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Authors
Franco, Alessandra
Kumar, Jeetendra
Lin, Gene
et al.

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Research Article

Pediatric tolerogenic DCs expressing CD4 and immunoglobulin-like transcript receptor (ILT)-4 secrete IL-10 in response to Fc and adenosine

Alessandra Franco[1], *Jeetendra Kumar*[1], *Gene Lin*[2], *Negar Behnamfar*[1], *Li-En Hsieh*[1], *Chisato Shimizu*[1], *Adriana H. Tremoulet*[1], *Jane C. Burns*[1] *and Joel Linden*[2,3]

[1] Department of Pediatrics, Division of Allergy, Immunology and Rheumatology, University of California San Diego, School of Medicine and Rady Children's Hospital, La Jolla, CA, USA
[2] La Jolla Institute for Allergy and Immunology, Division of Developmental Immunology, Athena Circle, La Jolla, CA, USA
[3] Department of Pharmacology, University of California San Diego, School of Medicine, University of California, San Diego, USA

We characterized a novel population of tolerogenic myeloid dendritic cells (tmDCs) defined as CD11c$^+$CD11b$^+$CD14$^+$CD4$^+$ and immunoglobulin-like transcript receptor (ILT)-4$^+$ that are significantly more abundant in the circulation of infants and young children than in adults. TmDCs secrete the immunosuppressive lymphokine interleukin (IL)-10 when stimulated with the heavy constant region of immunoglobulins (Fc) and express high levels of the adenosine A_{2A} receptor (A_{2A}R), which, when activated by adenosine, inhibits the release of pro-inflammatory cytokines from most immune cells. Here we show that stimulation of the A_{2A}R on tmDCs by regadenoson or N-ethylcarboxamidoadenosine (NECA) rapidly increases cyclic AMP accumulation and enhances IL-10 production under Fc stimulatory conditions. In co-culture experiments, tmDCs inhibit the differentiation of naïve T cells to a pro-inflammatory phenotype. In conclusion, although DCs are classically viewed as antigen presenting cells that activate T cells, we show an independent role of tmDCs in pediatric immune regulation that may be important for suppressing T cell responses to neoantigens in infants and young children.

Keywords: CD11b · CD11c · CD14 · Kawasaki disease · T cells

Additional supporting information may be found in the online version of this article at the publisher's web-site

Introduction

Little is known about the immune mechanisms by which infants and young children process the barrage of neoantigens that they encounter after birth without experiencing undue inflammation. Here we show an innate population of myeloid dendritic cells (DCs) that is very abundant in circulation in pediatric subjects and secretes the suppressive lymphokine interleukin (IL)-10 when stimulated by the heavy constant region of immunoglobulins (Fc).

The classic understanding of DCs is that they play a vital role in connecting the adaptive and innate immune systems by bringing foreign antigens to secondary lymphoid organs where they present

Correspondence: Dr. Alessandra Franco
e-mail: alfranco@ucsd.edu

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processed peptides bound to major histocompatibility complex (MHC) molecules to naïve T cells. When DCs process and transport antigens, they increase surface expression of MHC molecules and co-stimulatory signals such as CD86 that engage CD28 on T cells. Once activated, CD86$^+$ DCs migrate to secondary lymphoid organs [1, 2].

In the current study we describe the unique phenotype and functions of a novel population of tolerogenic myeloid DCs (tmDCs) that are abundant in healthy children and in children with acute inflammation compared to adults. These innate myeloid cells express high levels of the adenosine A_{2A} receptor ($A_{2A}R$) which, when stimulated, facilitates IL-10 secretion mediated by the Fc. Adenosine is known to regulate mouse and human macrophage and dendritic cell differentiation and function via Gs-coupled A2A and A2B adenosine receptors that stimulate intracellular cAMP accumulation [3].

Functional experiments suggest that tmDCs inhibit the differentiation of naïve T cells toward a pro-inflammatory phenotype. These findings indicate that circulating immunoglobulins play a role in the continuous stimulation of tmDC to maintain immune homeostasis and highlight a novel role of antibodies, adenosine and cAMP in immune regulation.

Results

Characterization of tolerogenic mDCs in pediatric and adult subjects

We previously reported that circulating myeloid DCs (mDCs) are abundant in infants and children with acute Kawasaki disease (KD) or with acute viral infections, and we defined a mature population of mDCs that expresses the monocyte marker CD14 and secretes IL-10 when stimulated with Fc [4]. Here we expand these findings by phenotypically characterizing and enumerating these tmDCs in various pediatric cohorts, including children of different ages who are healthy, have acute viral infections, systemic onset juvenile idiopathic arthritis, or acute KD.

In a cohort of 12 healthy children (cohort 1), we found that circulating myeloid cells, defined by the expression of CD11c and CD11b, ranged from 9.4 to 20% (median 17.75, IQR 14.80-19.95) of PBMCs (Fig. 1A). Myeloid cells that expressed the monocyte marker CD14 comprised from 32.5 to 72.7% of mDCs (median 51.10, IQR 39.73-62.13). Most CD11c$^+$CD11b$^+$CD14$^+$ cells were found to co-express the T cell co-receptor CD4 and the immunoglobulin-like transcript receptor (ILT-4). CD14$^+$ CD4$^+$ ILT-4$^+$ tmDCs comprised 78.6 to 96.8% of mDCs (median 91.05, IQR 88.47-94.65) and the majority expressed the maturation marker, CD86 (median 97.45, IQR 96.15-98.78). HLA-G, a minor HLA class I allele that binds ILT-4, was expressed on only a small percentage of tmDCs (median, 7.9, IQR 3.1-9.72). We therefore defined tmDCs as CD11c$^+$CD11b$^+$CD14$^+$CD4$^+$ILT-4$^+$.

Having characterized tmDCs in healthy children, we next enumerated tmDCs in PBMC derived from children with various acute illnesses (cohort 2): adenoviral infection ($n = 1$), acute viral syndrome ($n = 3$), or juvenile idiopathic arhritis (JIA) ($n = 1$). Numbers of CD11c$^+$ CD11b$^+$ myeloid cells in these cohorts were similar to healthy children (Fig. 1B) and ranged from 14.9 to 25.6% (median 20, IQR 15.40-24.70), CD11c$^+$ CD11b$^+$CD14$^+$ cells ranged from 24.9 to 80.4% (median 49.50, IQR 33.90-72.50), and CD14$^+$ CD4$^+$ ILT-4$^+$ tmDCs ranged from 80 to 87.5% (median 83.40, IQR 80.40-86.50). TmDCs were fully mature based on CD86 expression (median 81.40, IQR 76.75-93.95). Also in this cohort, HLA-G was expressed by a very small percentage of tmDCs (median 6.9, IQR 1.4-16.7).

