UNIVERSITY OF CALIFORNIA, SAN DIEGO

Characterization of a New Suppressor Myeloid Dendritic Cell Population in Pediatrics

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by

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ABSTRACT OF THE THESIS

Characterization of a New Suppressor Myeloid Dendritic Cell Population in Pediatrics

by

Robert Lee Padilla

Master of Science in Biology

University of California, San Diego, 2016

Professor Alessandra Franco, Chair
Professor Cornelis Murre, Co-Chair

Recently studies have shown that in subjects with acute Kawasaki disease (KD), an acute pediatric vasculitis of the coronary artery, there exists a population of myeloid dendritic cells (mDCs) that have the ability to secrete the suppressive cytokine interleukin (IL)-10. Enumeration of the mDC and the regulatory T cell (Treg) populations from 3 cohorts of pediatric subjects: acute KD, acute pediatric febrile controls, and healthy children have been analyzed with an expanded panel of surface

markers. From these cohorts of subjects, it was found that a newly described population of tolerogenic mDC is present in circulation and is defined as being $CD11c^{+}CD11b^{+}CD14^{+}ILT\text{-}4^{+}CD4^{+}$ and by the expression of differential amounts of HLA-G. This newly described population of mDCs express the DCs maturation marker CD86, which is a T cell co-receptor. An *in vitro* assay plating with these newly found tolerogenic mDC population produced IL-10 in a dose dependent fashion when stimulated with scalar doses of purified fragments of the heavy chain constant region of immunoglobulin (Fc). These tolerogenic mDC did not exhibit properties that enable them to polarize T cells into peripherally induced regulatory T cells (iTreg), but the Fc stimulated tolerogenic mDC demonstrated the ability to suppress T helper 1 (Th1) differentiation as demonstrated by reduced levels of interferon (IFN)γ mRNA transcript in naïve T cell co-culture in vitro. In pediatric subjects these mDC express the adenosine 2A receptor ($A_{2A}R$) which induces intracellular cAMP production, which in turn can cause production of IL-10 in these tolerogenic cells. Expression of CD31 on these tolerogenic mDC can offer a possibility for these cells to directly suppress inflamed tissue by being able to bind to CD38 on the inflamed endothelial cells.

This study identifies a new population of tolerogenic mDC in pediatrics who appears to compensate for the lack of a fully developed adaptive immune system in these young children.

I. INTRODUCTION

Dendritic cells (DCs) play a vital role in connecting the adaptive and innate immune system by bringing foreign antigens (Ag's), presented as peptide fragments by major histocompatibility complexes (MHC), to the secondary lymphoid organs where they are presented to T cells. Immature DCs, which are unable to stimulate T cells, perform phagocytic and pinocytic functions where they sample their surroundings in order to detect foreign Ag's by innate receptors, such as toll-like receptors (TLRs) (Hemmi and Akira 2005). Once DCs recognize a foreign Ag's they receive a signal to mature, where the DC then upregulates expression of MHC II and co-stimulatory signals for T cell activation, such as CD86 and begins migrating to the secondary lymphoid organs (Banchereau and Steinman 1998, Merad, Sathe et al. 2013). T cells are primed by recognizing MHC peptide complexes via their T cell receptor (TCR) on the surface of these activated DCs in the lymph nodes (Birnbaum, Mendoza et al. 2014). Activation of DCs that are canonically pro-inflammatory secrete IL-12 which is a key cytokine in polarizing naive T cells to the T helper (Th) lineages and $CD8^+$ cytotoxic T cells (CTL), which acts to rid the body of infections or newly occurring neoplasms (Heufler, Koch et al. 1996). CD31, a platelet endothelial cell adhesion molecule is expressed by endothelial cells, DCs, T cells and other cells of the immune system and allows these cells to interact with vascular endothelial cells which express CD38 (Marelli-Berg, Clement et al. 2013).

The common myeloid dendritic cell progenitor, which arises from hematopoietic stem cells in the bone marrow can identified by expression of the adhesion protein

CD34, has the ability to differentiate into many lineages of effector DC cells such as plasmacytoid (p)DCs and mDCs. mDCs are defined by the expression of the integrin molecules CD11c and CD11b, and are made up of three subsets of mDCs including $CD14^{+}$, $CD14^{-}$, and a population of $CD141^{high}$ cells. The $CD141^{high}$ mDCs function in priming strong Th1 responses due to production of IFN-ß (Jongbloed, Kassianos et al. 2010). CD14 is a co-receptor for lipopolysaccharide is a lineage marker for monocytes, that are early DCs precursors, and its expression on the mDC population has been linked to subsets of mDC with tolerogenic capabilities. CD4 and CD8α/α expression on DCs are molecules that allow for further characterization of DC populations as they are uniquely defined on DC lineages (Vremec, Pooley et al. 2000). Populations of mucosal $CD14^{-}$ mDC have been shown to express CD8α/α (Cerovic, Houston et al. 2015).

In the human decidua during pregnancy and in the periphery of adults, a population of $CD14^{+}$ mDCs have been identified and have the ability to secrete IL-10 and were subsequently termed DC-10. These DC-10 cells were described as co-expressing high levels of the immunoglobulin-like transcript (ILT)4 receptor and the non-polymorphic minor HLA class I allele HLA-G (Gregori, Tomasoni et al. 2010, Amodio, Mugione et al. 2013).

These cells can also be found in circulation of healthy adults and function to polarize Treg into the Tr1 lineage. ILT-4 is closely related to the killer-cell inhibitory receptors (KIR) and functions to regulate the immune response by inhibiting natural killer (NK) cell responses by inhibiting the cytolytic functions of the NK cells (Liang, Ristich et al. 2008, Liu, Wang et al. 2014).

HLA-G is the preferential ligand for ILT-4 activation which functions by recruiting SHP-1 and SHP-2 phosphatases activating a IL-6 STAT-3 pathway causing a reduction in the major histocompatibility complex (MHC) class II expression (Wu and Horuzsko 2009).

Previously, a population of $CD14^{+}$ mDCs with a similar phenotype and the ability to secrete IL-10 have been identified in subjects with KD, a self-limited vasculitis of the coronary artery in children (Burns, Song et al. 2013). KD is the leading cause of acquired heart disease in children in the developed countries, as it leads to an immune-mediated destruction of the arterial wall and myocardium (Kawasaki, Kosaki et al. 1974). It has been previously reported that IL-10 secretion from Treg that recognize the heavy constant region of the immunoglobulin (Fc) is critical for the pediatric KD subjects to not develop arterial abnormalities (Franco, Shimizu et al. 2010).

Treg function to suppress inflammation and lessen the effects of the immune response through secretion of IL-10 and TGFß (Jutel, Akdis et al. 2003). The Treg populations in humans and mice is composed of naturally derived (n)Tregs that positively recognize self-antigen in the thymus, and peripherally induced (i)Tregs which polarize from canonically pro-inflammatory T cells such as Th1 and Th17 under repeated antigenic stimulation (Xing and Hogquist 2012).

Naive T cells are identified by the expression of the CD45RA an isoform of CD45 that is a part of the TCR**,** known as the common leukocyte antigen and is a tyrosine phosphatase that regulates signaling in T cells (Berard and Tough 2002). Naïve

T cells have the ability to differentiate into effector T cell lineages such as helper T cells (Th)1, Th2, Th17, and Tregs (Zhu, Yamane et al. 2010, Duhen, Duhen et al. 2012).

The adenosine 2A receptor ($A_{2A}R$) is a G protein-coupled receptor that has been identified as having immuno-suppressive functions and has been found to be expressed by leukocytes in the spleen and thymus. Stimulation of the $A_{2A}R$ causes activation of adenylyl cyclase which acts to convert adenosine into cAMP, which then accumulates in the cytoplasm of cells. cAMP produced from activation of the $A_{2A}R$ has the ability to induce production of the immuno-suppressive cytokine IL-10 (Linden and Cekic 2012).

Very little is currently known about the immune system of children. In my laboratory, techniques have been developed to characterize and expand cells from a small number of precursors, allowing us to better understand pediatric immunity.

In this study a new population of tolerogenic mDCs that secrete IL-10, defined by specific markers and high expression of the adenosine 2A receptor ($A_{2A}R$), have the ability to inhibit pro-inflammatory T cell differentiation have been identified in 3 cohorts of pediatric subjects that include acute KD, acute pediatric febrile controls and healthy children.

II. RESULTS

Characterization of mDC in pediatric subjects

From the peripheral blood mononuclear cells (PBMC) of 14 pediatric subjects with acute Kawasaki disease (KD), ages 6 months – 11.9 years of age, comprised of 8 males and 6 females, flow cytometry (FACS) analysis was used to characterize a mDC population defined as being $CD11c^+CD11b^+CD14^+$ILT-4$CD4^+$. The mDC population previously defined as $CD11c^+CD11b^+CD14^+$ comprised 10.4 – 91.9% of the canonical mDC $CD11c^+CD11b^+$ population with a median of 72.6%. These $CD14^+$ mDC were further defined by expression of CD4 and ILT-4 levels, where it was seen that the $CD14^+$ mDC population co-expressed ILT-4 and CD4 in the range of 51.6 – 94.2% with a median of 83.1%. High expression of the T cell co-receptor CD86 was also observed on the $CD14^+$ mDC population at 75.4 ± 6.6% (Fig 1). This $CD14^+$ mDC population in the 2 week to 2 month post treatment KD subjects does not wane after immunotherapy like the other inflammatory cells and they still contribute up to 28.1 – 56.8% with a median of 32.5% of the mDC population, where the majority expressed CD4, ILT-4, and CD86 (Fig 2).

