic ITIM domain responsible for recruiting SHP phosphatase. PD-L1/PD-1 pathway has been reported to suppress adaptive T cell responses (Figure 3.14) (79, 210). In the presence of TGF-beta, PD-L1 signaling regulates generation of adaptive FoxP3+CD4+ Tregs both *in vitro* and *in vivo* (187). Furthermore, PD-L1/PD-1 inhibitory signal has been shown to mediate functional impairment of T cells in chronic viral infections. It is demonstrated that blocking PD-L1/PD-1 signaling pathway augmented the function of HIV-specific CD4 and CD8 T cells from HIV patients (80). In line with *in vitro* findings, in a mouse model of chronic LCMV infection, antibody blockade of PD-L1/PD-1 restored exhausted CD8 T cell function (211). We have previously demonstrated that blocking interaction of PD-L1 and its receptor PD-1 partially restores CD4+ T cell proliferation. We next examined whether PD-L1/PD-1 signaling is required for RSV-DC mediated Treg expansion.

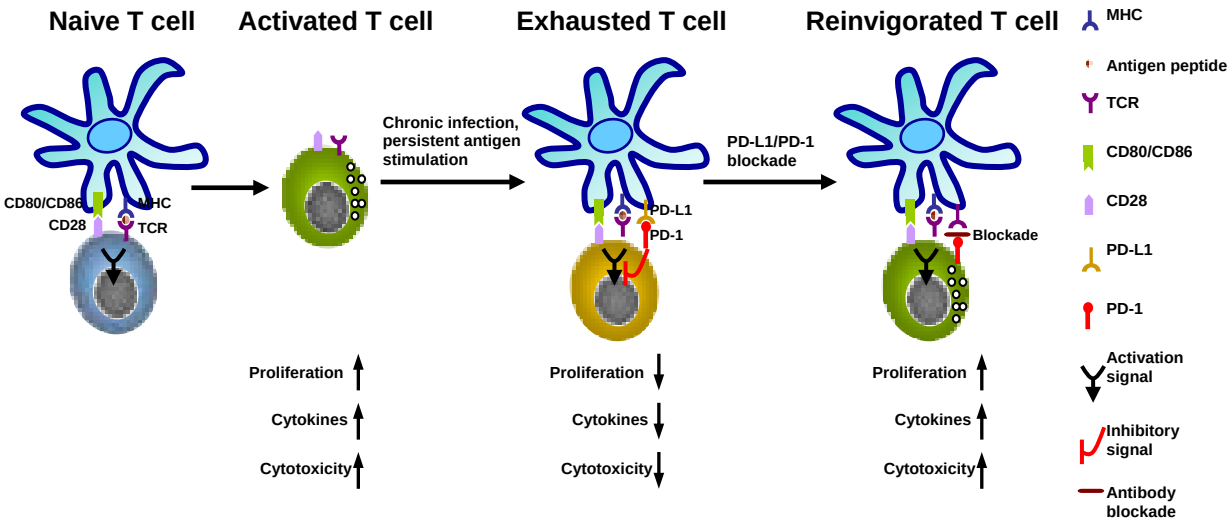


Figure 3.14: The PD-L1/PD-1 pathway contributes directly to T cell dysfunction and lack of viral control during chronic viral infection. Activated antigen-bearing dendritic cells have higher expression of CD80 and CD86 and stimulate the proliferation, cytokine production and cytotoxic activity of antigen-specific naive T cells. During chronic infection or in the presence of persisting antigen, T cells become 'exhausted' and lose the ability to proliferate. Exhausted T cells have high expression of PD-1 and receive a strong coinhibitory signal when engaging PD-L-expressing APCs. Blockade of interactions between PD-1 and its ligands can 'reinvigorate' T cells to expand their populations and regain effector functions, including cytokine production and cytolysis.

The appearance of tolerogenic function in mDCs occurs very rapidly following RSV exposure. In order to determine which PD-1 ligand(s) were responsible for the induction of Tregs by RSV-DCs, we performed a kinetic analysis of tolerogenic ligand expression following viral exposure. As shown in Figure 3.15, PD-L1 expression was upregulated within 2-3 hours of RSV exposure and persisted throughout the analysis. However, programmed death ligand homologue PD-L2 expression was not upregulated following RSV exposure when compared to the control. These data suggest that PD-L1 is the sole ligand for PD-1 specifically upregulated by RSV.