A third pediatric cohort consisted of 18 acute KD subjects prior to IVIG therapy (Fig. 1C). CD11c$^+$CD11b$^+$ myeloid cells ranged from to 3.4 to 46.6% (median 13.40, IQR 10.10-23.60). A high percentage of these cells were CD14$^+$, ranging from 51.6 to 95.5% (median 58.95, IQR 42.33-76.10). As observed in the other pediatric cohorts, a large percentage of CD11c$^+$ CD11b$^+$CD14$^+$ cells were CD4$^+$ILT-4$^+$ (median 83, IQR 69.82-92.30) and mature (CD86$^+$ median 85.10, IQR 68.20-93.93). As in the other two cohorts, HLA-G expression was negligible (median 5.90, IQR 1.70-14.45). We conclude that the numbers and cell surface markers of tmDCs are similar among pediatric cohorts regardless of the level of host inflammation.

In all pediatric cohorts (Fig. 1) 80–90% of CD11c$^+$CD11b$^+$ CD14$^+$ myeloid cells are tmDCs, as much as 7–8% of total PBMC. To determine if tmDCs are elevated in children vs. adults, we studied four healthy adults and enumerated their myeloid cells subpopulations. In contrast to the abundance of tmDCs in pediatric subjects, the levels in adults were much lower (median tmDCs = 3.7% of CD11c$^+$CD11b$^+$ myeloid cells and 0.5% of PBMCs) (Fig. 2). As in children, the majority of tmDCs were mature as evidenced by the expression of CD86. The percentage of PBMCs that were tmDCs in children, about 7.4% on average, was 15-fold higher that the percentage in adults. Individual FACS images from these cohorts are shown in Supporting Information Figs. 1–3.

Further phenotypical characterization of tmDCs

To further characterize the phenotype of the tmDCs, we evaluated markers of classical mDCs (CD8α, CD34, and BDCA-1) and CD31, which could be involved in homing to the vascular compartment. CD31 is a transmembrane protein that has immune receptor tyrosine-based inhibitory motifs (ITIMs) that bind to itself in a homophilic manner, or to CD38, a glycoprotein that is expressed on inflammatory cells, cardiac muscle, endothelial cells [5, 6] and myeloma cells, and has enzyymatic activity to convert extracellular NAD$^+$ to adenosine [7]. Tolerogenic mDCs were FACS-sorted from 2 acute KD subjects and then further characterized using monoclonal antibodies to CD31, CD8α, CD34, and BDCA-1 [8]. All of the FACS-sorted tmDCs expressed high levels of CD31, but did not express CD8α, which is commonly expressed by plasmacytoid and mucosal mDCs and functions to polarize naïve T cells to Th1 or CD8$^+$ cytotoxic T cell lineages [9] (Fig. 3). CD34, a marker expressed by hematopoietic stem cells [10] was expressed on a

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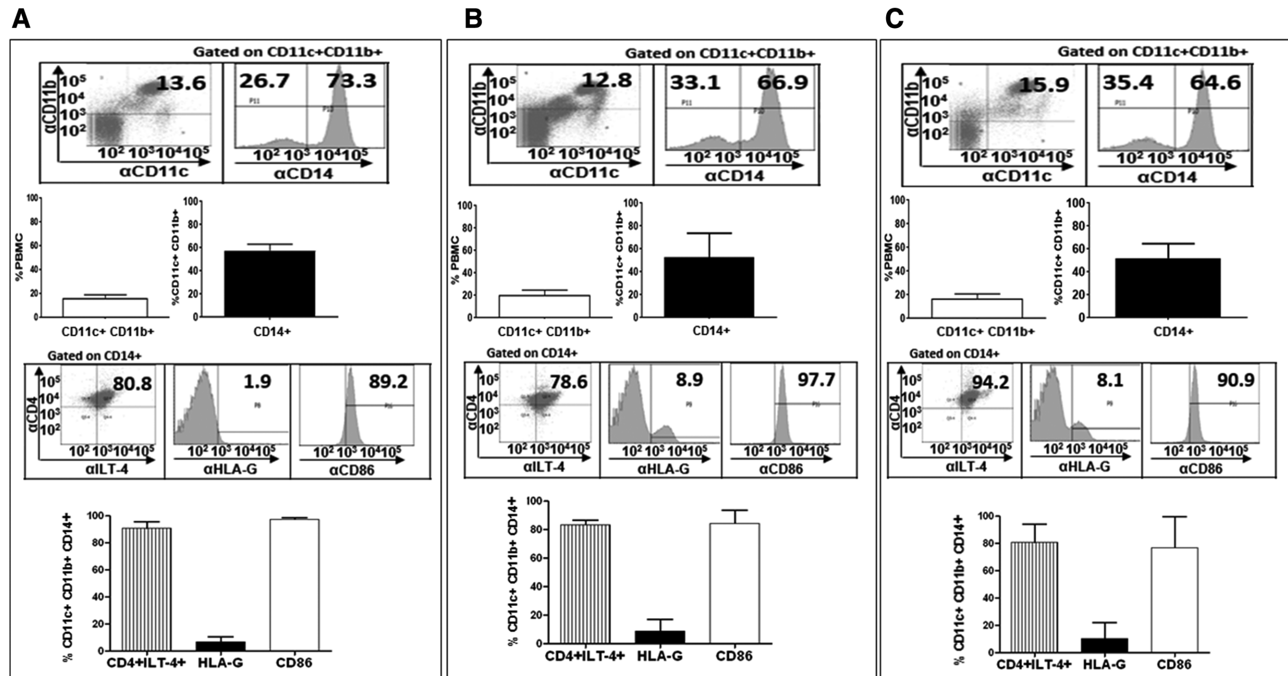

Figure 1. Characterization by flow cytometry of tolerogenic myeloid DCs (tmDCs) in pediatric subjects. Myeloid cells were enumerated from PBMCs by flow cytometry. Each panel shows two representative FACS plots from a single individual and bar graphs showing the median and IQRs of pooled data. The top panels show the frequencies of CD11b^{+}CD11c^{+} cells that are CD14^{-} or CD14^{+}. The bottom panels show the frequencies of CD11b^{+}CD11c^{+}CD14^{+} cells that are CD4^{+} and ILT-4^{+} or HLA-G^{+} or CD86^{+}. Enumeration of tmDCs was in (A) 12 healthy children; (B) 5 febrile children: 4 with acute viral infection and 1 with systemic onset juvenile idiopathic arthritis; and (C) 18 acute KD children sampled before IVIG therapy. FACS images are representative for each cohort. Bar graphs represent data pooled from each cohort (one experiment/subject).

small percentage of tmDCs as was the MHC class I-like molecule BDCA-1 (aka CD1c) that functions to present glycolipids to naïve T cells and is highly expressed by mature mDCs [11] (Fig. 3). CD34 and BDCA-1 were co-expressed by a small percentage of tmDC (<3%).