To see if this mDC population is a property of KD or is present in other acute febrile illnesses in pediatrics, 5 subjects, ages 6 months to 9.1 years, comprised of 1 male and 4 females, with acute febrile illnesses (1 adenovirus, 1 juvenile arthritis, 3 viral syndromes) were studied and it was found that the $CD14^+$ mDC population is present in circulation at levels similar to what has been reported in the acute KD subjects. The $CD14^+$ mDC population made up to 24.9 – 80.4% of the canonical mDC population in

the febrile control subjects with a median of 71.4% (fig. 3). To identify if this population of $CD14^+$ mDC is only present in acute febrile illnesses or in young children as a whole, a cohort of 12 healthy children, ages 1 to 8.5 years of age, comprised of 4 males and 8 females, were analyzed and found to contain in circulation the 32.5 – 72.7% of the $CD14^+$ mDC with a median of 41.7%. In healthy children these cells have been found to be $CD86^+$ as has been described during acute inflammation (fig. 4).

Variable levels of HLA-G expression on $CD14^+$ mDC from 3 cohorts of pediatric subjects

The non-polymorphic minor HLA class I allele HLA-G is the preferential ligand for ILT-4. Binding of HLA-G to ILT-4 has been shown to cause suppression of NK activity and down regulate other parameters of inflammation. In the acute KD subjects HLA-G expression was seen on the $CD14^+ILT\text{-}4^+CD4^+$ mDC population at variable levels with a mean of 14.5% of $CD14^+$ mDC with an SEM of 5.3%. The febrile control subjects $CD14^+ILT\text{-}4^+CD4^+$ mDC expressed HLA-G at a mean of 8.0 with an SEM of 1.1%. In healthy children $CD14^+ILT\text{-}4^+CD4^+$ mDC expressed HLA-G at a mean of 8.6% with a SEM of 3.8. These results suggest that these cells are phenotypically different from the DC-10 population that has been previously described (fig 5.).

Further characterization of mDC

To further define the phenotype of the $CD14^+$ mDC population described in pediatric subjects in figure 1, I further characterized these cells using four markers used to identify DC lineages. CD34, a marker expressed by hematopoietic stem cells, was analyzed on this $CD14^+$ mDC population in order to identify if this DC lineage is an

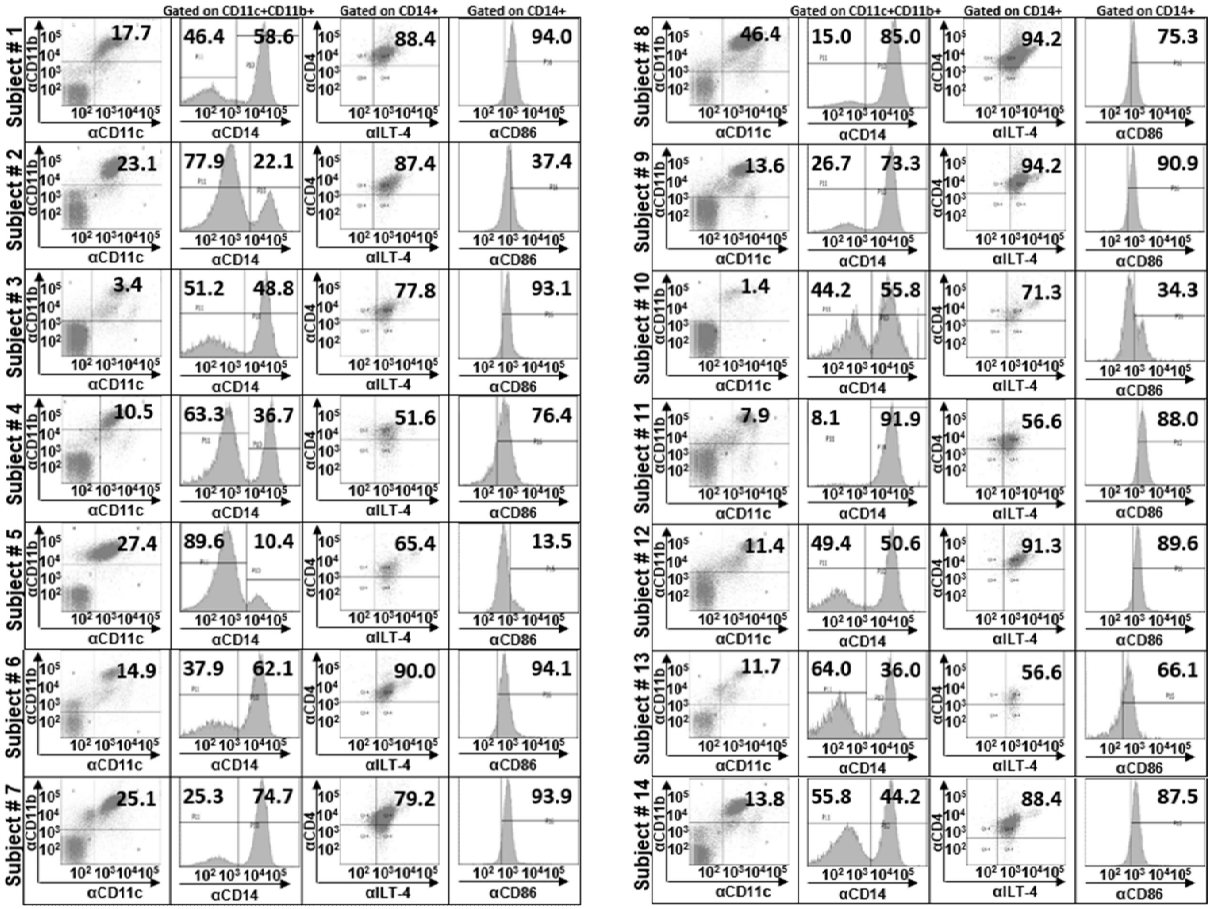

Figure 1. **Enumeration of a new subset of mDC were identified in 14 acute KD Subjects, 8 males and 6 females, with a median age of 2.3 years of mixed ethnicities.** PBMC were separated with Ficoll hypaque and stained with specific monoclonal antibodies (Methods section). Cells were gated on a $CD11c^+$ $CD11b^+$ double positive populations, canonical markers for mDC. From the $CD11c^+CD11b^+$ mDC gated populations we further gated CD14+ cells. $CD11c^+CD11b^+CD14^+$ were analyzed for ILT-4 and $CD4^+$ expression. $CD11c^+CD11b^+CD14^+CD4^+ILT\text{-}4^+$ activation and maturation stage has been defined by CD86 expression.

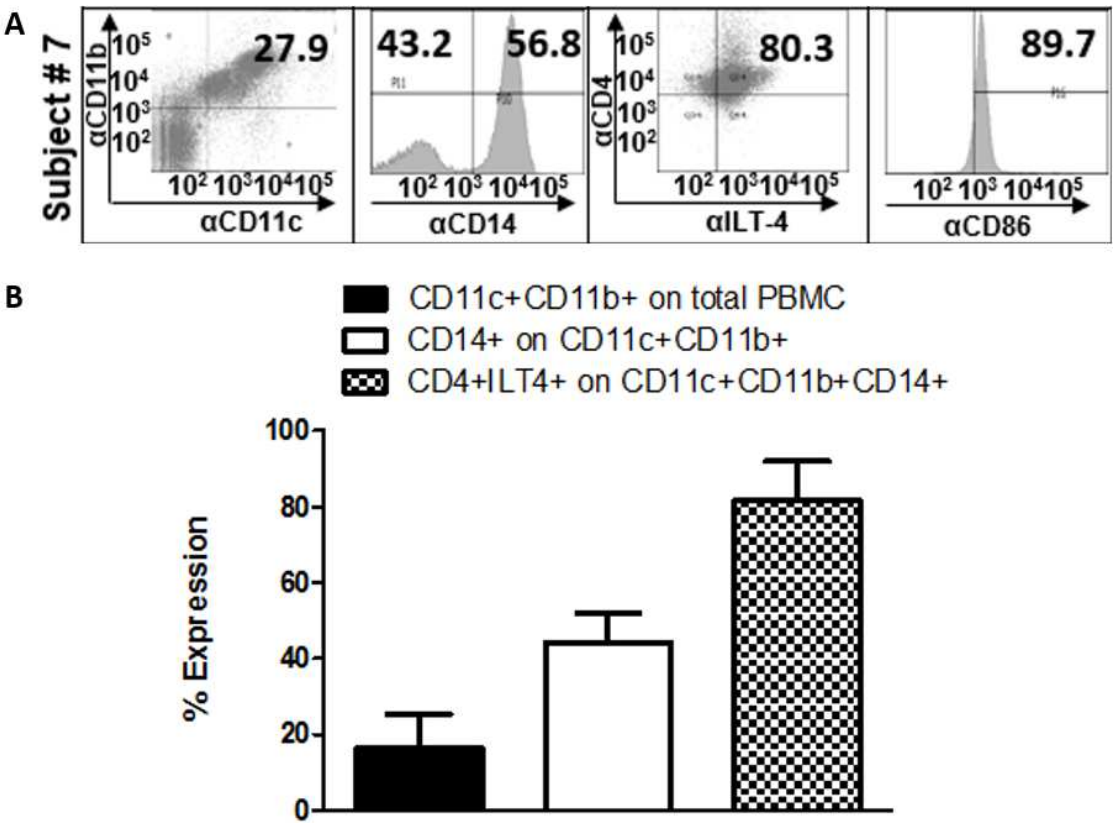

Figure 2. Enumeration of mDC population in 11 out of 14 subacute KD subjects previously studied in the acute phase of KD. Panel A. A representative subacute KD subject. Panel B) % total mDC, % CD14+ mDC, % CD14+ ILT-4+ CD4+ mDC. The data suggest that CD14+ ILT-4+ CD4+ mDC do not wane in the subacute phase after immunotherapy.