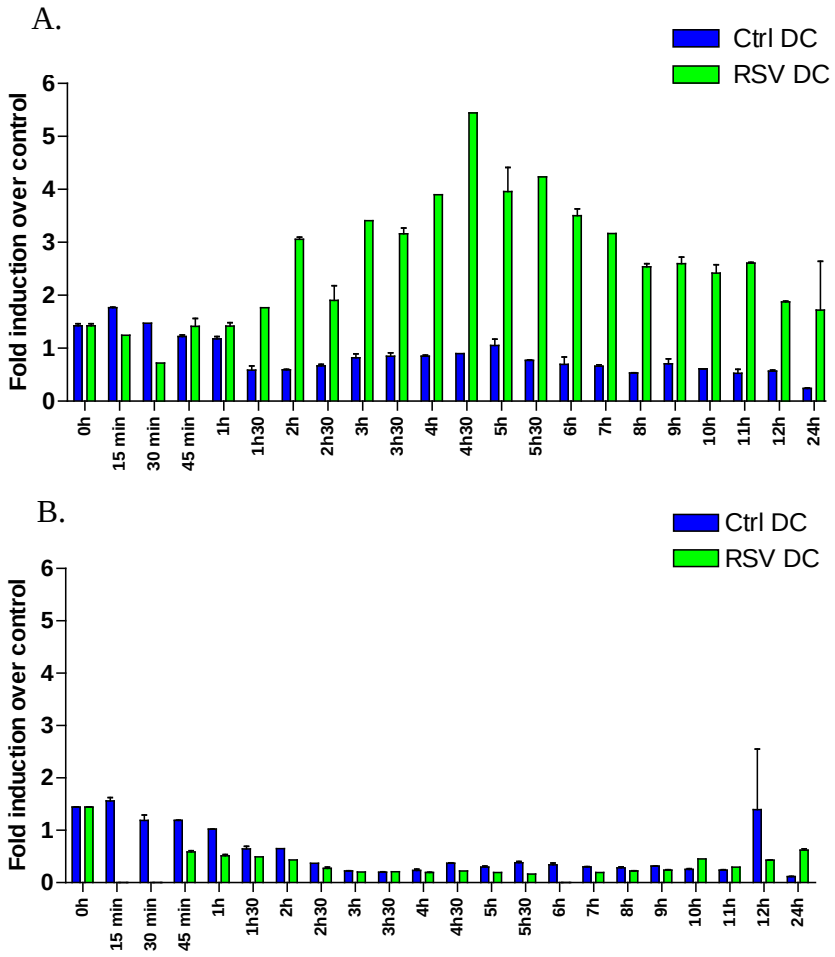


Figure 3.15: PD-L1 (A) and PD-L2 (B) mRNA expression kinetics in RSV-DCs vs. control DCs by Real-time RT-PCR.

To investigate the necessity of PD-L1/PD-1 signaling pathway to induce Treg expansion, we used PD-L1/PD-1 blocking antibodies to inhibit the receptor-ligand interaction. Similar to previous observations, in the presence of PD-L1 and PD-1 blocking antibodies, the alloproliferative capacity of RSV-T cells was partially restored. We have demonstrated above that RSV-DCs induce functional Tregs. To test whether this restoration of proliferation was due to impairment of Treg induction, we sorted the G1-T cell population from RSV-DC cultures in the presence or absence of PD-L1/PD-1

blockade, and tested their ability to suppress an on-going MLR. As shown in Figure 3.16, RSV-G1 T cells effectively suppressed CD4+ T cell allogeneic proliferation compared to control or Flu-G1 T cells. However, RSV-G1 T cells from PD-L1/PD-1 antibody treated co-cultures were no longer able to effectively suppress alloproliferation, indicating that the induction of Tregs from RSV-DCs requires PD-L1/PD-1 interaction.

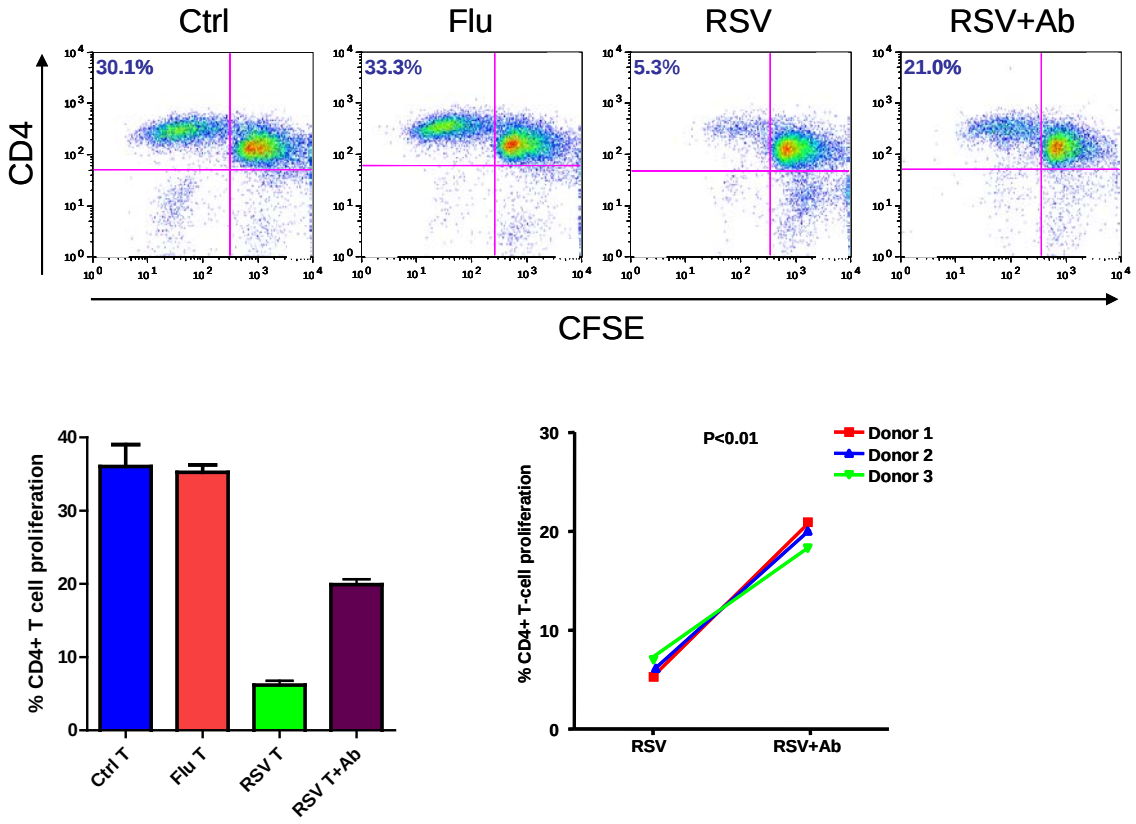


Figure 3.16: G1 T cell partially lost suppressive function upon PD-L1/PD-1 blockade. G1 T cells from PD-L1/PD-1 antibody treated RSV-DC co-culture partially lost their inhibitor function to suppress CD4+ T cell allo-proliferation. Bar graph summarizes data from n=3 independent experiments (RSV-RSV+Ab $p<0.01$, Ctrl-RSV+Ab $p<0.05$).

To confirm these observations, we performed a phenotypical analysis of the G1 T cell populations. As shown in Figure 3.17, PD-L1/PD-1 antibody blockade inhibited the

phenotypic development of Tregs as the expression of CD25, FoxP3, CTLA-4, PD-1 and Ebi-3 was comparable to the control.