Functional characterization of tmDCs

Tolerogenic mDCs were FACS-sorted from an acute KD subject and 2 healthy children to study their responses to stimulation with purified Fc (Fig. 4). We addressed Fc stimulation because: (i) our previous work showed that tmDCs secrete IL-10 in response to Fc [4], (ii) CD4 binds to the framework V regions of immunoglobulins [12, 13] suggesting that the expression of CD4 on tmDCs may function to attract immunoglobulins for Fc binding, and (iii) acute KD patients respond within 24 h to IVIG suggesting that rapid IL-10 secretion by tmDCs following Fc stimulation may explain, in part, the rapid decrease in systemic inflammation following IVIG treatment. The gating strategy for obtaining tmDCs from PBMCs is shown in Fig. 4A. After FACS-sorting, tmDCs were seeded in culture with 1–100 μg/mL of purified Fc for 24 h and the supernatants assayed for IL-10 secretion by ELISA. The tmDCs from two subjects (an acute KD and an healthy child) responded to Fc

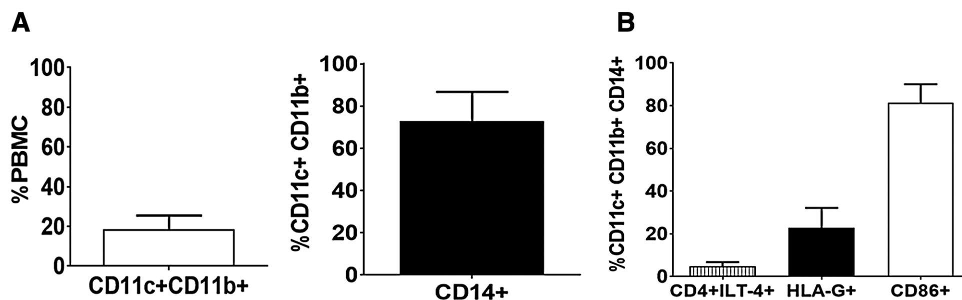

Figure 2. TmDCs are rare in healthy adults compared to children. Frequencies of PBMCs were characterized by flow cytometry from four individual healthy adults and plotted as the median ± IQR: (A) CD11c^{+}CD11b^{+} cells, median = 19.35; (B) CD11c^{+}CD11b^{+}CD14^{+} cells, median = 83.80; (C) CD11c^{+}CD11b^{+}CD14^{+}CD4^{+}ILT-4^{+} median = 3.7. As observed in tmDC from pediatric subjects, tmDC in adults were mature, CD86^{+} median = 88.0. Bar graphs represent data pooled from 4 subjects (one experiment/subject).

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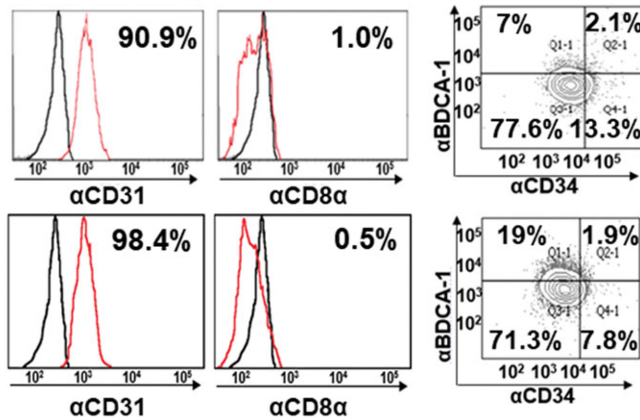

Figure 3. Further phenotypic characterization of tmDCs. Tolerogenic mDCs ($CD11b^+CD11c^+CD14^+CD4^+ILT\text{-}4^+$) were FACS-sorted from a convalescent KD subject and analyzed for the expression of CD31, CD8α CD34, and BDCA-1 (upper panel). Red lines show staining by the indicated antibodies and black lines by isotype controls. Similar results were obtained in a duplicate experiment form a second convalescent KD subject (lower panel).

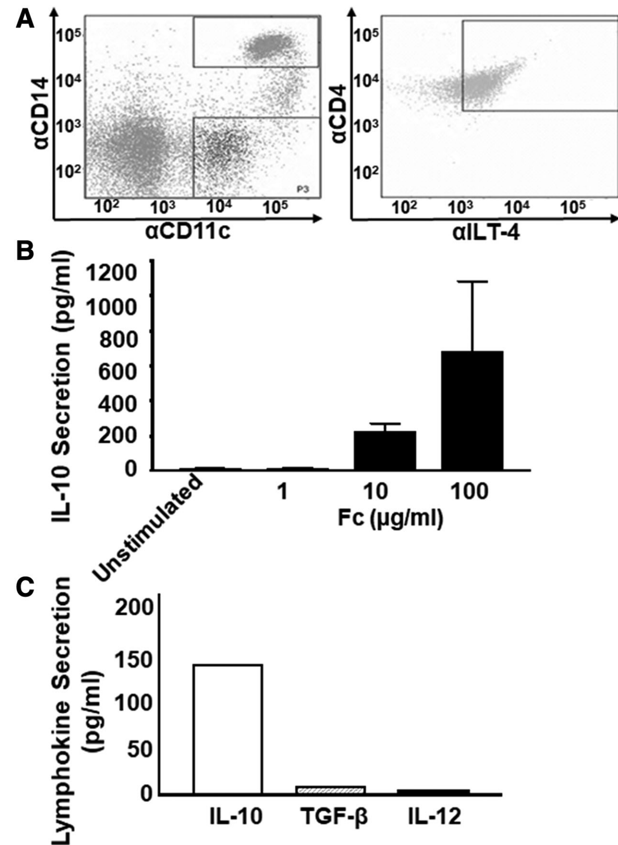

Figure 4. FACS-sorted tmDCs secrete IL-10 when stimulated with purified Fc. (A) FACS-sorting strategy used to collect tmDCs separately from 3 individuals. (B) IL-10 secretion was measured by ELISA 24 h after tmDCs from a healthy child and an acute KD child were cultured with the indicated doses of Fc. Each bar is the mean ± SD of duplicate children assayed in duplicate. (C) TmDCs from a second healthy child were FACS-sorted, stimulated with 10 μg/mL Fc, and evaluated by ELISA for IL-10, TGF-β and IL-12 in the same culture supernatant, each bar is the average of duplicate determinations from a single donor. Data shown in A is representative of three independent experiments. Data shown in B represent two independent experiments from two subjects. Data shown in C represent one independent experiment from one subject.

stimulation with dose-dependent secretion of IL-10 (Fig. 4B). IL-10 secretion in response to Fc stimulation was confirmed by testing FACS-sorted tmDCs from another healthy child (Fig. 4C). TGF-β and IL-12 were not detectable in the same culture supernatant (Fig. 4C).