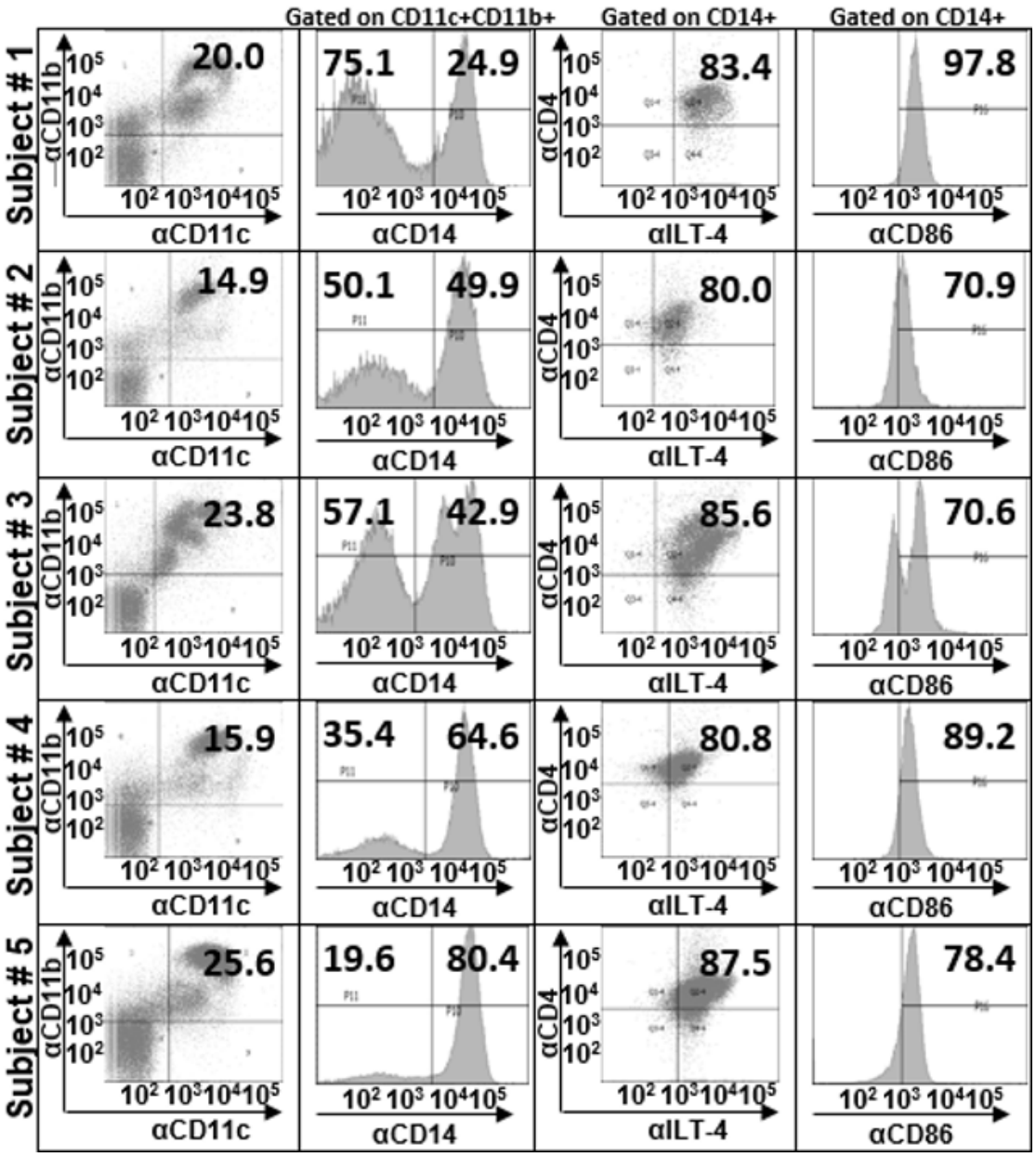

Figure 3. Enumeration of CD14+ ILT-4+ CD4+ mDC in PBMC from 5 pediatric febrile controls. Enumeration of the mDC population as identified in figure 1 was analyzed in 5 pediatric febrile controls, 1 male and 4 females, with a median age of 3.0 years of unknown ethnicities. Subject 1 was diagnosed with adenovirus, subject 2 was diagnosed with juvenile arthritis, and subjects 3-5 were diagnosed with viral syndromes. From the total PBMC's the enumerated mDC population constituted 10.2% of the total PBMC population.

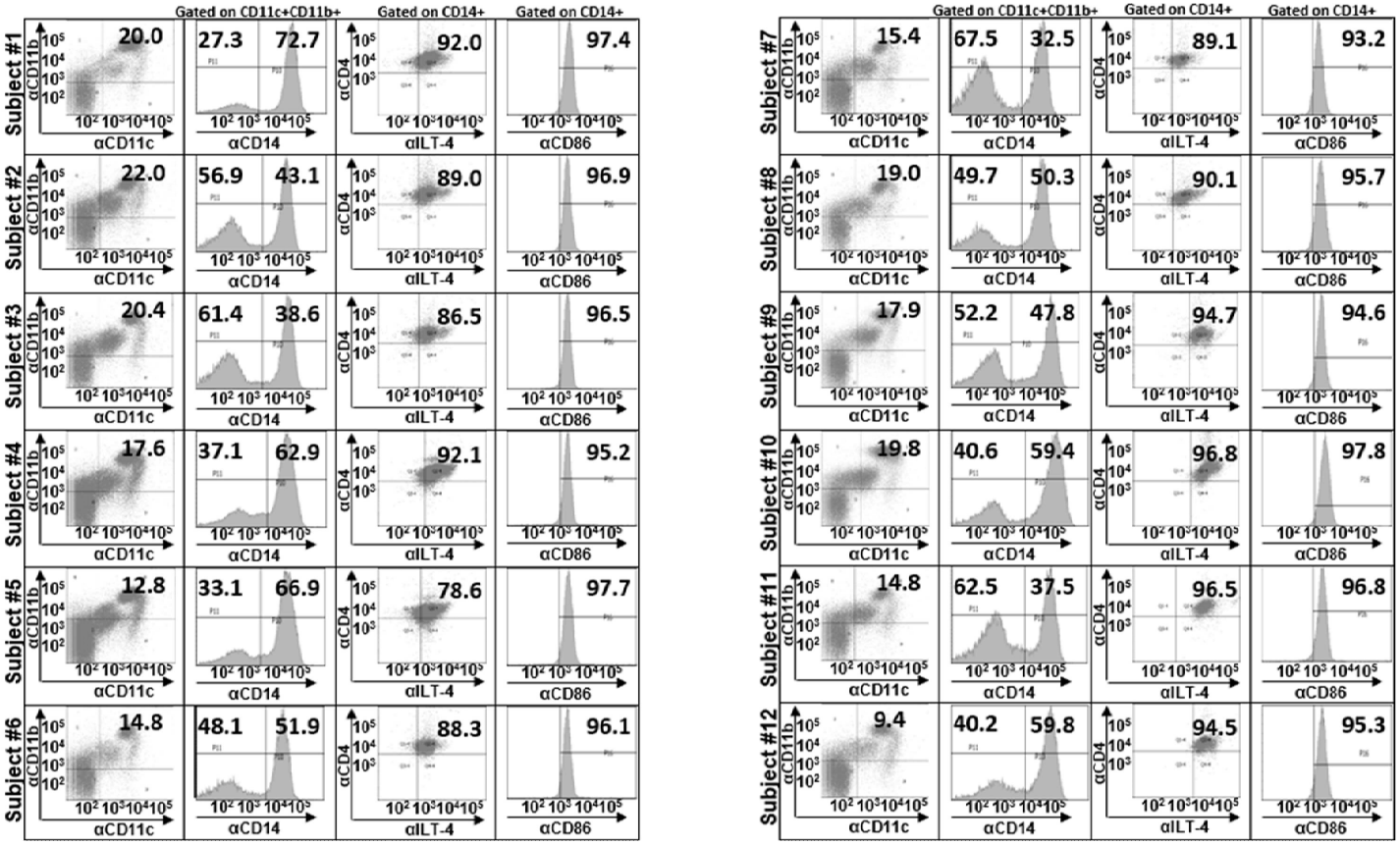

Figure 4. Enumeration of CD14+ ILT-4+ CD4+ mDC in 12 healthy children. Enumeration of the mDC population as identified in figure 1 was analyzed in 12 healthy children, 4 males and 8 females, with a median age of 3.8 years of mixed ethnicities. From the total PBMC's of these subjects the cells were gated on a $CD11c^+$ $CD11b^+$ population that includes the canonically described mDC. From the $CD11c^+CD11b^+$ mDC gated population we further gated these cells based on CD14 expression. The $CD11c^+CD11b^+CD14^+$ were then found to be ILT-4^+CD4^+. The $CD11c^+CD11b^+CD14^+CD4^+$ILT-$4^+$ population was found to be expressing CD86. From the healthy subjects the enumerated mDC population constituted 7.8% of the total mDC population.

