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*J Immunol* published online 16 March 2015
http://www.jimmunol.org/content/early/2015/03/14/jimmunol.1401434

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2015/03/14/jimmunol.1401434.DCSupplemental

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Regulatory T Cell–Derived Adenosine Induces Dendritic Cell Migration through the Epac-Rap1 Pathway

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Dendritic cells (DC) are one target for immune suppression by regulatory T cells (Treg), because their interaction results in reduced T cell stimulatory capacity and secretion of inhibitory cytokines in DC. We show that DC in the presence of Treg are more mobile as compared with cocultures with conventional CD4+ T cells and form DC–Treg aggregates within 2 h of culture. The migration of DC was specifically directed toward Treg, as Treg, but not CD4+ T cells, attracted DC in Boyden chambers. Treg deficient for the ectonucleotidase CD39 were unable to attract DC. Likewise, addition of antagonists for A2A adenosine receptors abolished the formation of DC–Treg clusters, indicating a role for adenosine in guiding DC–Treg interactions. Analysis of the signal transduction events in DC after contact to Treg revealed increased levels of cAMP, followed by activation of Epac1 and the GTPase Rap1. Subsequently activated Rap1 localized to the subcortical actin cytoskeleton in DC, providing a means by which directed locomotion of DC toward Treg is facilitated. In aggregate, these data show that Treg degrade ATP to adenosine via CD39, attracting DC to Treg. These effects were abrogated when CD39−/− Treg, which are not able to produce adenosine, were used in the DC–Treg interaction. Treg also decreased the migration of CD4+ T cells toward DC, as Treg, but not CD4+ T cells, attracted CD4+ T cells in Boyden chambers. Likewise, Treg deficient for CD39 or those treated with CD39 antagonists were unable to attract CD4+ T cells. These effects were restored when Treg were treated with ATP. Among inhibitory leukocytes, Treg constitute a well-defined subpopulation that is CD4+CD25Foxp3+ and exerts immunosuppressive actions on effector T cells and on APCs (3). For instance, we and others have shown that Treg are able to interconnect with DC via gap junctions (4, 5). After contact to Treg, DC produced reduced amounts of IL-1β and TNF-α, whereas the immunomodulatory cytokine IL-10 was upregulated. Furthermore, surface expression of immunosuppressive molecules such as B7H1 and B7H3 was augmented, resulting in a phenotype of DC that is not able to induce activation of effector T cells (1).

Recently, adenosine has been identified as an immunosuppressive factor, generated by Treg via the degradation of ATP by the sequential action of the ectonucleotidases CD39 and CD73 (6, 7). Naturally occurring Foxp3+ Treg constitutively express CD39 and CD73 (6–9), and in Foxp3+ CD4+ T cells the immune suppressor TGF-β, which has a profound role in generating “induced” Treg, upregulates CD73 expression. This renders the cells immunosuppressive by means of adenosine production (8). On the contrary, expression of CD73 is downregulated by proinflammatory cytokines such as IL-12 and IFN-γ, thus making the regulation of CD73 expression and the adenosine production thereof an important amplification loop for immune regulation (8).

Adenosine is well established as an immunosuppressive substance in the nervous and the immune systems. In the latter, adenosine has been shown in particular to downregulate the expression of T cell costimulatory molecules and the secretion of proinflammatory cytokines by DC (8, 10–12). Because DC and Treg do interact in vivo in peripheral tissues, as well as in LN, we aimed at investigating how Treg-derived adenosine is involved in guiding DC–Treg interactions and whether this interaction occurs randomly or directed. Using in vitro coculture systems, we show that DC in the presence of Treg-derived adenosine are highly motile and form clusters with Treg. These effects were abrogated when CD39−/− Treg, which are not able to produce adenosine, were used in the DC–Treg interaction.
cocultures. In Boyden chamber experiments, we further show that movement of the DC was directed toward the Treg. This was mediated by the A3 adenosine receptor (AR), because antagonists against this receptor blocked motility and aggregation of Treg and DC. Engagement of AR3 by Treg-derived adenosine in DC leads to activation of Rap1, eventually leading to reorganization of the actin cytoskeleton, and thus providing the molecular basis for increased motility and cluster formation. These data indicate that the production of adenosine by Treg is a critical factor for recruitment of DC, acting as a chemotactic agent that affects DC phenotype and function. The adenosine-regulated contact between Treg and DC may be one crucial mechanism by which the induction of immune responses in LN is regulated.