TmDCs inhibit naïve T cell differentiation into a pro-inflammatory phenotype

In order to assess the suppressive function of tmDCs, naive T helper cells defined as $CD4^+CD45RA^+CD127^-$ [14] were FACS-sorted to determine their lymphokine profiles when stimulated in co-cultures with FACS-sorted tmDCs. The experimental design was dictated by the limited number of naïve T cells that could be obtained from these pediatric subjects. Naive T cells were sorted by using a combination of monoclonal antibodies (Fig. 5A). First we addressed the suppressive function of tmDCs on IL-2 secretion by naive T cells. FACS-sorted $CD4^+$ naïve T cells were cultured for 24 h with anti-CD3 (0.1 μg/mL, 1 μg/mL) and 1 μg/mL of anti-CD28 either alone or in co-culture with tmDCs or canonical mDCs (control) stimulated with 10 mg/mL Fc at a 5:1 ratio (1.5×10^3 naïve T cells: 3×10^2 tmDCs or mDCs). After incubation, IL-2 was measured in T cell culture supernatants. The results suggest that tmDCs but not mDCs reduced IL-2 secertion by naive $CD4^+$ T cells (Fig. 5B). The tmDC cell number was very low in this experiment suggesting great potency for the suppression mechanism. Similar experiments with tmDCs and naive T cell co-cultures from two healthy children confirmed downregulation of IL-2 transcription and protein secretion (Fig. 5C). Of note, IFN-γ transcripts and IFN-γ measured in culture supernatants were also reduced by tmDCs in co-cultures (Fig. 5C). This demonstrates the regulatory function of Fc-stimulated tmDCs in suppressing the differentiation of naive T cells into a pro-inflammatory phenotype.

Characterization of A2AR expression on tmDCs

To further explore the biochemical mechanisms underlying the immunosuppressive function of tmDCs, we studied the expression of the $A_{2A}R$ receptor ($A_{2A}R$) that is found on most immune cells and supresses inflammation [15]. TmDCs from one acute and two paired acute and convalescent KD patients, two healthy children, and five healthy adults were studied by flow cytometry analysis for $A_{2A}R$ expression using a mouse-anti-human monoclonal antibody [16] (Fig. 6). Figure 6A shows representative $A_{2A}R$ expression in tmDCs from a healthy child, a KD patient sampled at acute and convalescent time points, and a healthy adult. Of interest, the density of $A_{2A}Rs$ was higher during the convalescent phase, two weeks after IVIG treatment, than during the acute phase, prior to IVIG. This finding was confirmed in a second KD patient (data not shown). The geometric mean fluorescence intensities

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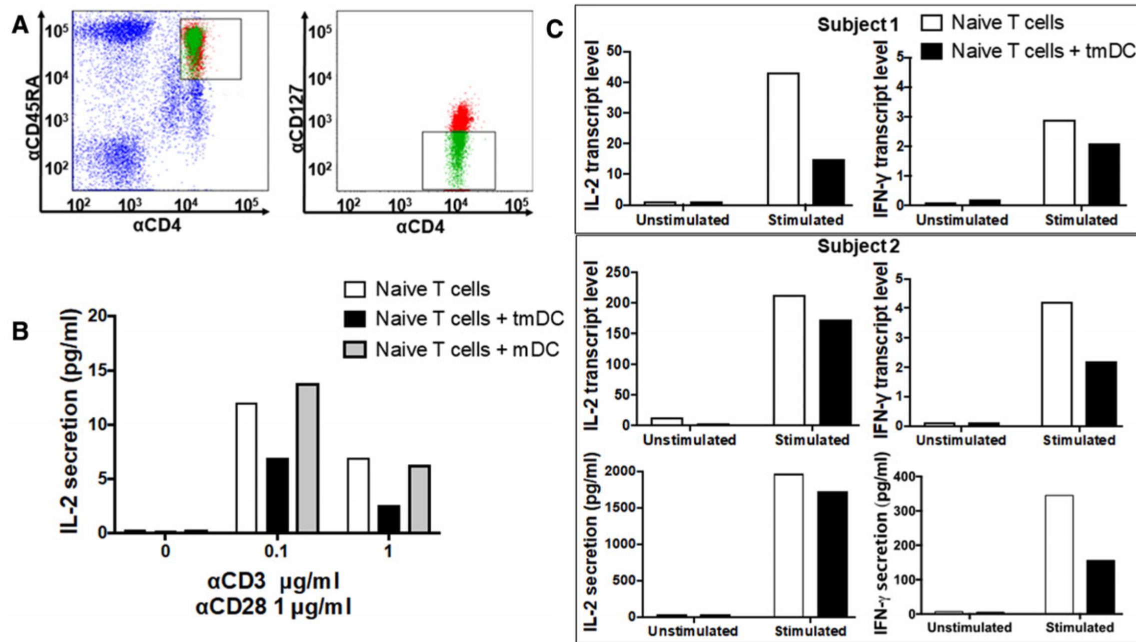

Figure 5. TmDCs stimulated with low-dose Fc inhibit IL-2 and IFN-γ transcription when co-cultured with naïve T cells activated by anti-CD3 and anti-CD28 monoclonal antibodies. (A) FACS-sorting strategy for naïve $CD4^+$ T cells from a healthy child: $CD4^+$ $CD45RA^+$ naïve T cells (left panel) were gated on $CD127^-$ cells (right panel). (B) IL-2 secretion by naive T cells derived from a healthy child were co-cultured with tmDCs or mDCs and measured in duplicate by ELISA. (C) IL-2 and IFN-γ transcripts measured by RT-PCR in lysates of naive T cell/tmDC co-cultures derived from two healthy children. IL-2 and IFN-γ were measured by ELISA in duplicate in culture supernatants. Data shown in A is representative of three independent experiments. Data shown in B represent one independent experiment from one subject. Data shown in C represent two independent experiments from two subjects.

for $A_{2A}R$ expression were higher in acute KD children compared to adults ($p < 0.0001$). These findings suggest that inflammatory signaling or IVIG may stimulate tmDCs to express higher levels of the $A_{2A}R$.