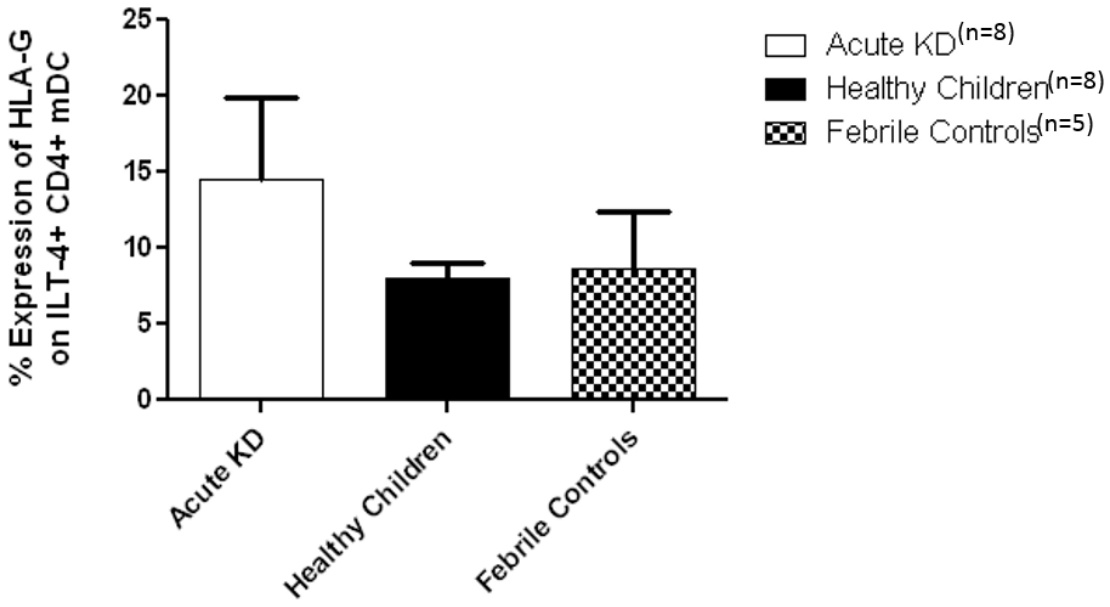

Figure 5. HLA-G expression on CD14+ ILT-4^{+}CD4^{+} mDCs from acute KD subjects (n=8), febrile controls (n=5) and healthy children (n=8). HLA-G+ expression has been measure in gated mDC populations. Data show the % HLA-G+ cells within the CD14+ ILT-4+ CD4+ mDC.

immature lineage of DC or if it is a mature lineage (Abbasian, Mahmud et al. 2006). The MHC I like molecule BDCA-1 (CD1c) functions to present glycolipids to naïve T cells and is expressed by canonical mDCs (Schroder, Melum et al. 2016). Expression of CD8α is commonly found on pDC and mucosal mDC, which function to prime naïve T cells to the Th1 lineage and CTL. Mucosal mDC populations lack CD14 expression, and CD8α expression is absent on the $CD14^+$ mDC population (Shortman and Heath 2010).

CD31, a transmembrane protein that has immunoreceptor tyrosine-based inhibitory motifs (ITIM) associated with it, expression has been identified on the surface of endothelial cells, B and T cell subsets, and is constitutively expressed on DCs (Baumann, Bailey et al. 2004). The ligands for CD31 include binding to itself in a homophilic manner, and CD38 which is a glycoprotein that is expressed in inflammatory cells, cardiac muscles, and throughout the endothelial side of arteries (Clement, Fornasa et al. 2014, Lee, Paudel et al. 2015)

The $CD14^+$ mDC population ($CD11c^+CD14^+CD4^+ILT\text{-}4^+$) were FACS sorted from 2 subacute KD subjects and then further characterized using ant-CD34, anti-CD31, anti-BDCA-1, and anti-CD8α monoclonal antibodies. The $CD14^+$ mDC from these subjects expressed high levels (90.2 – 98.7%) of CD31, had moderate expression (20.0 – 36.1%) of CD34, low expression (8.9 – 21.8%) of BDCA-1, and no expression (0.5 – 0.6%) of CD8α (Fig. 6).

$CD14^+ILT\text{-}4^+CD4^+$ mDC produced IL-10 when stimulated with purified Fc protein

The gating strategy for obtaining the tolerogenic mDC population from PBMC by FACS sorting is shown in panel A of figure 7 (Fig. 7A). 5.5 x 10^4 FACS sorted tolerogenic mDCs were plated in culture with scalar doses of purified Fc protein for 24 hours to stimulate their activation. Fc has been used to activate the FACS sorted mDC as the subjects respond very fast to doses of intravenous immunoglobulin (IVIG) therapy. The supernatant of this culture was then tested using ELISA and showed that IL-10 was secreted from the Fc stimulated $CD14^+ILT\text{-}4^+CD4^+$ mDC in a dose dependent manner. No IL-12 secretion was detected from Fc stimulated cell populations (Fig. 7B).

Characterization of regulatory T cells in pediatric subjects

In the lymph nodes DCs present Ag to naïve T cells which cause activation and maturation of the T cells, leading to proliferation and differentiation of the activated T cell (Erlebacher 2013). Naïve T cells express the surface marker CD45RA that enhances signaling through the T cell receptor which is downregulated after activation (Michie, McLean et al. 1992). During activation $CD4^+$ T cells have the ability to differentiate into one of the many helper T (Th) lineages including: Th1, Th2, Th17, follicular helper T cells (Tfh), and regulatory T cells (Treg) (Zhu, Yamane et al. 2010). pDC and the canonical BDCA-1 expressing mDC can cause naïve T cells to polarize to the Th1 lineage being effective during intracellular infections, $CD141^{High}$ mDC have a high potency for Th2 differentiation, but can also induce Th1 differentiation as well, and $CD14^+$ mDC can induce Th1 and follicular helper T cell differentiation (Durand and Segura 2015).

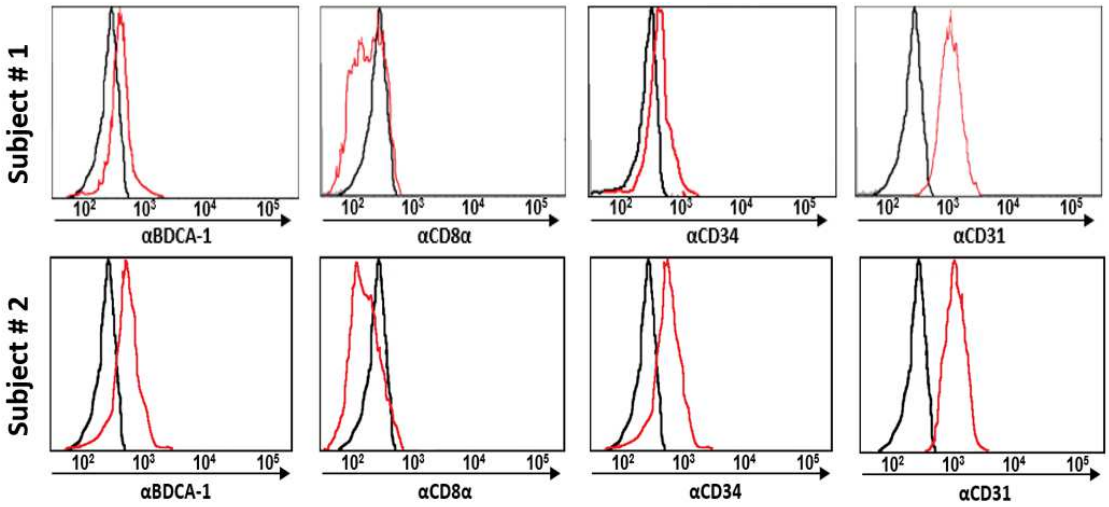

Figure 6. Phenotypical characterization of FACS sorted CD14+ ILT-4+ HLA-G+ mDC. From PBMC derived from 2 subacute KD subjects $CD14^{+}$ ILT-4+ CD4+ mDC have ben FACS sorted to evaluate BDCA-1, CD8α, CD34 and CD31 expression.