Materials and Methods
Mice and reagents
Wild-type mice were purchased from Janvier (Janvier Labs, Saint Berthevlin, France). CD39- (13) and AR2-deficient (14) mice were housed in the central animal facility of the University of Heidelberg. Media and supplements (Pen/Strep, HEPES, glutamine, FCS) were purchased from PAA (Cöle, Germany). Standard chemicals were purchased (not if stated otherwise) from Carl Roth (Karlsruhe, Germany). Adenosine and related chemicals were as follows: ATP, 5'-AMP; and adenosine, concentrations as indicated (Sigma-Aldrich, Taufkirchen, Germany): AR3 antagonist MRS1191 (Sigma-Aldrich); AR3 antagonist DPCPX, AR2A antagonist SCH-58261, AR2B antagonist SR142801 (all from Tocris, Wiesbaden, Germany); AR1 antagonist MS-201, AR1A antagonist SR140496A, all at 10 nM to 10 μM, Epac1 activator 8-CPT-2Me-cAMP, 200 μM, CD39 inhibitor sodium metatungstate (POM-1) 50–100 μM, as indicated (all from Tocris, Wiesbaden-Nordenstadt, Germany). The Epac1 inhibitor ESI-09 (200 μM) was obtained from Biolog (Bremen, Germany). Substances were diluted in either water or DMSO and respective solvent controls are referred to as “control” or “untreated,” respectively.

Preparation of cells, cocultures, and migration studies
Murine bone marrow-derived DC were prepared according to standard procedures. In brief, bone marrow cells were cultured in RPMI 1640 supplemented with GM-CSF and IL-4 (10 ng/ml each; eBioscience, Frankfurt, Germany) for 6 d with intermittent feeding. Nonadherent cells were harvested and used for further experiments. To obtain CD4+ T cells and Treg, respectively, we prepared single-cell suspensions from spleens by a collagenase digest (Collagenase IV; 800 U/ml; Cell Systems, Trois-Rivières, Canada) and running the cell suspension over magnetic beads by a collagenase digest (Collagenase IV; 800 U/ml; Cell Systems, Trois-Rivières, Canada). To enrich Treg, DC were labeled with FITC-phalloidin (1:1000; Enzo Lifesciences, Lörrach, Germany) and incubated for a further 15 min at 37°C, 5% CO2. Cells were fixed in 4% PFA/PBS (w/v) for 20 min at room temperature and washed several times with staining buffer (RPMI 1640, 50 mM glycine, 2% fetal calf serum, 10 mM Ethylenediaminetetraacetic acid (EDTA), 0.05% saponin). In a moist chamber, slides were incubated with anti-Rap1 Abs (Santa Cruz Biotechnology, Heidelberg, Germany) for 30 min followed by incubation with Alexa 549-labeled goat anti-rabbit Abs (1:200; Diana, Hamburg, Germany) for 30 min. After three washes, FITC-phalloidin was added to the slides for 20 min, which were then washed and mounted (Aquamount; Dako, Hamburg, Germany) with coverslips. Specimens were examined using a confocal microscope (Zeiss, Jena, Germany) or epifluorescence microscope (Perkin Elmer, Rodgau, Germany).

Statistical analysis
Student t test was used as indicated in the figure legends.
Results

DC and Treg form clusters in vitro

To investigate interactions between DC and Treg, we set up cocultures of the respective isolated cells and after 2 h we observed spontaneous aggregation of cells in culture dishes containing DC and Treg (Fig. 1A, Supplemental Video 1). After overnight culture, even larger aggregates formed. In contrast, in cocultures of DC with CD4+ T cells, no clusters formed (Supplemental Video 2). Likewise, in single-cell cultures of CD4+ T cells, Treg, or DC alone, respectively, no aggregation was recorded (data not shown).