Role of cAMP in IL-10 secretion

To explore the effects of $A_{2A}R$ signaling on IL-10 secretion by mDCs, we FACS-sorted classical $CD14^-$ mDCs and $CD14^+$ tmDCs

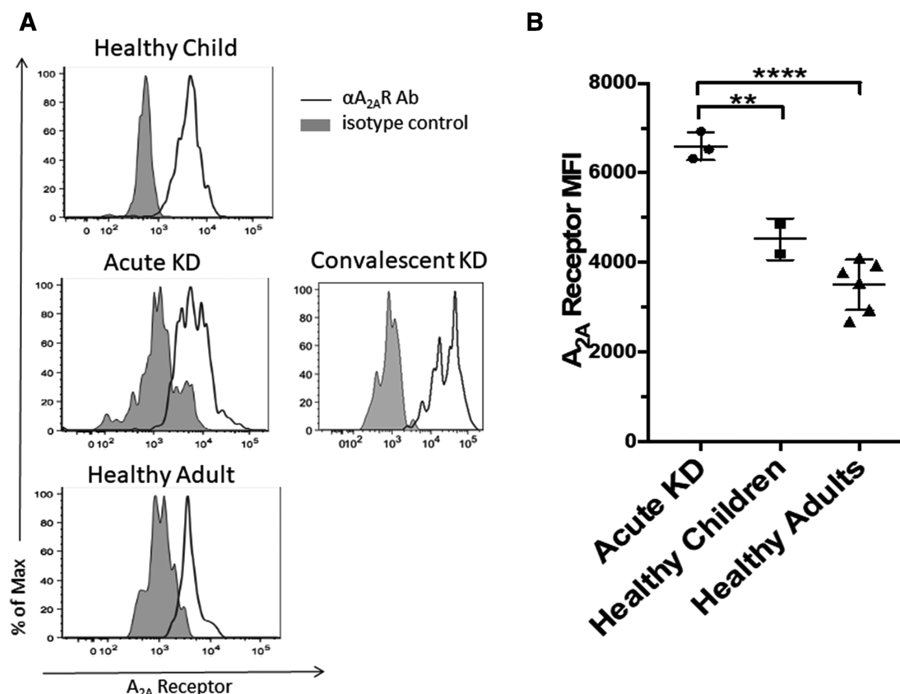

Figure 6. Adenosine A_{2A} receptors are expressed by tmDCs at higher levels in KD children than in adults. (A) Representative histograms of of $A_{2A}R$ expression measured by flow cytometry. (B) Geometric Mean fluorescence intensity (MFI) of $A_{2A}R$ expression in tmDCs from acute KD patients, healthy children, and healthy adults. Each symbol represents one patient. Data shown in A is representative of one healthy child, an acute and convalescent KD and one healthy adult. Data shown in B show $A_{2A}R$ expression in three acute KD, two healthy children, and six healthy adults. Each subject has been studied in an independent experiment. Analysis of variance with post-hoc Tukey's multiple comparisons test, $^{**}p < 0.01$, $^{****}p < 0.0001$).

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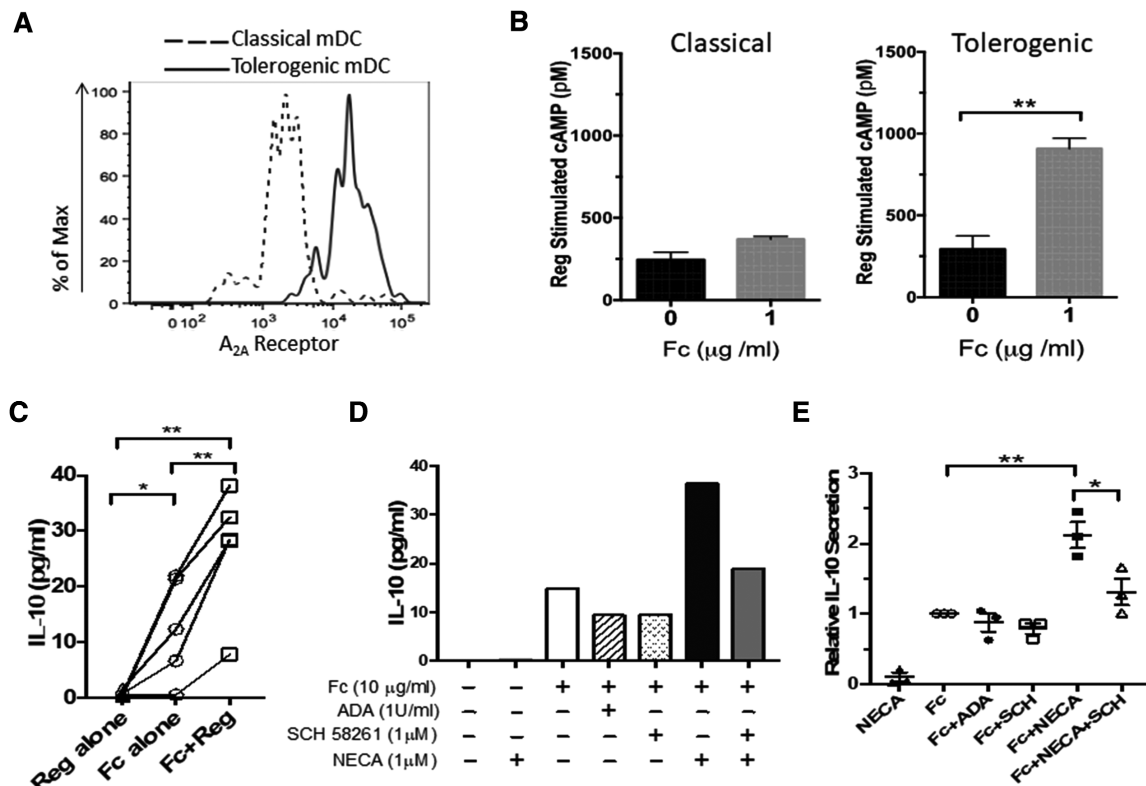

Figure 7. Adenosine A_{2A} receptor expression and function in tmDCs. (A) A_{2A}Rs are highly expressed by tolerogenic but not classical CD14⁻ mDC from a convalescent KD patient. (B) Classical mDCs and tmDCs sorted from a subacute KD patient were incubated overnight without or with 1 μg/mL Fc, and then for 15 min with the selective A_{2A} receptor agonist, regadeonoson (1 μM). Intracellular cAMP production was determined by immunoassay of triplicate samples. (C) TmDCs were sorted from five convalescent KD patients and inclubated overnight with 1 μM regadenoson alone, 10 μg/mL Fc alone, or both. IL-10 in samples from five individuals exposed to different treatments were analyzed by paired Student's t-tests. (D) Fc-stimulated or non-stimulated PBMCs from one convalescent KD patient were incubated as indicated with no additions, adenosine deaminase (ADA), SCH58261, or NECA. IL-10 secretion was quantified by ELISA. (E) Relative IL-10 production in response to 10 μg/mL Fc-stimulated PBMCs in the presence or absence of ADA, SCH58261, or NECA from three convalescent KD children. Relative IL-10 secretion in response to each condition was normalized to the amount of IL-10 secreted in the presence of Fc alone. Graph show median of three independent experiments where one subject/experiment has been studied. Data were analyzed by Tukey's multiple comparisons test. $^{*}p < 0.05$, $^{**}p < 0.01$.