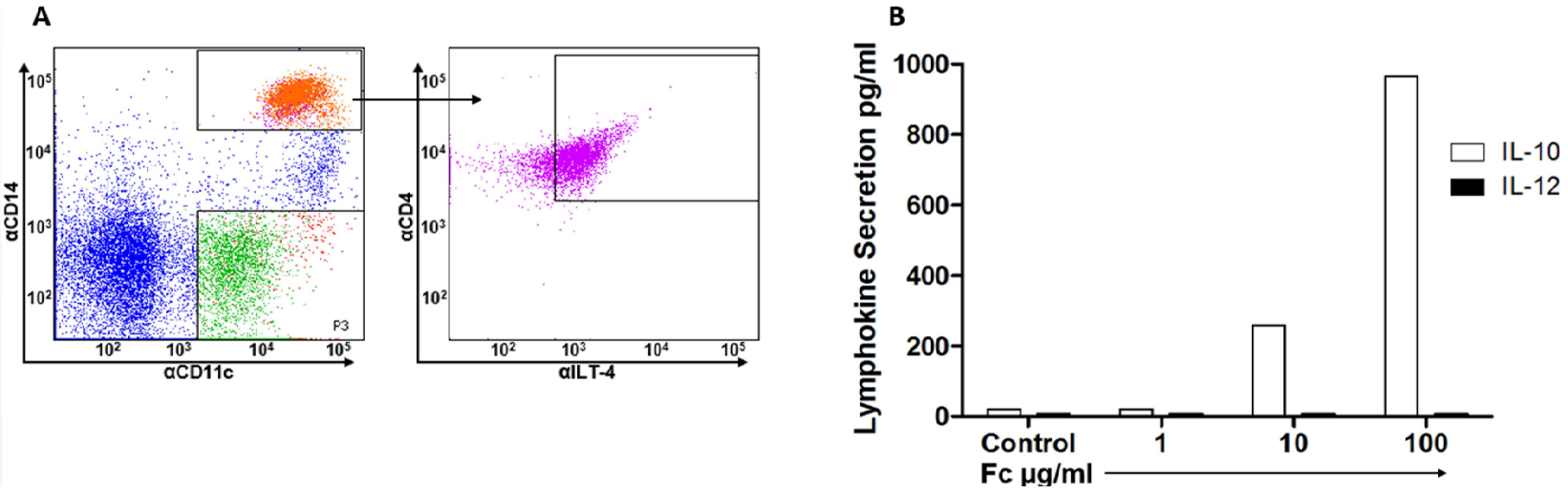

Figure 7. Lymphokine profile of CD14+ILT-4+ CD4+ FACS sorted mDC from a subacute KD subject. Panel A. gating strategy for FACS sorting of CD14+ ILT-4+ CD4+ mDC. **Panel B.** Lymphokine profile of CD14+ILT-4+ CD4+ stimulated for 24 hours with purified Fc fragments.

to maintain these regulatory features for long periods of time without the need for stimulation (Mills 2004, von Boehmer and Daniel 2013). Regulatory T cells (Treg), defined as being $CD4^{+}CD25^{High}$ and expressing the transcription factor FOXP3, play a critical role in suppressing the immune response during an infection (Fontenot, Gavin et al. 2003). Tregs perform their suppressive function by secreting IL-10 and transforming growth factor (TGF)ß. There are two types of Tregs that have been described in humans and mice: natural (n)Treg which are derived by recognition of self-antigen in the thymus, and induced (i)Treg that induce expression of FOXP3 in pro-inflammatory T cells in the periphery (Lin, Chen et al. 2013). IL-2 and IL-7 in the periphery can act in a co-stimulatory manner to induce FOXP3 expression in $CD4^{+}CD25^{-}$ cell population, thus producing iTregs that are $CD4^{+}CD25^{high}CD127^{+}$ from other pro-inflammatory T helper lineages (Long and Buckner 2008).

From the same cohort of 14 pediatric subjects with acute KD in figure 1 nTreg and iTreg were enumerated with specific monoclonal antibodies using FACS analysis. In these subjects the Treg population constituted 0.009 – 1.41 % of the total PBMC population. The majority of the Treg population seen in these subjects were nTreg as seen by the lack of Treg expressing CD127 (median = 86.8%) and lack of CD45RA expression (median = 54.7%). These nTreg cells in acute KD express HLA-DR (median = 62.6%), a canonical activation marker for human T cells (Fig. 8a).

For the febrile subjects studied in figure 3 the Treg population constituted 0.03 – 0.55% of the total PBMC. The majority of the subjects Treg cells were also all nTreg defined by lack of CD127 expression (median = 77.6%) and lack of CD45RA (median

= 68.2%). These nTreg expressed HLA-DR as those in the acute KD subjects (median = 45.4%) (Fig. 8B). From the cohort of healthy children studied in figure 4 the Treg population constituted 0.03 – 0.49% of the total PBMC, and all of the Treg's were nTreg for all the subjects as defined by lack of CD127 expression (median = 90.3%) and lack of CD45RA expression (median = 88.5%). These nTreg's also expressed HLA-DR like those in the acute KD subjects (median = 42.2%) (Fig. 8C).

Naïve T cell polarization

T cells are produced in the thymus and are considered naïve until they encounter an MHC-peptide complex which has high affinity for its T cell receptor (TCR). The MHC-peptide complex is presented by a professional antigen presenting (APC) cell such as a DC along with co-stimulatory molecules such as B7 which binds to CD28 on the naïve T cell. The naïve T cell that recognizes its specific Ag in an MHC-peptide complex signals through the TCR which involves the CD3 complex. Naïve T cells are distinguished from activated T cells by the expression of CD45RA, CCR7, and CD62L which are all down regulated in activated effector T cells (Berard and Tough 2002).

Helper T cells (Th) are the main effector T cell lineages for $CD4^+$ expressing T cells. Th1 are polarized in the presence of IL-12 and function in a pro-inflammatory manner to rid the body of pathogens by production of IFNγ, TNFα and other pro-inflammatory cytokines. Development of Th2 requires IL-4 and function to fight off extracellular parasites by production of IL-4, IL-5, and IL-13. IL-23 is required for Th17 differentiation and is involved in enhancing the innate response through recruitment of innate cells and IL-17 induced production on ß-defensins and antimicrobial peptides

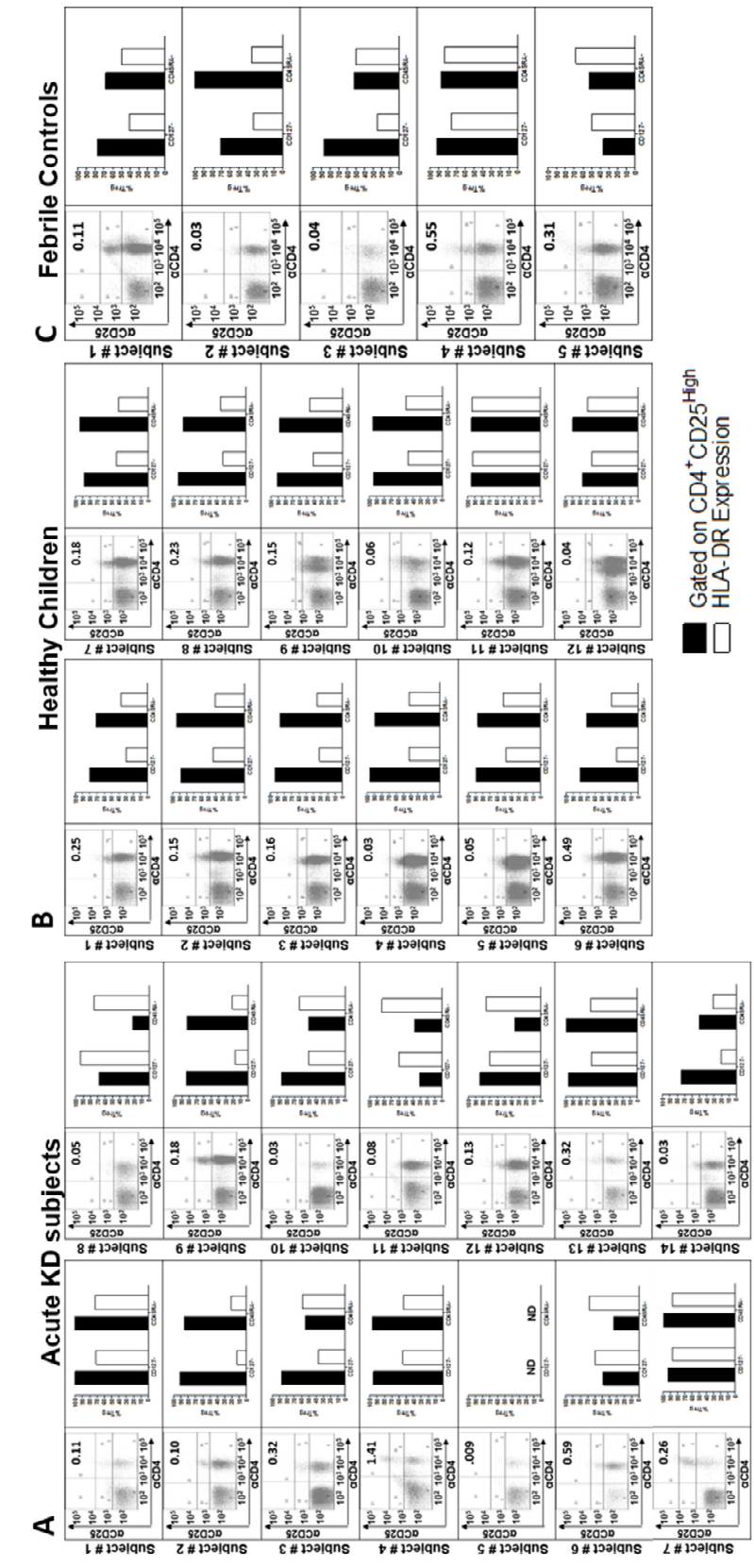

Figure 8. Enumeration of nTreg and iTreg in acute KD subjects (Panel A), febrile controls (panel B) and healthy children (Panel C). The regulatory T cell population was defined from a lymphocyte gate as being $CD4^{+}CD25^{High}$. From the $CD4^{+}CD25^{High}$ population CD45RA and CD127 expression were looked at to determine if the regulatory T cell populations were natural (nTreg) or inducible regulatory T cells (iTreg). $CD45RA^{-}$ and $CD127^{-}$ nTreg were studied for expression of HLA-DR to determine their activation.