Because DC mature in vitro and the expression of surface molecules is changing during maturation, we next tested differently matured DC in DC–Treg cocultures for their capacity to induce cell clustering. At first we determined the maturation state of the DC. As depicted in Fig. 1B, DC were cultivated for different time periods, and surface expression of maturation markers was assessed by FACS. As expected, expression of the maturation markers CD80 and CD86 increased too. When these differently matured DC were tested in cocultures with Treg, cell clustering occurred already when DC were immature and/or semimature (days 6 and 7; Fig 1C), whereas clusters of DC and conventional CD4+ T cells only formed with mature (day 8) DC. We therefore used in all subsequent experiments day 6 DC. We next analyzed whether DC stick to Treg specifically or whether they interact randomly with isolated T cells in general. For that end we cocultured DC together with a mixture of fluorescently labeled Treg and conventional CD4+ T cells. In these cultures (Fig. 1D), rapid formation of aggregates between DC and Treg (displayed in red) occurred. In contrast, conventional CD4+ T cells were mainly excluded from the aggregates. Therefore, we concluded that DC form clusters exclusively with Treg, but not with CD4+ T cells.

Production of adenosine by Treg is necessary for formation of DC–Treg clusters

One major difference in the expression of surface molecule between Treg and conventional CD4+ T cells is the constitutive expression of CD39 and CD73 by Treg. These two ectonucleotidases degrade ATP and release adenosine. To confirm production of adenosine by Treg, we cultivated Treg and conventional T cells in medium containing ATP with or without CD39 inhibitor (POM-1). After 2 h of incubation, the concentration of adenosine in tissue culture supernatants was determined by HPLC. Fig. 2A shows that incubation of Treg with ATP induces significant production of adenosine. When POM-1 was added to the cultures, adenosine production by Treg was blocked, indicating that the majority of the adenosine produced by Treg is derived from CD39-driven ATP degradation. Unstimulated CD4+ T cells did not express significant amounts of CD39, and thus did not produce adenosine in large quantities as compared with Treg.

We next added POM-1 to DC–Treg cocultures and observed abrogation of DC–Treg cluster formation (Fig. 2B). In addition, we measured the content of ATP in DC–Treg and DC–CD4+ T cell cocultures, respectively, and observed reduced ATP levels in DC–Treg cultures. In contrast, in DC–CD4+ cultures, the amount of ATP remained stable (Fig. 2C). Therefore, we concluded that Treg degrade ATP to adenosine, which is involved in inducing DC–Treg aggregation.

To further exclude off-target effects of POM-1 and to identify the source of adenosine, that is, being Treg-associated CD39, we performed aggregation experiments with cells isolated from CD39-deficient (CD39−/−) mice (Fig. 2D). We cultured CD39−/− Treg together with DC from wild-type mice and deficient mice and vice versa. When analyzing formation of aggregates after 2 h, we observed cell clusters only in cultures containing wild-type Treg, whereby the expression of CD39 by DC was dispensable. In contrast, Treg derived from CD39−/− mice, which are unable to produce adenosine, failed to aggregate with DC. Thus, because genetic ablation of CD39 exclusively on Treg but not on DC abrogated the clustering process, we concluded that production of adenosine by Treg via CD39 is crucially involved in inducing DC–Treg interactions.

Treg-derived adenosine has chemotactic activity on DC

In DC–Treg cocultures, we initially observed that cluster formation was preceded by rapid and enduring DC movement, leading to large aggregates. To quantify the movement of DC in cocultures, time-lapse video analysis was applied to follow the tracks of individual DC. We found (Fig. 3A) that in the presence of Treg, DC were more motile as compared with DC in coculture with CD4+ T cells. This increased motility was abrogated when the CD39 antagonist POM-1 was applied to the cultures.