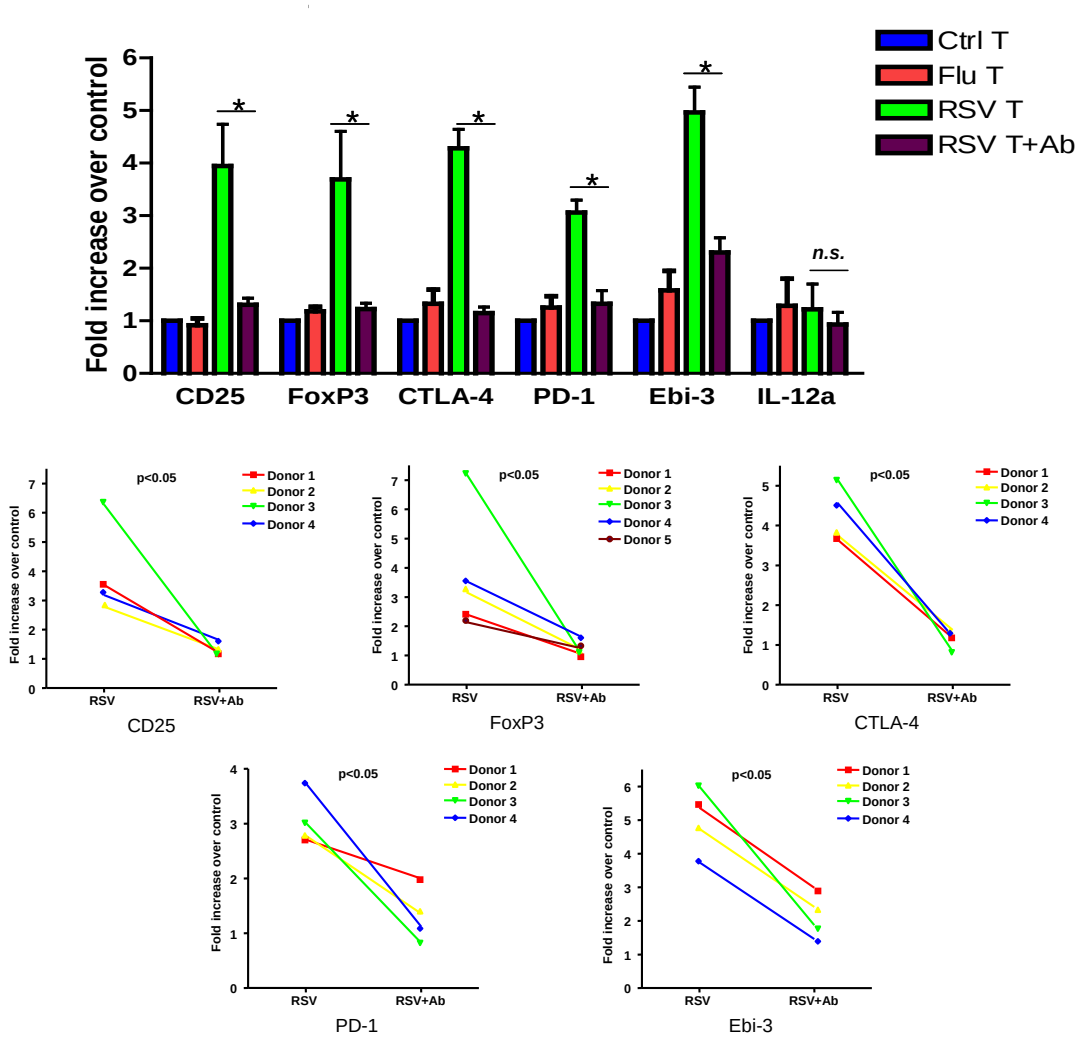


Figure 3.17: Analysis of Treg markers upon PD-L1/PD-1 blockade by RT-PCR. CD25, FoxP3, CTLA-4, PD-1, Ebi-3 and IL-12a expression in mRNA level upon PD-L1/PD-1 blockade. Antibody blockade significantly reduce the expression of Treg markers in RSV-G1 T cells. Bar graph summarizes n=4 or 5 independent experiments.

Since PD-L1/PD-1 blockade inhibited the development of functional Tregs, we also tested whether anergy in the G0 population was reversed. The expression of Cblb and Itch in G0-T cells was assessed by Real-time PCR. As shown in Figure 3.18, the expression of T cell anergy markers restored to the basal level upon PD-L1/PD-1

blockade, indicating that in the absence of Treg induction the G0 population was no longer anergized. Collectively, these data indicate that the rapid induction of PD-L1 on mDCs following RSV exposure is required for the expansion of functional regulatory T cells.

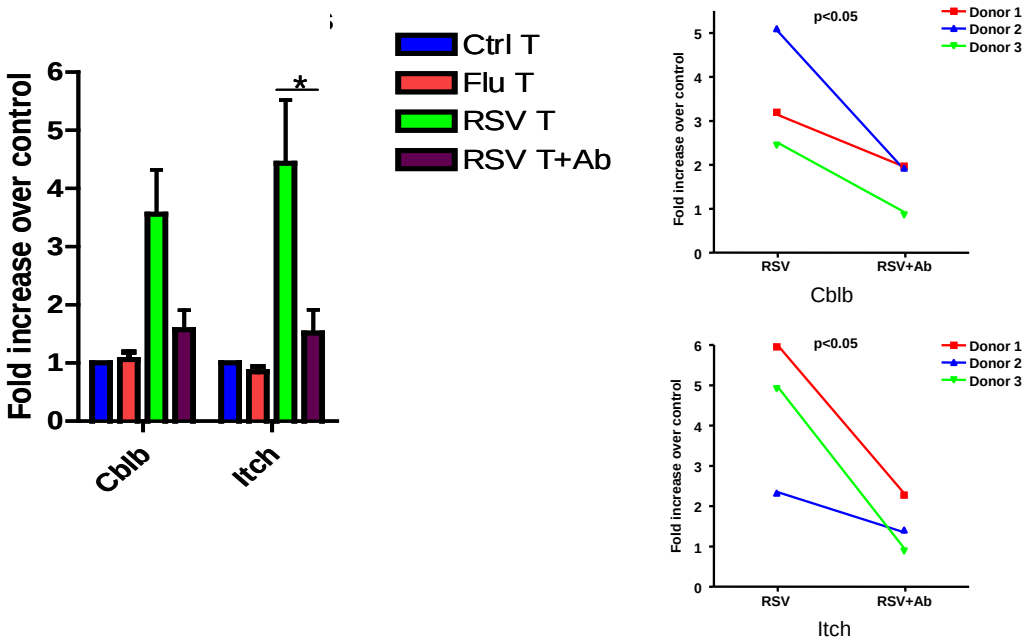


Figure 3.18: Analysis of T cell anergy markers Cblb and Itch expression in mRNA level upon PD-L1/PD-1 antibody blockade, upregulated Cblb and Itch expression restored to a level comparable to control or Flu T cells. Bar graph summarizes n=3 independent experiments.