(Femke Broere 2011). These effector T cells can develop into long lived memory T cells which reside in the lymph nodes waiting for a secondary exposure to their specific pathogen. Memory T cells have two lineages: one being central memory who do not perform effector functions and express CD45RO, CCR7, CD62L, and CD127, the other being effector memory who provide the specific T cell response and express CD45RO and lack CCR7 and CD62L (Femke Broere 2011).

To see how the tolerogenic mDC population affects the differentiation of $CD4^+$ T cells, naïve T cells ($CD4^+CD45RA^+CD127^-$) were FACS sorted along with the tolerogenic mDC population ($CD11c^+CD14^+ILT\text{-}4^+CD4^+$). The gating strategy for the naïve T cells included gating on the $CD4^+CD45RA^+$ population first and then gating on $CD4^+CD127^-$ in order to obtain the naïve T cells (Fig. 9a). The naïve T cells were then either plated alone or in a co-culture with the tolerogenic mDC cells in 10:1 ratio (2.5×10^4 naïve T cells: 2.5×10^3 tolerogenic mDC). The naïve T cells were stimulated with scalar doses of agonistic anti-CD3 (1µg/ml, 10µg/ml, and 100µg/ml) and 1µg/ml of agonistic anti-CD28 to stimulate their activation alone or in co-culture with tolerogenic mDC stimulated with Fc protein for activation. Cell cultures were then incubated for 24 hours. After the incubation the cells were then analyzed by qRT-PCR for IFNγ transcript levels. The naïve T cells that were plated in co-culture with the tolerogenic mDC expressed significantly less IFNγ than the naïve T cells alone, where the amount of IFNγ mRNA expression was seen in a dose dependent fashion with respect to the anti-CD3 concentration (Fig. 9b).

Adenosine-2A receptor expression on tolerogenic mDC leads to cAMP accumulation in the cytoplasm

Stimulation of the adenosine-2A receptor ($A_{2A}R$), a G protein-coupled receptor, has immuno-suppressive activity which inhibits inflammatory responses produced during an infection due to activation of adenylyl cyclase causing an increase of intracellular cAMP (fig 10). The $A_{2A}R$ is expressed by leukocytes and its activation has been shown to reduce inflammation in many inflammatory conditions (Zhang, Li et al. 2016). Activation of $A_{2A}R$ cause a G protein-dependent adenylate cyclase to activate leading to an increase of intracellular cAMP (Kim, Sun et al. 2012).

Tolerogenic mDC from subacute pediatric KD subjects were FACS sorted and found to express high levels of the adenosine 2A receptor ($A_{2A}R$) (Fig. 11). To examine the functionality of tolerogenic mDC by activation of $A_{2A}R$ tolerogenic mDC and canonical mDC populations were FACS sorted. $CD14^+$ and $CD14^-$ mDC were stimulated with Fc protein and allowed to incubate for 24 hours. After Fc stimulation the 2 mDC populations were then incubated with the $A_{2A}R$ agonist regadenson (1μM). The functional $A_{2A}R$ activity was seen in the Fc stimulated tolerogenic DC, but not in the $CD14^-$ mDC based on the level of intracellular cAMP accumulation (Fig. 12).

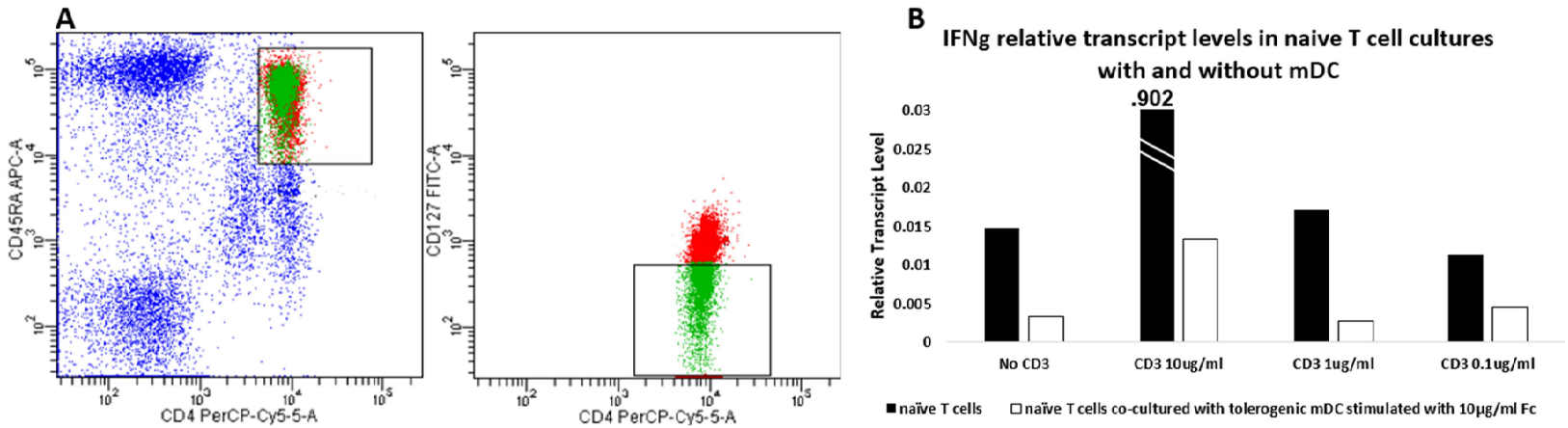

Figure 9. Inhibition of Th1 polarization of naïve T cells by Fc-stimulated CD14+ ILT-4+ CD4+ mDC. Panel A. Gating strategy for the FACS-sorting of naïve CD4+ T cells from a healthy child. $CD4^{+}CD45RA^{+}$ double positive (left in panel A) were then gated on $CD127^{-}$ cells. 2.5 x 10^4 of the sorted naïve T cells were then plated with scalar doses of agonistic anti-CD3 (.1µg/ml, 1µg/ml, and 10µg/ml) and 1µg/ml of anti-CD28, either alone or in a co-culture with 2.5 x 10^3 of the sorted mDC that were stimulated with 10µg/ml of purified Fc protein. The data shown is the relative mRNA transcript level if IFNγ in the naïve T cells after 24 hours in culture.

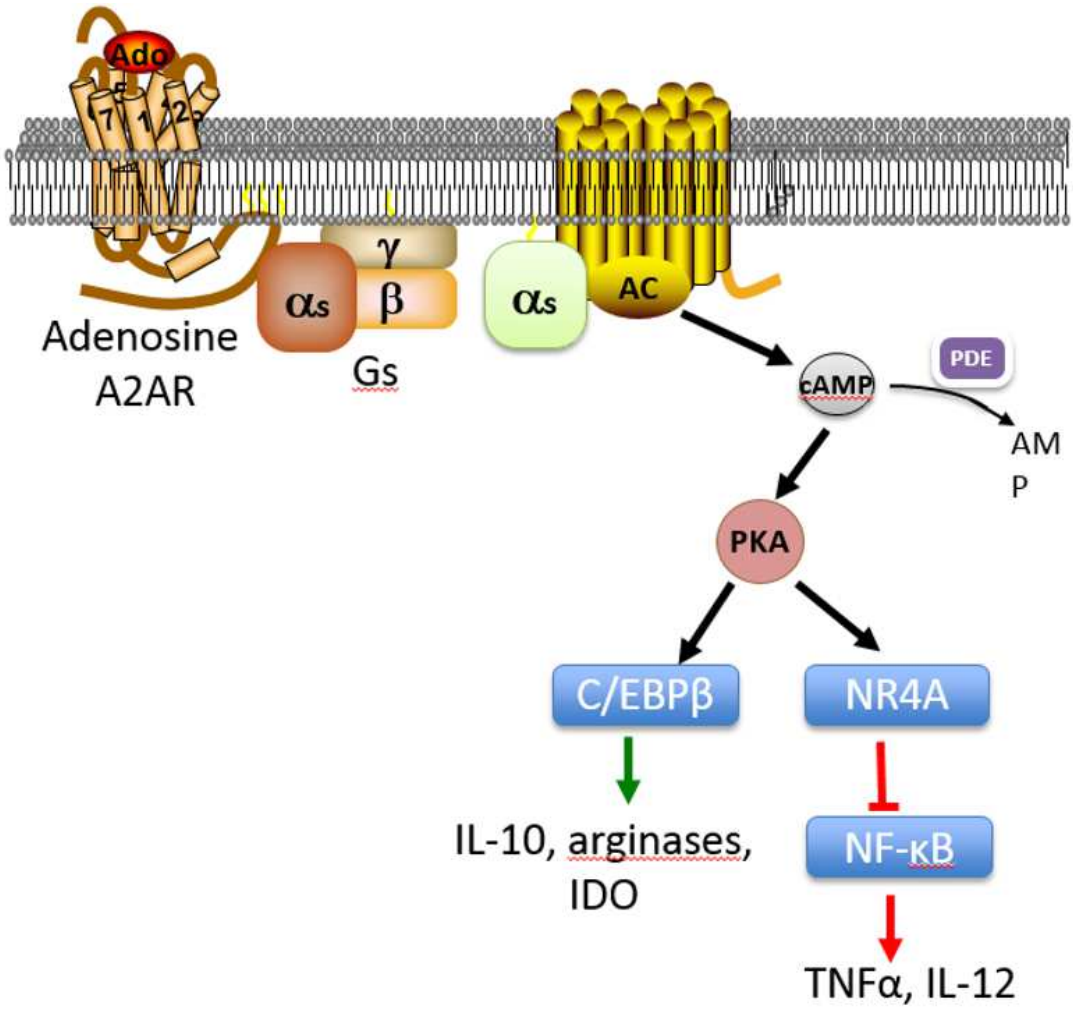

Figure 10. Adenosine 2A receptor signaling pathway. The cartoon shows activation of adenylyl cyclase (AC) producing intracellular cAMP which induces a PKA pathway that leads to IL10 secretion.