Furthermore, we set up cocultures of DC and CD39−/− Treg and followed the individual tracks of the DC in vitro (Fig. 3B). Similar to POM-1 treatment, movement of the DC stalled in the presence of Treg devoid of CD39. Degradation of ATP to ADP and AMP is critically dependent on CD39 expression, but the final step of adenosine generation, that is, the conversion of AMP to adenosine, is facilitated by CD39 (8). Therefore, to test involvement of CD73 in adenosine generation by Treg, we added AMP to the cultures of CD39−/− Treg, which serves as substrate for CD73 and can be degraded to adenosine. In this study, we found (Fig. 3B) that migration of DC was comparable with that observed with wild-type Treg. Thus, these data indicate that Treg, via the sequential degradation of ATP to adenosine by CD39 and CD73, stimulates the motility of DC.

To test whether adenosine does not only stimulate “random” movement of DC but also exerts chemotactic activity, we performed experiments in Boyden chambers and analyzed migration of DC. In Fig. 3C, we show that DC exhibited increased movement toward adenosine. Likewise, enhanced migration of DC toward Treg, but not toward conventional CD4+ T cells was recorded. In contrast, this enhanced migration was reduced to control levels after incubating Treg with the CD39 antagonist POM-1. These data indicate that Treg-derived adenosine is chemotactic for DC. Because artificial membranes used in vitro in Boyden chambers only partially mimic tissues, we next performed gel invasion assays (Fig. 3D). Collagen gels, enriched in adenosine, were prepared and placed in culture vessels containing DC. After 2 h of incubation at 37°C, we found that most of the DC had actively “invaded” the gel containing adenosine. In contrast, gels prepared without adenosine were devoid of DC. Therefore, these data indicate that adenosine has chemotactic activity for DC.

The ARA2A mediates clustering of DC and Treg

Adenosine can act via four known receptors and we next wanted to analyze which type of AR is responsible for mediating the effects of adenosine on DC movement. To this end we performed PCR to test for expression of mRNA for AR in DC (Fig. 4A). We found strong mRNA expression for the ARA2A and the ARA3. However, with primers specific for ARA1, only trace amounts of PCR products can be found, and similarly the ARA2B-specific PCR yielded only low amounts of product. As a control, we performed PCR also with ARA2A−/− DC and found no PCR product with the specific primers, whereas expression of mRNAs for other AR was normal. Therefore, we conclude that DC can express the ARA2A, ARA2B,
and the AR_A3, whereas the expression of the AR_A1 is questionable due to nearly absent AR_A1-specific mRNA. To identify the receptor responsible for induction of DC–Treg clustering, we added graded doses of AR antagonist’s specific for the different AR to DC–Treg cocultures. After 2 h of coculture, only the antagonist of the AR_A2A was able to block DC–Treg aggregation (Fig. 4B). In contrast, other AR antagonists had no effects. This effect was detectable in concentrations of 10 nM to 10 μM (data not shown), which comprises the range of receptor-specific actions of the antagonists.

To further exclude off-target effects of the AR antagonists and to confirm our conclusion that AR_A2A mediates the observed effects on DC, we set up cocultures of Treg and respective DC derived from AR_A2A−/− mice. In this study, we show impaired aggregation and reduced migration of the DC as compared with wild-type DC (Fig. 4C). Therefore, we concluded that adenosine induces DC–Treg aggregation by engaging the AR_A2A on DC.

Because the AR_A2A has been described to signal via cAMP, we subsequently determined cAMP levels in DC after 60 min incubation with Treg or conventional CD4+ T cells, respectively. In this study, we recorded substantially increased levels of cAMP in DC derived from Treg cocultures. In contrast, DC reisolated from cultures with conventional CD4+ T cells showed only marginally increased levels of cAMP as compared with control DC (Fig. 4D).