RSV Exposed Mouse BM-DCs are Tolerogenic and are Able to Induce Treg Expansion

RSV-DCs demonstrate potent tolerogenic function through the expansion of Tregs. The mouse system offers an opportunity to test these tolerogenic DCs' function *in vivo*, so we first tested whether RSV-murine-DCs have similar tolerogenic phenotype and function. Following 7 days' culture of BALB/c bone marrow cells in the presence of murine GM-CSF and IL-4, we examined the differentiation efficiency of DCs. DC surface markers MHC class II, CD80 and CD11c were examined by flow cytometry. As

shown in Figure 3.19, in the 84.3% live cells, around 73.4% are class II+/CD11c+ cells. This indicates that around 84.3%X73.4%=61.9% of all cells in the culture are DCs. Similar percentage is observed in cells treated with Flu or RSV for 18h (date not shown). Because our human DC study indicates that PD-L1/PD-1 signaling plays an important role in tolerogenic function, we examined the expression of PD-L1 in all three conditions. As shown in Figure 3.20, RSV-mouse DCs showed a significant upregulation of PD-L1 expression when compared to control or Flu conditions, consistent with the human study.

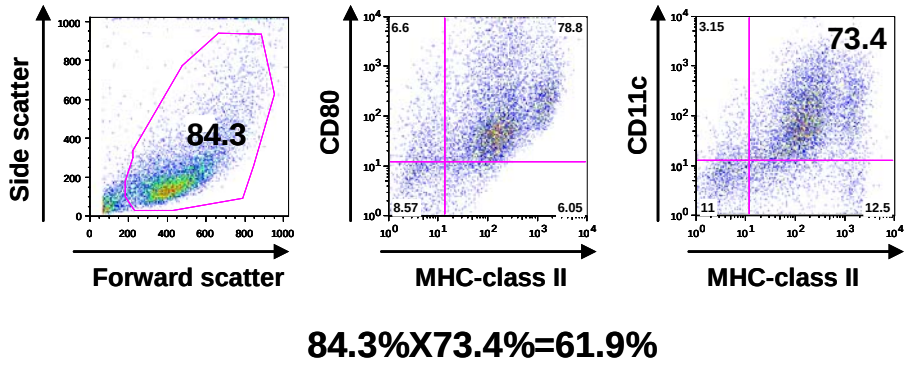


Figure 3.19: The differentiation of DCs from mouse bone marrow cells. More than 60% of cells in the culture are MHC-II+/CD11c+ DCs.

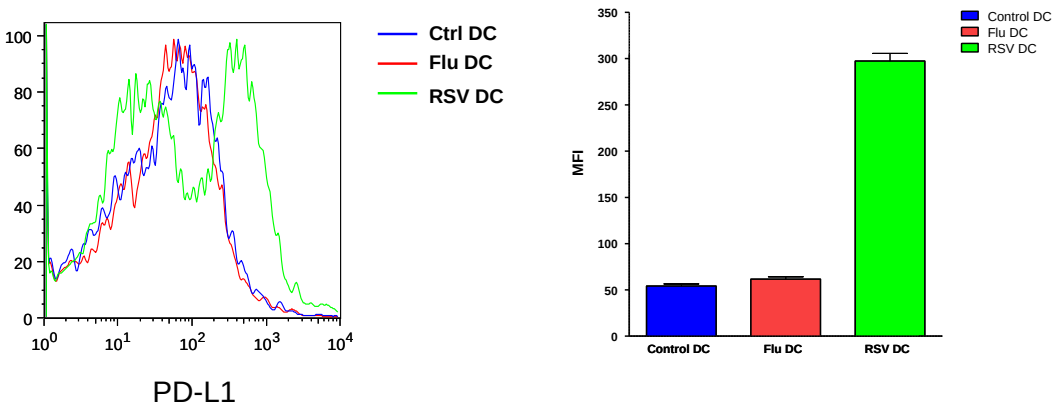


Figure 3.20: The expression of PD-L1 in mouse DCs. Upon exposure for 18h, RSV-DCs show a significant upregulation of PD-L1 on their surface, bar graph summarizes N=5 independent replicates ($p<0.05$).

To analyze the capacity of mouse DCs to prime mouse CD4+ T cell alloproliferation, BALB/c BM-DCs were co-cultured with CFSE-labeled C57BL/6 CD4+ T cells at the ratio of DC:T cell=1:20 for 6 days. At Day 6, cells were stained with anti-mouse CD4 antibody and analyzed the proliferation by CFSE dilution. As shown in Figure 3.21, in contrast to control or Flu DCs, RSV exposed DCs significantly inhibited the alloproliferation of CD4+ T cells, similar to what is observed in human study. These indicate that RSV-exposure renders mouse DCs incapable of driving alloproliferation.