from convalescent KD patients. TmDC exhibited higher A_{2A}R expression compared to canonical $CD14^{-}$ mDCs (Fig. 7A). To examine the effect of Fc stimulation followed by A_{2A}R agonist stimulation in tmDCs and mDCs, we measured cAMP accumulation in response to treatment for 15 minutes with an A_{2A}R agonist, 1 μM regadenoson, with or without prior treatment for 24 h with 1 μg/mL Fc (Fig. 7B). Functional A_{2A}R activity based on cAMP accumulation was increased by Fc pretreatment of tmDCs but not classical $CD14^{-}$ mDCs. These data are consistent with higher functional A_{2A}R expression on tmDCs compared to $CD14^{-}$ mDC. We next sought to determine if there is an interaction between Fc and A_{2A}R activation to stimulate IL-10 production in tmDCs (Fig. 7C). On average, regadenoson enhanced IL-10 secretion greater than 2-fold when added with 10 μg/mL Fc (Fig. 7C), but did not influence greater IL-10 release in response to 100 mg/mL Fc (data not shown). In order to further characterize the response of tmDCs to adenosine receptor agonists, PBMCs (400,000 cells/well) isolated from a convalescent KD patient were seeded onto a 96-well flat bottomed plate with medium containing 1 U/mL adenosine deaminase (ADA) together with 10 μg/mL of Fc, 1 μM of the non-selective adenosine receptor agonist, NECA ± 1 μM of the A_{2A}R-selective antagonist SCH58261. Cells were incubated at 37°C for 24 h and medium from each well was assayed for IL-10 by ELISA. Figure 7D shows IL-10 production by tmDCs derived from a single individual and Fig. 7E shows the average responses of 3 individuals with IL-10 production normalized to treatment with 10 μg/mL Fc alone. Fc-stimulated IL-10 production was slightly, but not significantly reduced by the addition of ADA or SCH58261, suggesting that cultured tmDCs produce some endogenous adenosine that can partially activate A_{2A}Rs to facilitate Fc-mediated IL-10 secretion. NECA (1 μM) produced a large enhancement of the effect of 10 μg/mL Fc to stimulate IL-10 production that was mostly blocked by 1 μM SCH58261. This suggests that adenosine enhances Fc-mediated IL-10 production by activating the A_{2A}R and less so via other adenosine receptor subtypes, such as the A_{2B}R.

Discussion

Here we show that a unique population of tmDCs secrete IL-10 in response to Fc stimulation and are abundant in the circulation of

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children. Their unique phenotype includes the expression of the T cell co-receptor CD4, ILT-4, high levels of the anti-inflammatory adenosine receptor, $A_{2A}R$, and CD31. CD34 and BDCA-1 are co-expressed in a small percentage of tmDCs (Fig. 3) suggesting that the new nomenclature used to describe monocytes and DC types in adults [17] does not include this predominantly pediatric myeloid population. In adults, tmDCs can be detected in the peripheral blood, but their number is 15-fold lower than in children (Fig. 2). In adults, regulatory myeloid DCs that arise from the stroma of the spleen and liver have been previously reported [18, 19] and match the reclassified cDC2 population [17]. These important studies demonstrate that one function of adult tolerogenic mDCs is to inhibit the proliferation of pro-inflammatory T cells. We did not examine the effects of tmDCs on the proliferation of naive T cells due to cell number limitations. Rather, we measured the effects of tmDCs on naive T cell polarization in co-cultures to evaluate their effects on secretion of IL-2 and IFN-γ (Fig. 5). The supression of IL-2 production in co-cultures with tmDCs suggests that naive T cell expansion is affected as well.

We also show that tmDCs express higher levels of the immunosuppressive $A_{2A}R$ than classical mDCs. Pediatric tmDCs are phenotypically different than recently described tolerogenic fetal cDC2 cells [20] but appear to be functionally similar, as fetal cDC2 cells suppress ariginase-2 that is induced by cAMP [21].

These observations raise interesting questions about the role of innate immunity in “teaching tolerance” in children who are encountering the universe of new antigens. Our results suggest that innate cells play an important role in immune regulation, by-passing the immaturity of the T cell repertoire that is undergoing selection in infants and children. Self/non-self discrimination by the immune system, and tolerance to foreign antigens that enter through the gut and airways may be modulated by tmDCs, thereby helping infants and young children to adapt to their microbiome. We would like to highlight the concept that DC function goes far beyond antigen presentation to T cells and that the innate compartment in children is very diverse and complex. In our studies of children with an acute inflammatory condition, KD, these cells may contribute to the supression of inflammation and likely play a role in the therapeutic immunosuppressive response to IVIG [4, 22]. Circulating immunoglobulins certainly play a role in stimulating tmDCs, and IVIG may boost their IL-10 secretion by binding to Fc receptors. It is interesting to note that the anti-inflammatory response to IVIG in patients with acute KD is rapid (on the order of 6–12 h) consistent with the 24 h time course used in the functional experiments reported here.

Although there are many similarities between pediatric tmDCs and DC-10 cells found in the human decidua during pregnancy and in the peripheral blood of adult subjects [23–25], there are phenotypic and functional differences. Phenotypically, DC-10 cells co-express high levels of the ILT-4 receptor, and the non-polymorphic minor HLA class I allele HLA-G [24]. Although pediatric tmDCs also are ILT-4^{+}, they express very little HLA-G (Fig. 1). ILT-4 and HLA-G are killer-cell inhibitory receptors (KIR) and function to regulate the immune response by inhibiting natural killer (NK) cells and by reducing inflammation by stimulating IL-10 production [26]. We found that HLA-G is expressed on T cells (data not shown), suggesting that tmDCs may modulate T cell function in part due to ILT-4-HLA-G interactions. Functionally, tmDCs inhibit the differentiation of naïve T cells into pro-inflammatory T cells (Fig. 5). This suppressive role of tmDCs may have profound implications for the control of inflammation. This function is likely carried out in the lymph nodes and may serve to prevent expansion of pro-inflammatory T cells in various disease states.