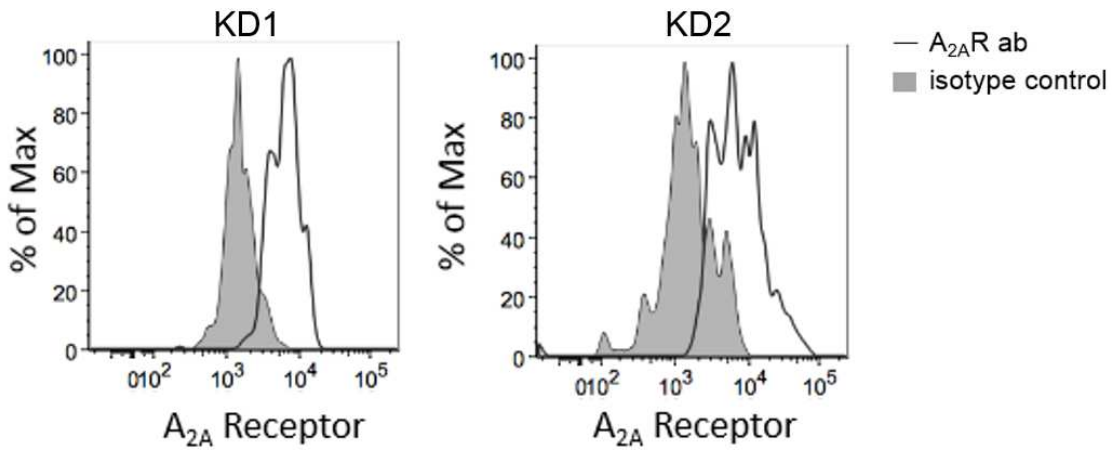

Figure 11. A2AR expression in FACS sorted CD14+ ILT-4+ CD4+ mDC from 2 acute KD subjects. A2AR expression has been measure with an anti-A2AR specific monoclonal antibody (empty histograms). Isotype control is also shown (filled histogram).

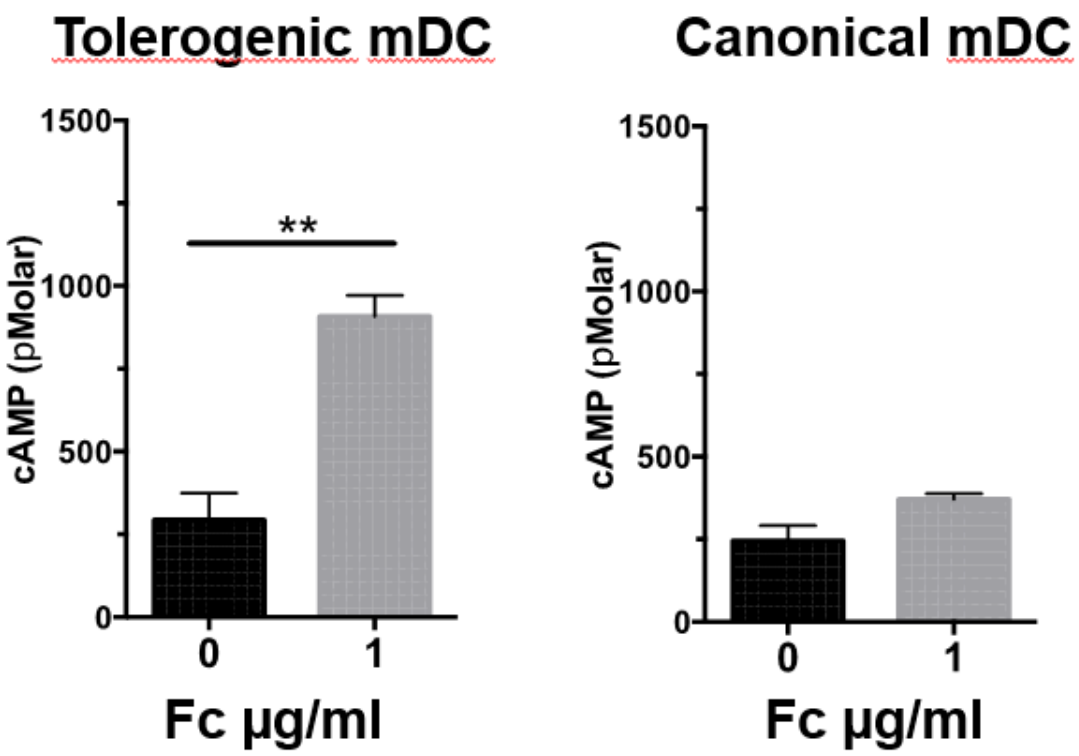

Figure 12. cAMP accumulation in the cytoplasm of FACS-sorted CD14+ ILT-4+CD4+ mDC and canonical CD14- mDC. FACS-sorted CD14+ CD4+ ILT-4+ mDCs and CD14$^-$ mDC derived from PBMC of a subacute KD subject were tested for intracellular cAMP levels using TR-Fret assay with Perkin Elmer's UltracAMP kit.

DISCUSSION

In the present study, I report that in pediatric subjects there exists a newly defined population of suppressor myeloid dendritic cells. Previous studies in my laboratory, which identified the presence of an IL-10 secreting mDC lineage in pediatric KD subjects, opened the door to further characterize and understand the function of this suppressor mDC lineage (Burns, Song et al. 2013). This suppressor mDC population has been characterized as being $CD11c^{+}CD11b^{+}CD14^{+}$ ILT-$4^{+}CD4^{+}$.

The $CD14^{+}$ILT-$4^{+}CD4^{+}$ mDC lineage defined here was determined to be a mature lineage and not an early progenitor due to the expression of CD86, normally expressed by mature DCs, and the low expression of the hematopoietic stem cell marker CD34 (fig. 6). It has been previously reported that mDCs that do not express CD14 were positive for CD8α/α expression, I therefore analyzed the expression of CD8α on the $CD14^{+}$ILT-$4^{+}CD4^{+}$ mDC population described in this study that has not been found to not be expressed. However, CD4 was found to be expressed by the majority of the CD14+ mDC in our pediatric cohorts (fig. 6).

In figure 6 it is also shown that these tolerogenic mDC express the surface protein CD31. The expression of CD31 on this cell population can allow for these cells to cognately interact with endothelial cells in inflamed vessels by binding to CD38 (Deaglio, Aydin et al. 2010). This can be a mechanism of direct suppression of inflammation in KD by the tolerogenic mDC due to their ability to secrete IL-10 in close proximity to inflamed vascular compartments.

HLA-G, which is the preferential ligand for ILT-4, was expressed in a percentage of the $CD14^{+}ILT\text{-}4^{+}CD4^{+}$ mDCs in all three cohorts of subjects (fig. 5). Signaling through ILT-4, by tyrosine phosphorylation of the ILT-4 receptor recruits SH2-containing tyrosine phosphatases, SHP-1 and SHP-2, which then activate the IL-6-STAT3 pathway. Although it has been shown that activation of ILT-4 can cause down regulation of MHC II expression lessening their ability to activate pro-inflammatory T cells (Liang, Ristich et al. 2008), more recently ILT-4 has been seen to play an active role in the inhibition of the cytotoxicity of NK cells (Anderson and Allen 2009). ILT-4 has been shown to be involved in the regulation of a variety of inflammatory cells throughout the body (Shiroishi, Tsumoto et al. 2003). Our study, the cohorts of pediatric subjects in this study showed no correlation with the number of tolerogenic mDC in the pediatric subjects and the number of NK/NKT cells in circulation.

The expression the $A_{2A}R$ on this tolerogenic mDC population in children is expressed at a significantly higher level, as shown in figure 11. Because stimulation of the $A_{2A}R$ causes activation of adenylyl cyclase, which in turn cause an increase of intracellular cAMP, the accumulation of cAMP in the cytoplasm of these tolerogenic mDC seen in figure 12 can be the mechanism by which these tolerogenic mDC induce production of IL-10 (fig. 10) (Linden and Cekic 2012).