Thus, these data suggest that adenosine signals in DC by elevating the intracellular concentration of cAMP. In an attempt to delineate the signaling pathways downstream of cAMP, we investigated the role of the first-choice signal mediator, that is, CREB, in DC after contact with Treg. Surprisingly, we could not detect any substantial modulation of CREB activity, regardless of whether DC were cocultivated with Treg or conventional CD4+ T cells (Fig. 4E). Therefore, the further intracellular signaling, downstream of cAMP, seemed to be independent of the classical CREB pathways. In addition to CREB, cAMP can also activate Epac1, which transduces signals along via Rap1. To prove direct involvement of Epac1 in cluster formation, we set up DC–Treg cocultures in the presence of the Epac1 antagonist ESI-09 (Fig. 4F) and observed abrogation of DC–Treg clustering, whereas in control cultures, large aggregates were formed. Thus, Treg-derived adenosine activates Epac1 in DC for further signal transduction.

**Treg-derived adenosine activates Rap1 and affects the actin cytoskeleton in DC**

The major substrate of Epac1 is the small GTP binding protein Rap1. Epac1 induces binding of GTP to Rap1, leading to activation. To test involvement of Rap1 in adenosine-induced signaling events in DC, downstream of the cAMP-Epac1 axis, we cultivated DC either alone or in the presence of adenosine, 2-Chloro-Ado (the stable analog of adenosine), or the Epac1 activator 8-CTP-2ME-cAMP, respectively. After 30 min, cell lysates were prepared and quantitative pull-down assays with subsequent Western blotting analyses were performed. These assays show (Fig. 5A) that activated Rap1-GTP is induced in DC after treatment with adenosine or with the Epac1 activator 8-CTP-2ME-cAMP. In further experiments, cocultures of DC and Treg were prepared as described before and the cells were separated 30 min after coculture.
Thereafter cell lysates were prepared and Rap1-GTP was determined. A significant induction of GTP-activated Rap1 also could be recorded in cocultures of DC with Treg, whereas DC alone and DC cocultured with conventional CD4⁺ T cells showed only medium levels of Rap1-GTP. Thus, these data indicate that Treg-derived adenosine induces Rap1 activation via an adenosine-cAMP signal transduction cascade.

It has been shown that Rap1 translocates within the cells to components of the cytoskeleton and/or the plasma membranes after activation. We therefore performed fractionation experiments to test for differential localization of Rap1 in DC after coculture with different T cell populations. DC were reisolated after 1 h of coculture with Treg or CD4⁺ T cells, respectively, and membrane and cytoplasmic fractions were prepared. Those fractions were tested for Rap1 by Western blotting. In Fig. 5B, we show that Rap1 is predominantly found in the membranous fraction of DC isolated from cocultures with Treg. In contrast, in DC alone or in DC derived from cocultures with CD4⁺ T cells, most of the Rap1 is present in the cytoplasm. A redistribution of Rap1 was also apparent when DC from different cocultures were examined by immunofluorescence (Fig. 5C). In this study, a colocalization of Rap1 with subcortical actin fibers in the veils of the DC was noticed in DC–Treg cocultures as opposed to control cultures showing a more spotted pattern of actin and Rap1. Because Rap1 has reported effects on the stability of actin fibers, we next measured the content of F-actin in DC in response to coculture with Treg and CD4⁺ T cells, respectively. Fig. 5D shows that Treg stabilize the content of F-actin in DC as compared with conventional CD4⁺ T cells. Thus, these data show that Rap1 is a central adenosine-driven switch in DC leading to remodeling of the actin cytoskeleton and its functions during migration of DC and during establishment of cellular contacts.

Discussion

In contrast with solid organs that are located at particular sides of the body and are surrounded by a defined cellular environment, the immune system is not localized to a defined cellular structure. Its cellular components, that is, the lymphocytes, are highly motile and can virtually be found in all remote tissues of the body. Thus, motility and directed migration is an integral part of the immune cell functions. In this article, we report that Treg can attract DC by means of adenosine that is produced by hydrolysis of ATP by the ectonucleotidases CD39 and CD73.