The unique expression of the T cell co-receptor CD4 on tmDC links to previous work on domains for CD4 binding within the immunoglobulin framework [12, 13]. CD4 expression on tmDCs may serve to faciliate immunoglobulin binding and allow Fc stimulation. TmDCs are likely to operate in secondary lymphoid organs where they perform immune surveillance and they may mobilize to tissues under inflammatory conditions. Our data suggest that tmDCs down-regulate the polarization of naive T cells toward a pro-inflammatory phenotype (Fig. 5).

Another interesting feature of tmDCs is their high expression of CD31. CD31, also known as platelet endothelial cell adhesion molecule 1 (PECAM-1), is a cell adhesion molecule that allows interaction of many circulating cell types with vascular endothelial cells that express CD38 [6]. CD31 is also a key inhibitor of the development of pro-inflammatory DCs [8]. This suggests that tmDCs may play an additional role in maintaining vascular homeostasis through their interaction with endothelial cells via CD31-CD38 binding. The potential binding of tmDCs to endothelial cells may serve to modulate inflammation in the vascular compartment.

Here we show that Fc stimulates IL-10 production by tmDCs and this production is enhanced by activation of $A_{2A}Rs$ that are highly expressed on tmDCs, but not on classical $CD14^{-}$ mDCs (Fig. 6). It has been reported previously that IVIG triggers the production of IL-10 in bone-marrow derived macrophages by binding to FcγRI and stimulating ERK1/2 phosphorylation [27]. Adenosine production in inflamed tissues results in $A_{2A}R$ activation and cyclic AMP production [15]. CCAAT/enhancer-binding proteins (C/EBPs) have been shown to be involved in the regulation of cAMP-dependent gene expression [28]. C/EBP mediates constitutive and most cAMP-stimulated IL-10 transcription in myelomonocytic cell lines [28]. An important role of $A_{2A}R$ activation in IL-10 production is suggested by the observation that myeloid-selective deletion of the $A_{2A}R$ in *lysM*Cre-$A_{2A}R^{f/f}$ mice reduced by over 95% production of IL-10 by tumor-infiltrating myeloid cells [15, 29]. $A_{2A}R$ activation also enhances IL-10 production by *E. Coli*-challenged macrophages, and this effect is mediated by C/EBPβ binding to the IL-10 promoter [30]. TmDCs may be especially sensitive to $A_{2A}R$ stimulation due to their high expression of $A_{2A}Rs$. By activating protein kinase A in tmDCs, $A_{2A}R$ activation may function to suppress the production of proinflammatory cytokines by increasing the production of NR4A transcription factors that reduce NF-κB activation [31] as well as by enhancing IL-10 production.

The high levels of $A_{2A}R$ expression on tmDCs may also contribute to the control of vascular inflammation [32]. Adenosine inhibits platelet degranulation and suppresses the expression of

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adhesion molecules by activating $A_{2A}R$ and $A_{2B}R$ not only on platelets, but also on endothelial and vascular smooth muscle cells. The interaction between CD31 on tmDCs and CD38 on endothelial cells, in addition to mediating cell–cell interactions, may influence adenosine signaling because CD38 has enzymatic activity and converts extracellular NAD^+ to ADP-ribose, which can be further metabolized to AMP and adenosine by CD203a and CD73, respectively [7]. Thus, a tight association via CD31-CD38 of tmDCs and endothelial cells may enhance the local production of adenosine due to an ecto-enzyme cascade. The resulting locally produced adenosine may occupy adenosine receptors on platelets, tmDCs and endothelial cells to inhibit platelet degranulation and endothelial adhesion and enhance IL-10 production.

We recognize both strengths and limitations to our work. We describe a novel human population of DCs that regulate inflammation through secretion of IL-10 and modulates the polarization of naïve T cells toward an inflammatory phenotype. However, working with cells from young children has inherent limitations with respect to blood volumes and availability of normal controls.

Our work suggests that tmDCs teach tolerance to innate and adaptive immune cells in children after birth and may play an important role in suppression of inflammation in both health and disease. Future studies will investigate the link between Fc-mediated IgG stimulation and $A_{2A}R$ activation in immune regulation by tmDCs.

Materials and methods

Study population

Heparinized blood samples (2-9 mLs) were obtained from three pediatric cohorts: (i) healthy children, (ii) children with acute inflammatory conditions, and (iii) acute KD subjects sampled prior to treatment with IVIG and healthy convalescent KD subjects 2 weeks to 3 months after IVIG treatment. The first cohort included 15 healthy children: immune monitoring (characterization and enumeration of immune cell subsets by flowcytometry) was performed in 4 males and 8 females, aged 1 to 8.5 years. $A_{2A}R$ expression was measured in cells from 1 male and 1 female, aged 2 and 4 years, respectively. Two healthy males age 5 and 9.3 years and 1 healthy 3 year old female were enrolled for functional experiments with FACS-sorted tmDCs and naïve T cells in vitro. The second cohort included 5 children with acute febrile illnesses, 1 male and 4 females, aged 1.5 to 5.5 years and diagnosed with adenovirus infection ($n = 1$), viral syndrome ($n = 3$), and systemic onset juvenile idiopathic arthritis (soJIA) ($n = 1$). The third cohort included 18 subjects with KD, 12 males and 6 females, aged 0.5 to 11.9 years, who were enrolled for immune monitoring. In 3 additional untreated acute KD subjects (1 male and 2 females aged 4.1 to 10.4 years) we studied $A_{2A}R$ expression on tmDC. We also performed functional studies with FACS-sorted tmDCs and mDC-T cell co-cultures and $A_{2A}R$ functional studies from convalescent KD subjects without arterial complications, 7 males and 4 females aged 1.6 to 4.3 years. All KD subjects were treated with intravenous immunoglobulin therapy (IVIG) during the acute illness. The study protocol for pediatric subjects was approved by the Institutional Review Board at UCSD (IRB 140220). Pediatric subjects were enrolled at Rady Children's Hospital, San Diego, following written parental informed consent and patient assent as appropriate. Eight healthy adult donors, 3 males and 5 females aged 21 to 60 years were recruited at the Scripps Research Institute (IRB 146346 and UCSD 101213X).

Dendritic cell characterization

DC populations were defined by binding of the following monoclonal antibodies (mAb) to cell surface markers and analyzed by flow cytometry: anti-human CD11c-APC, mouse IgG1κ, clone B-ly6, anti-human CD11b-APC-Cyanin 7 (Cy7), mouse IgG1κ, clone ICRF44, anti-human CD14-phycoerythrin (PE) Cy7, mouse IgG2aκ, clone M5E2, anti-human CD86-fluorescein isothiocyanate (FITC), mouse IgG1κ, clone 2331 (FuN-1), anti-human CD4-AlexaFluor 700, mouse IgG1κ, clone RPA-T4 from BD Biosciences; 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. Data were acquired with FACS ARIA II and analyzed using FACSDiva (BS Biosciences, San Jose, CA) software.