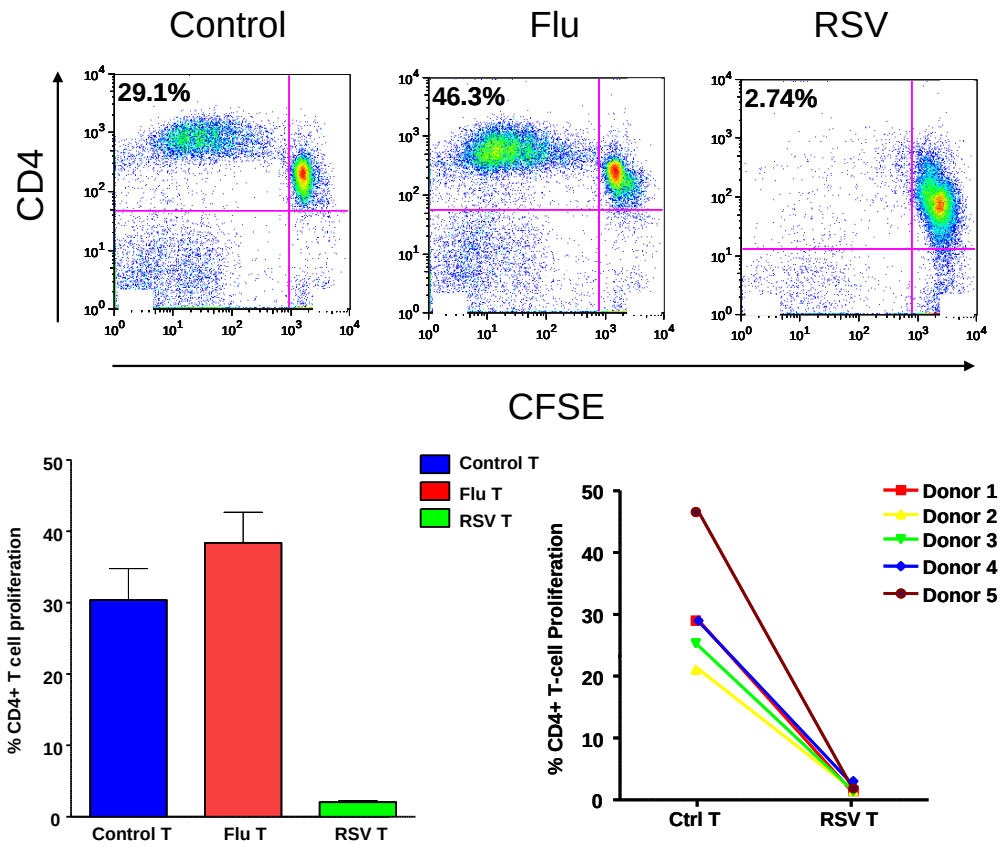


Figure 3.21: Allogeneic proliferation of C57BL/6 T cells upon stimulation with control, Flu or RSV-exposed BALB/c BM-DCs. RSV-DCs are able to significantly inhibit the alloproliferation of CD4+ T cells when compared to control or Flu-DCs. Bar graph and line graph summarize N=5 independent replicates ($p<0.05$).

In our observation from human studies, another feature of RSV-DCs is that they are potent inducers of regulatory T cell expansion. To examine the percentage of Tregs in the DC-T cell co-culture, we analyzed the expression of CD4, CD25 and FoxP3 using a Mouse Treg Staining Kit from eBiosciences. As shown in Figure 3.22, RSV-T cells contain significantly more CD4+/CD25+/FoxP3+ Tregs when compared to control or Flu-T cells. This indicates that RSV-mouse DCs are able to induce the expansion of Tregs, similar to the observation in the human study.

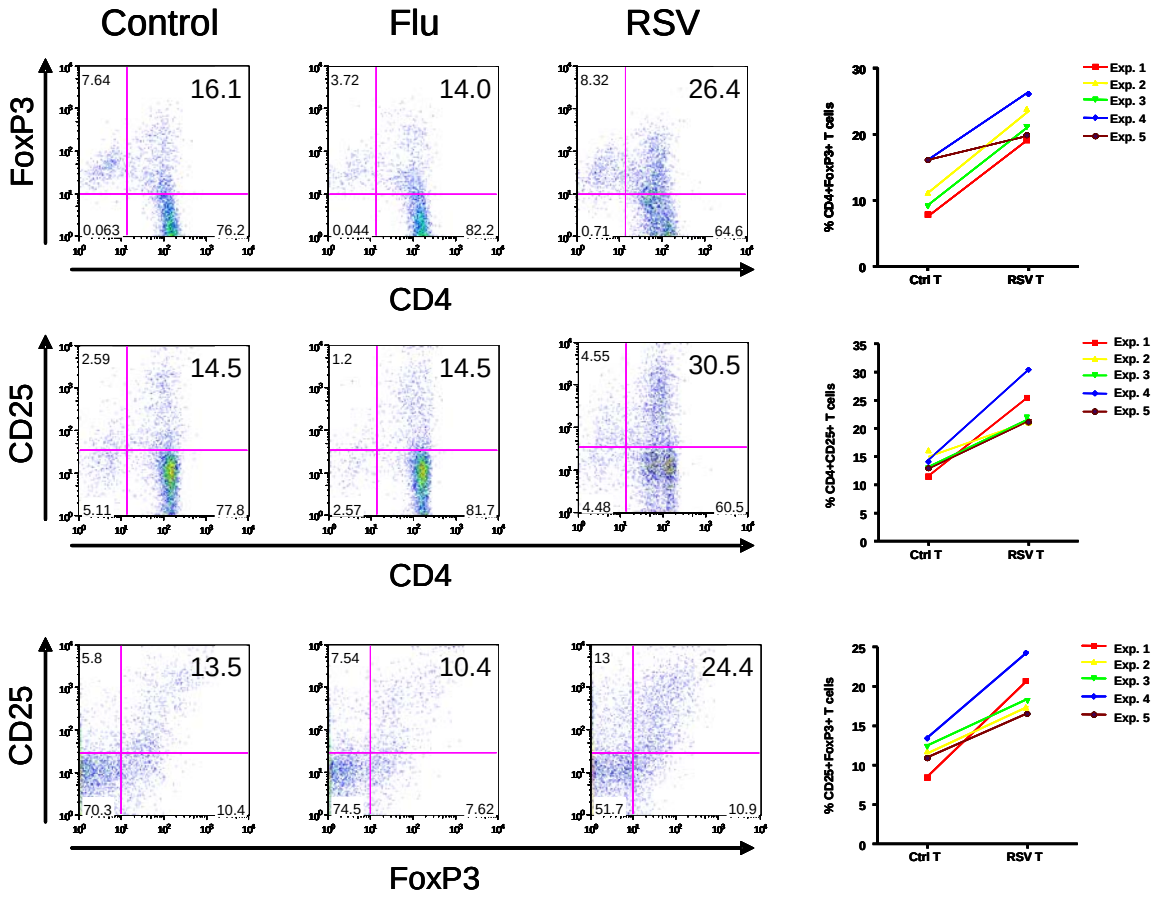


Figure 3.22: Treg populations in control, Flu and RSV conditions. Compared to control or Flu T cells, RSV T cells contain more CD4+CD25+FoxP3+ cells, indicating the expansion of Tregs in RSV condition. Line graph summarizes N=5 independent replicates ($p<0.05$).