Adenosine has long been identified as acting as an immunosuppressive agent that downregulates production of proinflammatory cytokines by DC (8, 12) and monocytes, suppresses Ag presentation (10), shapes the polarization of T cells (11), and prevents the influx of proinflammatory leukocytes into inflamed tissues (6). But its ability to act as chemoattractant for DC has not been elucidated in detail yet. Nevertheless, the role of purines in affecting cell motility in general has been established for neurons, endothelial cells, leukocytes, and even cancer cells. For instance, microglia cells of the brain convert ATP to adenosine, resulting in enhanced migration and outgrowth into the brain (16), and che-

![FIGURE 2](http://www.jimmunol.org/)

**FIGURE 2.** Adenosine production by Treg is necessary to induce clustering. (A) Treg and CD4⁺ T cells were incubated in complete medium supplemented with ATP as indicated. To some cultures the CD39 antagonist POM-1 in different concentrations was added. After 2 h incubation, tissue culture supernatants were collected and analyzed for adenosine by HPLC. (B) Cocultures of DC and Treg were prepared in 24-well plates. To some cultures POM-1 was added. After 1 h incubation, photomicrographs were taken with magnifications as indicated. (C) Cocultures with different ratios of DC and Treg or CD4⁺ T cells, respectively, were incubated for 2 h. Thereafter aliquots of the respective tissue culture supernatant were analyzed for ATP using a commercially available assay. Shown are the mean values ± SD of quadruplicates. The asterisk marks a significant (*p < 0.05) difference. (D) DC and Tregs were prepared from control (wild-type [wt]) and CD39-deficient mice (CD39⁻/⁻) and subjected to cocultures. After 2 h incubation, photomicrographs were taken with an inverted microscope.
FIGURE 3. Treg stimulate migration of DC. (A) Cocultures of DC with Treg, with Treg and POM-1 or CD4+ T cells, respectively, were subjected to time-lapse video microscopy for 30 min. Thereafter individual tracks of DC in the culture were analyzed. (B) Cocultures of DC with CD39−/− Treg ± AMP (10 μM) as indicated. Track analysis was performed as in (A). Shown are the tracks of three individual DC for 30 min in one typical experiment of five. Distance is indicated in pixels. (C) The lower compartment of a Boyden chamber was loaded with medium, CD4+ T cells, adenosine, Treg, or Treg and POM-1, respectively. A total of 1 × 10^5 DC was subjected to the upper compartment. After 2 h incubation, DC that migrated into the lower compartment were counted. Displayed are the mean values ± SD of four experiments. The asterisk indicates a significant difference (*p < 0.01). (D) Collagen gels (5 mm × 5 mm) with or without adenosine were prepared and placed into culture vessels containing DC. After 2 h incubation, photomicrographs were taken, focusing on the gel-medium border. Arrows indicate DC outside the gel (left) and inside the gel (right).
motaxis of neutrophils toward the chemoattractant peptide fMLF depends on adenosine (17, 18). Furthermore, endothelial cell migration and consequently angiogenesis is stimulated by ATP (19), and motility of breast and lung cancer cells is regulated by ATP as well (20, 21). Interestingly, in these reports, the initial purine released into the environment was ATP and not adenosine.

![FIGURE 4. Adenosine signals through AR\textsubscript{A2A} in DC. (A) mRNA from wild-type and AR\textsubscript{A2A}\textsuperscript{−/−} DC was prepared and analyzed by PCR for expression of mRNA for AR\textsubscript{A1}, AR\textsubscript{A2A}, AR\textsubscript{A2B}, and AR\textsubscript{A3}, respectively. (B) Antagonists against all four AR were added to DC–Treg cocultures (all 10 μM). After 2 h incubation, photomicrographs were taken. (C) Treg were cocultivated with wild-type DC or DC derived from AR\textsubscript{A2A}\textsuperscript{−/−} mice, respectively, followed by track analysis of the DC as described in Fig. 3. (D) DC were either cultivated alone (DC) or together with CD4\textsuperscript{+} T cells or Treg, respectively. After 1 h, lysates of the reisolated DC were prepared and analyzed for cAMP content by a commercially available assay. Shown are the mean values of quadruplicates ± SD of a typical assay. The asterisk indicates a significant difference (*p < 0.01). (E) DC, Treg, CD4\textsuperscript{+} T cells, and adenosine, as indicated, were cocultured and treated as in (D), and CREB/pCREB concentrations were determined by ELISA. Lysates provided by the supplier served as positive and negative controls. Shown are the mean values of quadruplicates ± SD of a typical assay. (F) DC were cocultured with Treg, and the Epac1 inhibitor ESI-09 was added to one set of cultures. After 2 h incubation, photomicrographs were taken.](http://www.jimmunol.org/)