My findings suggests that there is a definitive role of the tolerogenic mDC population in redefining the ability of the innate immune system in pediatrics in order to regulate inflammation, mediated by high levels of IL-10 secretion. In children this tolerogenic mDC lineage does not have the ability to promote Tr1 lineage

differentiation, but it can inhibit Th1 lineage differentiation from naïve T cells by inhibiting upregulation of IFNγ. The expression of $A_{2A}R$ on this tolerogenic mDC lineage, which when stimulated causes accumulation of cAMP, could describe a HLA-G independent mechanism of inducing IL-10 secretion from these cells.

High amounts of $A_{2A}R$ expression on these tolerogenic mDC in pediatrics may indicate an alternative mechanism of inducing IL-10 production in these cells.

MATERIALS AND METHODS

Study population

14 subjects with acute Kawasaki disease (KD), 8 males and 6 females, aged 0.5 to 11.9. KD subjects were treated with intravenous immunoglobulin (IVIG) therapy. Additional immunotherapy as infliximab, atorvastatin or anakinra has been administered in cases of IVIG resistance or development of coronary arteries abnormalities (CAA). Heparinized blood samples (2-9mls) were obtained prior to treatment at the time of diagnosis (acute) and 2 weeks to 2 months after therapy (subacute). 5 subjects with other acute febrile illnesses, 1 male and 4 females, with a median age of 3 years were studied as febrile controls. These children have been diagnosed with adenovirus infection (1 subject), viral syndrome (3 subjects) and juvenile idiopathic arthritis (JIA). 12 healthy children 4 males and 8 females, aged 1 to 8.5, were studied as asymptomatic controls. All KD subjects, healthy children and children with febrile illnesses, were enrolled at Rady Children's Hospital, San Diego, following written parental informed consent and patient assent as appropriate.

Dendritic cell (DC) characterization by surface staining

DC populations were defined by a combination of monoclonal antibody (mAb) markers by cell surface staining and analyzed by flow cytometry: CD11c-APC, mouse IgG1κ, clone B-ly6, anti-human CD11b-APC-Cyanin 7 (Cy7), mouse immunoglobulin IgG1κ, clone ICRF44, anti-human, anti-human CD14-phycoerythrin (PE) Cy7, mouse IgG2aκ, clone M5E2, anti-human CD86-fluorescein isothiocyanate (FITC), mouse

IgG1κ, clone 2331 (FuN-1) from BD Biosciences and anti-human CD85d [Ig-like transcript 4 (ILT-4)] peridinin chlorophyll (PerCP)-elfuor 710, mouse IgG2aκ, clone 42D1, anti-human human leukocyte antigen (HLA)-G PE, mouse IgG2aκ, clone 87G from eBioscience and anti-human CD4-AlexaFluor 700, mouse IgG1κ, clone RPA-T4 from BD Bioscience. Data were acquired with FACS ARIA II and analyzed using FACSDiva (BS Biosciences, San Jose, CA) software.

T cell lineage cell characterization by surface staining

Regulatory T cells (Treg) lineages (nTreg and iTreg) and natural killer (NK) cells were also enumerated and characterized in acute KD and subacute subjects, febrile controls and healthy children using the following mAb detected by cell surface staining: anti-human CD8-AlexaFluor 700, mouse IgG1κ, clone RPA-T8 from BD Bioscience. Anti-human CD4-PerCP-Cy5.5, mouse IgG1κ, clone RPA-T4, anti-human CD25-PE, mouse IgG1κ, clone BC96, anti-human CD45RA-APC, mouse IgG2bκ, clone HI100, anti-human CD56 (NCAM) PE-Cy7, mouse IgG1κ, clone CMSSB, anti-human CD127 (IL7-receptor) FITC, mouse IgG1κ, clone eBioRDR5 and anti-human HLA-DR-APC-H7, mouse IgG2aκ, clone G46-6 from eBiosceince. Anti-human HLA-DR-APC-H7, mouse IgG2aκ, clone G46-6 (BD Bioscience) has been used to define the activation state of Treg and NK cells. Data were analyzed using FACSDiva software.

Phenotypical and functional characterization of FACS-sorted tolerogenic mDC

Tolerogenic mDC previously defined were sorted from PBMC derived from a subacute KD subject using BD FACSAria II with bioBUBBLE using the following combination of mAb's: anti-human CD4-AlexaFluor 700, mouse IgG1κ, clone RPA-

T4, anti-human CD11c-APC, mouse IgG1κ, clone B-ly6, anti-human CD14-PE-Cy7, mouse IgG2aκ, clone M5E2, anti-human CD86-FITC mouse IgG1κ, clone 2331 (FuN-1) from BD Biosciences and anti-human CD85d (ILT-4) PerCP-elfuor 710, mouse IgG2aκ, clone 42D1 from eBioscience. After cell sorting, tolerogenic mDC were divide in two preparations to: a) further define their phenotype and maturity using the following cell surface markers: anti-human CD1c-FITC [blood dendritic cell antigen 1-phycoerythrin (BDCA-1-FITC)], mouse IgG2a, clone AD5-8E7 from magnetic affinity cell sorting (MACS) (Miltenyi Biotech, San Diego, CA, USA), anti-human CD8α-APC, mouse IgG1κ, clone RPA-T8 from eBioscience, and anti-human CD34-AlexaFluor 700, mouse IgG1κ, clone 581 from Biolegend. The data was analyzed using FACSDiva software. b) In vitro cultures to measure their lymphokine's profile under stimulating conditions. Cells were re-suspend in complete RPMI, 5% AB human serum and plated in 96 U bottomed plates at 5.5 x 10^3 cells/well and stimulated with purified Fc fragments (Meridian Life Sciences Inc., lot 5A02215). Cell cultures were incubated for 24 hours at 37°C, 5.5% CO_2, supernatants collected to measure IL-10 and IL-12 secretion by ELISA (BD Bioscience) according to manufacture instructions.

Naïve T cell polarization in co-culture with Fc-stimulated tolerogenic mDC

Naïve T cells defined as being $CD4^+CD45RA^+CD127^-$ were sorted using BD FACSAria II using the following combination of specific mAbs: anti-human CD4-PerCP-Cy5.5, mouse IgG1κ, clone RPA-T4, anti-human CD45RA-APC, mouse IgG2bκ, clone HI100, anti-human CD127 (IL7-receptor) FITC, mouse IgG1κ, clone eBioRDR5 from eBioscience. Tolerogenic mDC ($CD11c^+$, $CD14^+$, $CD4^+$, ILT-4^+) were

also sorted using BD FACSAria II with bioBUBBLE using the following combination of mAb's: anti-human CD4-AlexaFluor 700, mouse IgG1κ, clone RPA-T4, anti-human CD11c-APC, mouse IgG1κ, clone B-ly6, anti-human CD14-PE-Cy7, mouse IgG2aκ, clone M5E2, anti-human CD86-FITC mouse IgG1κ, clone 2331 (FuN-1) from BD Biosciences and anti-human CD85d (ILT-4) PerCP-elfuor 710, mouse IgG2aκ, clone 42D1 from eBioscience. The cells were sorted using BD FACSAria II with bioBUBBLE from peripheral blood mononuclear cells (PBMC). The sorted naïve T cell population were re-suspended in complete RPMI 5% human AB serum, plated at the concentration of 2.5 x 10^4 cells/well and stimulated with agonistic anti-CD3, mouse IgG1κ, clone UCHT1 from BD Bioscience in a concentration curve (10μg/ml, 1μg/ml, 0.1μg/ml and unstimulated) and agonistic anti-human CD28, mouse IgG1κ, clone CD28.2 from eBiosceince at a concentration of 1μg/ml either alone or in co-culture at a 10:1 ratio with Fc-stimulated tolerogenic mDCs. 24 hours after cultures cells were frozen in TRIzol (Thermo Fisher) and then Quantitative Reverse Transcriptase–Polymerase Chain Reaction (qRT-PCR) was used to test interferon (IFN)γ transcript levels. RNA was extracted from using TRIzol (Thermo Fisher) following manufacture's instruction. cDNA was made using SuperscriptIV (Thermo Fisher) following manufacture's instruction. Levels of IFNγ transcript were measured using TaqMan Assays (Thermo Fisher). IFNγ transcript levels were normalized to the expression level of TATA box binding protein-associated factor, RNA polymerase I, B (TAF1B).

A_{2A}R expression on tolerogenic mDC and Intracellular cAMP production in tolerogenic mDC

Tolerogenic mDCs from 2 acute KD subjects identified by flow cytometry analysis were studied for $A_{2A}R$ expression by using the mouse monoclonal antibody 7F6-G5-A2, IgG2a generated in Dr. Linden's laboratory. Function $A_{2A}R$ activity was measured downstream of receptor activation by intracellular cAMP accumulation using TR-Fret assay with Perkin Elmer's UltracAMP kit. Cells were harvested, centrifuged at 300g x 5 minutes and carefully removed with a pipette and incubated with stimulation bugger and 1μM regadenoson, ADA, and rolipram for 15 minutes at 37°C. Cells were then lysed with ice cold perchloric acid, neutralized with potassium carbonate, incubated on ice for 30 minutes, centrifuged at 10,000 rpm in a microfuge at 4°C fir 10 minutes and then the lysate was collected. 20μl of the lysates were used for cAMP assays in triplicate wells on a 96 well plate together with cAMP standards. The cAMP assay was performed using Perkin Elmer's UltracAMP kit as directed.

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