In summary, RSV exposed mouse BM-DCs have a similar phenotype to human tolerogenic DCs, which are characterized by the inhibition of CD4+ T cell alloproliferation and the expansion of CD4+CD25+FoxP3+ Tregs.

Discussion

Tregs represent an important attenuating arm of the adaptive immune response. The potency of Treg suppression ensures that tolerance to self-antigens is maintained. Given this functional potency, it is no surprise that pathogens have evolved mechanisms to induce, recruit or expand Tregs in order to shut off host immune activation. For example, *Leishmania* infection leads to induction of Treg cells that help to maintain the balance of the host immune response against the pathogen and the pathogen's persistence in the host. Depletion of Treg cells leads to clearance of *Leishmania* in the host (212, 213). Treg cells also play an important role in chronic viral infections. They can be induced to subdue the antiviral immune response, which allows for persistent viral infection. This is demonstrated during hepatitis C virus (HCV) infection in humans and chimps (214) and during Friend leukemia virus infection in mice (215). Recently, Tregs have been found to be enriched following RSV infection in mouse (198). These studies led us to investigate the role of Tregs in RSV mediated suppression of T cell activation *in vitro*. We demonstrated that RSV DC primed T cells showed an increased proportion of $CD25^{hi}$/$FoxP3^{hi}$/CTLA-4^{hi}/GITR+ T cells. Further investigation indicated that functionaly potent Tregs exist in RSV-G1 T cell population of RSV DC esposed cells. The expansion of Tregs by RSV-DCs helps to explain why very few RSV-DCs are able to suppress the ability of unexposed DCs to drive MLR. In our model, RSV-DCs

propagate tolerogenic signals through expansion of Tregs. These Tregs are capable of inducing anergy in naïve and effector T cells. Tregs may then confer infectious tolerance to convert unexposed DCs to a tolerogenic state.

Previous studies indicated that in the presence of TGF-beta, PD-L1 signaling regulates the generation of adaptive FoxP3+CD4+ Tregs both *in vitro* and *in vivo* (187, 188). Blockade of the PD-1/PD-L1 pathway abrogates Treg-mediated immunoregulation in graft-versus-host disease and skin transplant models, suggesting that the PD-1/PD-L1 pathway is required for Treg suppression of the alloreactive responses (216). In our study, we further demonstrated an important role of PD-L1/PD-1 signaling in RSV-DC mediated induction of Treg expansion. Upon blockade of PD-L1/PD-1 signaling, RSV-G1 T cells are not longer able to suppress MLR effectively and Treg marker expression was downregulated. These observations point to a specific adjuvant based approach to the design of an effective RSV vaccine strategy. PD-L1/PD-1 signaling is required for RSV-DC mediated inhibition of T cell response through expansion of Tregs, suggesting that targeting PD-L1/PD-1 may be an effective approach to boost T cell response against RSV infection, similar to what is observed in HIV and LCMV infection (80, 211).

Lastly, we found that RSV exposure renders mouse BM-DCs tolerogenic characterized by inhibition of allogeneic CD4 T cell proliferation and upregulation of inhibitory PD-L1 molecule. Much like their human counterparts, these RSV-mouse-DCs are able to expand a population of CD4+CD25+FoxP3+ regulatory T cells. These mouse studies not only validated our observation in human cells, more importantly, they also open up the possibility of studying the effect of tolerogenic DCs *in vivo*. The molecular

basis of tolerence induced by RSV may form the foundation for novel therapys for the treatment of autoimmune diseases and organ transplantion.

CHAPTER FOUR

Conclusions and Future Studies

In this dissertation, I demonstrated three novel findings regarding how dominant immune tolerance is induced by RSV. First, RSV exposure polarizes DC maturation toward a tolerogenic state. These DCs secrete high amount of IL-10 and display upregulated surface immune inhibitory molecules. A striking discovery is that RSV-DCs not only themselves can suppress T cell alloproliferation, but they also are able to suppress other un-infected DCs' T cell priming capacity. A mechanistic study further demonstrates that autocrine IL-10 is required for RSV-mediated DC tolerogenic conversion and PD-L1/PD-1 interaction is required for tolerogenic DC function. To quatitatively evaluate the tolerogenic potency of RSV-DCs, we did a direct comparison of RSV-DCs with other known pharmacologically induced tolerogenic DCs and demonstrate that RSV-DCs are far more tolerogenic than pharmaco-DCs in their abilities to suppress T cell alloproliferation.

Secondly, I found that RSV-DCs propagate their tolerogenic signalings through the expansion of regulatory T cells. RSV-T cells contain a higher percentage of CD25+/FoxP3+/CTLA-4+/GITR+ Tregs. These Tregs exist mainly in the G1 (first generation of proliferation) population and they are able to inhibit T cell alloproliferation *in trans.* Furthermore, these Tregs function to induce conventional CD4+ T cell anergy. These anergized cells are unresponsive to a strong secondary re-stimulation, secrete less T cell effector cytokine IL-2 and express upregulated T cell anergy specific markers. A subsequent study indicates that PD-L1/PD-1 signaling is required for Treg expansion and

anergy induction, blocking PD-L1/PD-1 signaling partially restores T cell alloproliferative capacity.

Lastly, I demonstrated that RSV works similarly in mouse cells to induce DC tolerogenic and Treg expansion. Based on these findings, subsequent work can be done to evaluate the function of RSV-induced tolerogenic DC *in vivo.* There are several cucirmstances in which inhibition of antigen-specific T cell activation is desired, such as autoimmune diseases and organ transplantation. Thus, RSV-DCs can be adoptively transferred into murine models of autoimmune diseases or organ transplantion to suppress antigen-specific immune activation. Hopefully, RSV protein based therapeutic strategies can be developed for the treatment of such diseases.