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and only after ATP was converted by CD73/CD39 to adenosine, chemotactic activity developed in an autocrine fashion. Because DC express only sparse amounts of CD73, and thus are unable to produce significant amounts of adenosine by degradation of ATP by themselves, it is conceivable that ATP in the tissue environment is degraded by Treg, and thus acts mostly paracrine on DC.

Motility of cells in general is dependent on the rearrangement of the cytoskeleton and requires a coordinated action of actin fibers and adhesion molecules. For directed cell migration, as opposed to “random walking,” cells have to become polarized, that is, the actin cytoskeleton has to establish cytoplasmic protrusions into one direction and receptors as sensors have to be located here to direct the cell movement. As for adenosine, so far the AR A3 (17, 22) and the AR A2A (23) have been demonstrated to exhibit gradient-dependent redistribution in the cell membranes and to cause directed migration of neutrophils. Moreover, data on immature human monocyte-derived DC (moDC) have shown that adenosine acts immunosuppressive and chemotactic on DC, most

FIGURE 5. Activation and translocation of Rap1 in DC by adenosine. (A) DC were cultured in presence of adenosine, 2-chloro-adenosine, 8CPT-2Me-cAMP, or were left untreated. In some experiments, cocultures of DC with CD4+ T cells or Treg were prepared. After 1 h culture, DC were isolated, lysates were prepared, and activated Rap1-GTP was determined by quantitative pull-down assays. (B) DC isolated from cocultures as indicated were subjected to differential detergent fractionation to yield the cytoplasmic fraction and the membranes. Subsequently, the content of Rap1 was determined by Western blotting. (C) DC were cultivated on coverslips together with Treg or CD4+ T cells. After fixation, cells were stained for actin with FITC-labeled phalloidin (green) and with Abs specific for Rap1 (red), and confocal pictures were taken with a 63× objective. (D) Cocultures of DC and Treg or DC and CD4+ T cells were fixed with PFA and stained for CD4, CD11c, and actin, using specific Abs and FITC-phalloidin as indicated. In FACS analysis, single CD4+ T cells were gated out and the remaining cells were analyzed for F-actin content by measuring FITC-phalloidin content. A typical histogram overlay is shown, with the numbers in the graph displaying the mean FITC fluorescence.
likely by engaging G or G0 protein–coupled AR\textsubscript{A1} and AR\textsubscript{A3} (24, 25). This is partially corroborated by results from Schnurr et al. (26), showing a migratory response of plasmacytoid DC toward adenosine; but as opposed to moDC, this effect was mediated by AR\textsubscript{A1}.

In moDC, expression of the AR\textsubscript{A3} is strongly regulated, whereby it is only expressed by immature moDC and downregulation occurs during maturation (24, 27). Although we did not perform a detailed expression analysis of AR in murine DC, we found similar to Panther et al. (24) strong expression of AR\textsubscript{A2A} and AR\textsubscript{A3}. However, according to our data, the effects on migration of murine DC were mediated by AR\textsubscript{A2A}. Thus, different subtypes of human and murine DC have a diverse repertoire of different AR, and their effects may vary depending on the expression and maturation status.