In some experiments tmDCs were FACS-sorted from PBMCs for further phenotypical characterization using the following combination of monoclonal antibodies: 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, from BD Biosciences and anti-human CD85d (ILT-4) PerCP-elfuor 710, mouse IgG2aκ, clone 42D1 from eBioscience. After cell sorting, tmDCs were further studied by flow cytometry using the following cell surface markers: anti-human CD31 PE-Cy7, mouse IgG1k, clone WM-59, anti-human CD8α-APC, mouse IgG1κ, clone RPA-T8 from eBioscience, anti-human CD1c-FITC [blood dendritic cell antigen 1] (BDCA-1)], mouse IgG2a, clone AD5-8E7 from Miltenyi, and anti-human CD34-AlexaFluor 700, mouse IgG1κ, clone 581 from Biolegend. The data were analyzed using FACSDiva software.

Functional assays with FACS-sorted mDC

Tolerogenic and $CD14^-$ mature mDCs were FACS sorted by using specific monoclonal antibodies and resuspended in complete RPMI, 5% AB human serum. Cells were seeded at 25×10^3 cells/well in 96 U bottomed plates (Falcon) and stimulated with purified Fc fragments (Meridian Life Sciences Inc., lot 5A02215). Cell cultures were incubated for 24 h at 37°C, 5.5% CO_2, and supernatants collected to measure IL-10, TGF-β and IL-12 secretion by ELISA (BD Bioscience) according to manufacturer instructions.

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Co-culture of tmDCs and T cells

CD4+ naïve T cells defined as CD4+CD45RA+CD127− were FACS-sorted from PBMC using a BD FACSAria II: 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. TmDCs (CD11c+, CD14+, CD4+, ILT-4+) were also sorted using BD FACSAria II with bioBUBBLE using the following combination of monoclonal antibodies: 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, and anti-human CD85d (ILT-4) PerCP-elfuor 710, mouse IgG2aκ, clone 42D1 from eBioscience. Anti-human CD11b-APC-Cy7, IgG1k, was used in combination with the antibodies to FACS-sort classical CD11c+ CD11b+ CD14− CD4−ILT-4− classical mDCs. FACS-sorted naïve T cells were re-suspended in complete RPMI 5% human AB serum, plated at the concentration of 25 $\times$ 10^4 cells/well either alone or in co-culture (10:1 ratio) with tmDCs (25 $\times$ 10^3) and stimulated with anti-CD3, mouse IgG1κ, clone UCHT1 from BD Bioscience at the following concentrations: 10, 1, 0.1 μg/mL; and anti-human CD28, mouse IgG1κ, clone CD28.2 from eBiosceince at a concentration of 1 μg/mL. Twenty four hours after culture, cells were frozen in TRIzol (Thermo Fisher). RNA was extracted using TRIzol (Thermo Fisher) and cDNA was made using Superscript IV (Thermo Fisher) following the manufacturer's instruction. Levels of IL-2 and interferon (IFN)γ transcripts were measured using TaqMan Assays (Thermo Fisher) and normalized to the expression level of TAF1B. IL-2 and IFN-γ were measured in culture supernatants by ELISA (eBioscience) according to manufacturer's instructions.

A2AR expression and function

Tolerogenic mDCs were collected from acute KD subjects prior to therapy and from healthy children and adults, and evaluated by flow cytometry for $A_{2A}R$ expression using the mouse anti-human monoclonal antibody 7F6-G5-A2, IgG2a [16, 33]. Cells were permeabolized for $A_{2A}R$ analysis to detect the intracellular epitope recognized by the anti-receptor antibody. Functional $A_{2A}R$ activity was measured downstream of receptor activation based on intracellular cAMP accumulation in FACS-sorted mDCs and tmDCs populations. In functional experiments, tmDCs were pretreated with Fc for 24 h and washed prior to stimulation with other agents for 15 min in buffer containing 1 μM of the selective $A_{2A}R$ agonist, regadenoson, the nonselective agonist, NECA, the selective $A_{2A}R$ antagonist, SCH58261, 1 U/mL adenosine deaminase (ADA, to deaminate endogenous adensoine), and 10 μM rolipram, a cAMP phosphodiesterase inhibitor. Cells were lysed with ice-cold perchloric acid, neutralized with potassium carbonate, incubated on ice for 30 minutes, and centrifuged at 10 000 rpm in a microfuge for 10 min. Twenty microliters of supernatants were used for triplicate cAMP assays in 96-well plates using time-resolved FRET (Perkin Elmer UltracAMP). To address the role of Fc stimulation in combination with adenosine agonists/antagonists, PBMCs derived from venous blood of convalescent KD children were isolated and 400 000 cells were seeded into wells of a 96-well flat bottomed plate in RPMI 1640 medium with 5% human AB serum, and incubated with or without Fc (10 mg/mL), adenosine deaminase (ADA)(1U/mL) (Roche), non-selective adenosine receptor agonist, NECA (Sigma-Aldrich), or selective $A_{2A}R$ antagonist, SCH 58261 (Sigma-Aldrich) for 24 h at 37°C. Medium from each well was collected and the amount of IL-10 secreted under the various conditions was measured by ELISA.

Statistics

Results were analyzed as medians with interquartile range (IQR). Comparison across treatment groups for $A_{2A}R$ expression or IL-10 production used analysis of variance with post-hoc Tukey's multiple comparison test.

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Authors contributions: A.F. designed, directed, supported the experimental work and wrote the manuscript. J.K. participated to all the experimental activities directed by Dr. Franco. G. L. studied $A_{2A}R$ the expression and function in tmDCs under the direction of Dr. Joel Linden. N. B. studied the tmDC in adults and participated to the FACS-sorting experiments to address tmDC functions together with L.E.H. C.S. performed RT-PCR of tmDCs and T cell co-cultures. A.H.T. and J.C.B. provided pediatric clinical samples. J.C.B. and J.L. helped editing the manuscript.

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Abbreviations: **A_{2A}R**: adenosine A_{2A} receptor · **Fc**: heavy constant region of immunoglobulins · **ILT-4**: immunoglobulin-like transcript receptor-4 · **IVIG**: intravenous immunoglobulins · **KD**: Kawasaki disease · **tmDCs**: Tolerogenic myeloid dendritic cells

Full correspondence: Dr. Alessandra Franco, Department of Pediatrics, Division of Allergy, Immunology and Rheumatology, University of California San Diego, School of Medicine, 9500 Gilman drive, La Jolla 92093-0641, USA
Fax: +1-858-246-0156
e-mail: alfranco@ucsd.edu

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