Based on the above findings, I proposed a model of RSV-mediated immune suppression (Figure 4.1). RSV infection of respiratory mucosa induces secretion of chemokines by the mucosal epithelial cells to attract immature DCs to migarate to the site of infection. At the respiratory mucosa, RSV exposure induces DCs to secrete large amount of IL-10. Autocrine IL-10 renders DC tolerogenic and upregulates surface immune inhibitory molecules, such as ILTs and PD-L1. RSV-DCs then migrate to the local lymphoid organs where they encounter naïve T cells. Interaction of PD-L1 on RSV-DCs and PD-1 on Tregs triggers the expansion of Tregs. These functional potent Tregs in turn inhibit not only T cells that directly interact with them, but also inhibit other un-infected DCs T cell priming capacity.

Taken together, these studies demonstrate how RSV subverts host adaptive immune response by induction of immune suppressive tolerogenic DCs and regulatory T cells. These findings will contribute to current understanding of tolerogenic DC biology

and can be further exploited to develop cell-based immune suppressive agents for the treatment of autoimmune diseases and organ transplant rejection.

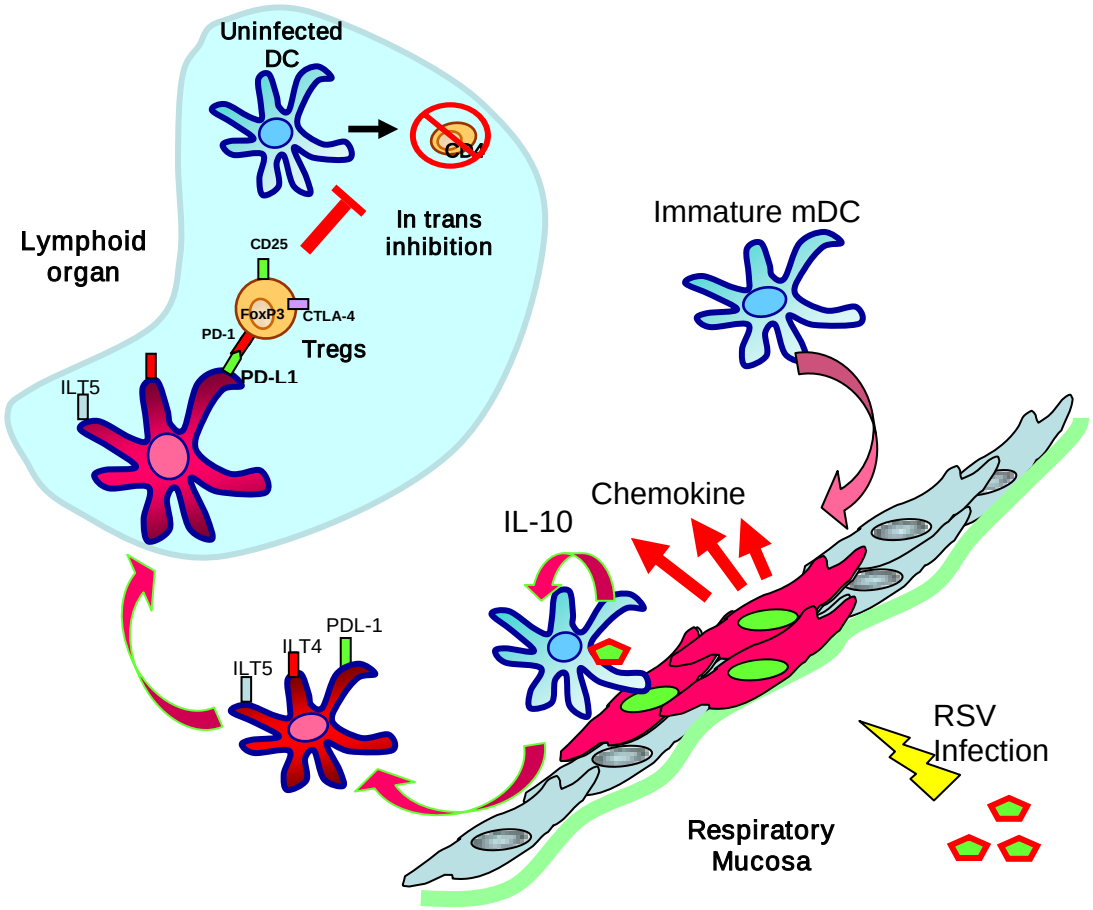


Figure 4.1: Model of RSV-mediated subvertion of host adaptive immune response.

APPENDIX

APPENDIX

Bench Protocols

Purification of Human Myeloid DCs from Peripheral Blood

1. Purchase one bag of "buffy coat" (leukocyte enriched peripheral blood) from local blood bank (Carter Blood Care, Bedford TX).

2. Dilute 30ml of buffy coat in 150ml of EDTA-PBS, making a total volume of 180ml.

3. Prepare 6 50ml Falcon tubes, each add 15ml of Ficoll.

4. Carefully lay the diluted blood onto the top of the Ficoll solution, and then spin down 2000rpm for 15min without brake.

5. After the density centrifugation, collect the buffy middle layer into 3 50ml tubes, wash off the excess Ficoll by adding EDTA-PBS to the maximum volume of the tube and spin down 1300rpm for 5min.

6. Discard the supernatant, resuspend and wash the pellet by adding EDTA-PBS to the maximum volume. Spin down 1000rpm for 5min to get ride of platelets.

7. Discard the supernatant, combine 3 tubes of PBMCs into 1 tube, and count the number of the cells.

8. Prepare cells in EDTA-PBS with 2% FCS at a concentration of $1X10^8$/ml.

9. Add antibody cocktail from commercially purchased human DC enrichment kit (Dynabeads, Invitrogen), antibody volume=cell number in million X 0.5 (ml). Mix cells with antibody well and incubate at 4°C for 20min.

10. During the antibody labeling, wash Dynabeads in the kit with EDTA-PBS-2%FCS. Beads volume=antibody volume X 5. Add same volume of EDTA-PBS-2%FCS, mix well, put the tube into a magnetic field for 3min to pull aside the beads and discard the supernatant. Repeat the beads washing once. After 20min's antibody binding, wash off excess antibody by adding EDTA-PBS-2%FCS to maximum volume followed by spin down at 1200rpm for 5min.