As a major switch in DC for signaling induced by adenosine, we identified the Epac-Rap1 axis. Epac is a cAMP-dependent guanine-nucleotide-exchange factor for small GTPases Rap1 and Rap2, which exert effector functions upon GTP binding (28). This system signals independent from CREB; however, interconnection with protein kinase A signaling has been described. It is active in a variety of cells, and Epac-Rap activation provides a link to three main adrenergic signaling cascades in cells, affecting the mAKAP and the Rac/Ras pathways, and even more interestingly, it regulates cell adhesion and motility in different cell types (29, 30). In DC, Rap1 signaling has so far only been linked to reduced cytokine production (31), but our observation of Rap1 being involved in guiding chemotaxis in leukocytes is supported by analogous investigations in monocytes. In this study, TLR-induced chemotaxis (32) is guided by Rap1, and global activation of Rap1 affects MAC-1-dependent adhesion (33) and reorganization of the actin cytoskeleton (34). Of note, engagement of serotonin receptors activates Rap1 via cAMP and induces enhanced migration of the monocyctic cell line U937 (35). Because serotonin, similar to adenosine, has widely been described to convey signals in nervous tissues, the Epac-Rap1 pathway might reflect one signaling pathway by which immune cells may integrate signals derived from neurotransmitters. Beyond its function in mediating cell motility, Rap1 can be linked directly to immunosuppressive functions as it suppresses release of cytokines in DC (31) and is involved in conveying signals leading to abrogation of immune synapses between APC and T cells (36). This is in agreement with previous studies showing that Treg are indeed able to suppress DC functions, such as Ag presentation and cytokine production, and that cAMP levels, which induce activated Rap1, are elevated in DC after contact to Treg (37). Therefore, the activation of Rap1 pathways in DC by Treg-derivased adenosine may at first lead to initiation of DC–Treg contacts through chemotaxis and in a second step may induce a suppressive phenotype of DC once DC–Treg aggregates have formed.

It is known that purines such as ATP may serve as a “find me” signal for leukocytes, independently from the presence of Treg (17). Because under noninflammatory conditions, extracellular ATP concentrations are kept low and only after necrotic cell death increased ATP levels and substantial gradients of the metabolite adenosine are present. Thus, adenosine-induced chemotaxis may be one mechanism by which DC are normally recruited for clearance and detoxification of dying cells in peripheral tissues. However, the same chemotactic signals may also be used by Treg to exert immune suppression. For instance, if only minor tissue damage is induced, little amounts of released ATP can effectively be converted by CD39/CD73 Treg to adenosine, which are more sensitive to ATP as compared with conventional CD4\textsuperscript{+} T cells (38). That can attract DC, resulting in interaction between Treg and DC, inducing downregulation of DC activation and dampening of the induction of immune reactions. Thus, this mechanism may play a role in preventing overboading immune reactions in response to minor injury or spontaneous cell death.

In summary, our data show that adenosine derived from degradation of ATP by Treg acts chemotactic for DC by inducing Epac-Rap1–dependent signaling events. This is an important pathway to mobilize and attract immature DC via their AR\textsubscript{A2A}. In light of results published by Onishi et al. (39), showing that Treg may shield DC from effector T cells by simply outcompeting effector T cells for binding to MHC class II molecules on their surface, the drawing of DC into Treg–DC contacts also reduces the possibility for immunostimulatory interactions between DC and effector T cells, and thus may help to prevent activation of effector T cells by DC.

Acknowledgments

We thank M. Thome for excellent technical assistance. Confocal microscopes were provided by the Nikon Imaging Center at the University of Heidelberg.

Disclosures

The authors have no financial conflicts of interest.

References

Legend for supplemental Data S1 and S2

**S1:** DC were cultivated together with isolated Treg in 24 well plates and placed into an environmental chamber mounted to an inverted microscope (Leica) equipped with a digital camera (Diagnostic Instruments). Over a period of 45 min every 30 sec one picture was taken. The sequence of pictures was converted into a movie file using adequate software (Metaview). The movie comprises a 30 min period of a typical experiment.

**S2:** DC were cultivated together with isolated CD4$^+$ T cells in 24 well plates and placed into an environmental chamber mounted to an inverted microscope (Leica) equipped with a digital camera (Diagnostic Instruments). Over a period of 45 min every 30 sec one picture was taken. The sequence of pictures was converted into a movie file using adequate software (Metaview). The movie comprises a 30 min period of a typical experiment.