11. Resuspend the cells, bring the cell concentration to $5X10^7$/ml and then mix the beads with the cell suspension. Incubate cells with beads on a cold-room rotor for 15min.

12. After 15min incubation, add EDTA-PBS-2%FCS to the volume of 50ml, then aliquot into 5ml tube for subsequent 3min magnet depletion. All antibody-beads conjugated cells will be pull aside in the magnet field and discarded.

13. Count the unbound cells, stain them with antibodies: Lin-1-FITC, CD123-PE, HLA-DR-quantum red and CD11c-APC for 30min on ice.

14. Wash off excess antibody; resuspend cells in 2ml EDTA-PBS-2%FCS for FACS sorting.

15. On a FACS-Aria cell sorter, gate and collect LINEAGE-neg, HLA-DR+, CD11c+, CD123- cells. The purity of these mDCs is no less than 97%.

Purification of Human CD4+ T Cells and Stain with CFSE

1. Collect PMBCs as described in the last section and suspend them at a concentration of $5X10^7$/ml in PBS-2%FCS.

2. Add EasySep Negative Selection Human CD4+ T cell Enrichment Cocktail at a concentration of 50ul/ml cells, mix well, incubate at room temperature for 10min.

3. Mix EasySep Magnetic Nanoparticles to ensure they are in a uniform suspension by vigorously pipetting up and down for more than 5 times. Add nanoparticles to the antibody-labeled cells at a concentration of 50ul/ml cells, mix well, incubate at room temperature for 10min.

4. After 10min beads incubation, add 25% more PBS-2%FCS to the cell suspension and then aliquot into 5ml tubes at 2ml/tube.

5. Apply magnet field to the tubes for 5min to pull aside beads-bound cells.

6. Collect unbound cells and count the purified CD4+ T cell number.

7. For CFSE staining, wash the enriched cells with excess plain PBS, spin down at 1200rpm for 5min.

8. Discard supernatant, resuspend the cells in PBS-0.01%FCS at the concentration of 2-5 million/ml. Keep the cells in hood at room temperature.
9. In another sterile tube, add equal volume of 0PBS-0.01%FCS and dilute CFSE dye to a concentration of 2.5-4.0 uM.

10. Mix tubes of liquid from step 8 and 9 together so that the final concentration is 1.25-2.0 uM.

11. Stain the cells undisturbed at room temperature for 7-10 min. Stop the reaction by adding 20% volume of FCS.

12. Spin down the cells at 1200rpm for 5min, resuspend the cells in cRPMI medium for subsequent experiments.

Purification of Total mRNAs from Cells

1. Spin down cells at 1200rpm for 5min to remove the supernatant. Resuspend the cell pellet in 350ul cell lysis buffer (RLT buffer from QIAGEN RNeasy Micro Kit plus 1% of beta-Mercaptoethanol).

2. Add 350ul of 70% ethanol to the homogenized lysate and mix well by pipetting.

3. Apply the sample, including any precipitate that may have formed, to an RNeasy MinElute Spin Column in a 2ml collection tube. Close the tube gently, centrifuge for 15s at 10,000rpm. Discard the flow through.

4. Add 350ul buffer RW1 to the RNeasy MinElute Spin Column. Centrifuge for 15s at 10,000rpm to wash the column. Discard the flow through.

5. Add 10ul DNase I stock solution to 70ul Buffer RDD. Mix well by gently inverting the tube. Then pipet DNase I incubation mix (80ul) directly onto the RNeasy MinElute silica-gel membrane, and place on benchtop at room temperature for 15min.

6. Pipet 350ul Buffer RW1 into the RNeasy MinElute Spin Column, and centrifuge for 15s at 10,000rpm. Discard the flow-through and the collection tube.

7. Transfer the RNeasy MinElute Spin Column into a new 2ml collection tube. Pipet 500ul Buffer RPE onto the RNeasy MinElute Spin Column. Centrifuge for 15s at 10,000 to wash the column. Discard the flow-through.

8. Add 500ul of 80% ethanol to the RNeasy MinElute Spin Column. Centrifuge for 2min at 10,000 to dry the silica-gel membrane. Discard the flow-through and the collection tube.

9. Transfer the RNeasy MinElute Spin Column into a new 2ml collection tube. Open the cap of the spin column, and the centrifuge for 13,000rpm for 5min. Discard the flow-through and the collection tube.

10. To elute the RNA, transfer the spin column to a new 1.5ml collection tube. Pipet 20ul RNase-free water directly onto the center of the silica-gel membrane. Centrifuge for 1min at 13,000rpm to elute.

Reverse Transcript Total RNAs into cDNAs

1. Prepare 2X RT master using components supplied in High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems).

Component	Volume (ul)
10X RT Buffer	2.0
25X dNTP Mix (100mM)	0.8
10X Random Primers	2.0
MultiScribe™ Reverse Transcriptase	1.0
RNase Inhibitor	1.0
Nuclease-free H_2O	3.2
Total per Reaction	10.0

2. Mix 10ul of 2X RT master mix with 10ul of RNA sample. Avoid any bubble.

3. Load the tubes into a PCR machine. The program set is as following.

	Step 1	Step 2	Step 3	Step 4
Temperature (C)	25	37	85	4
Time (Min)	10	120	5	Forever

4. Measure the concentration of resultant cDNAs.

Real-time PCR Analysis of Gene Expression Profile

1. Prepare components for Real-time PCR reaction. Primers and probes for specific genes and TaqMan 2X Universal PCR Master Mix are purchased from Applied Biosystems. Load components into each well in a 96-Well Optical Reaction Plate (Applied Biosystems).

Component	Volume (ul)
TaqMan 2X Master Mix	10.0
cDNA	1.5
Primers and Probes	1.0
Nuclease-free H_2O	7.5
Total Per Reaction	20

2. Briefly centrifuge the 96-well reaction plate at 500-600rpm to homogenize the mixture and avoid bubble.

3. Run the sample plate in ABI PRISM® 7900HT Sequence Detection System, set GAPDH as endogenous control. After Real-time PCR is finished, analyze and export the resultant data by SDS RQ manager software.

4. Data are transformed and presented by the following formula:

   Relative Expression= $1.8^{(\text{CT endogenous control-CT gene of interest})}$

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