Adenosine is produced as a metabolic byproduct and exerts dose-dependent suppression of T-cell activation. Naive T cells are maintained in a quiescent state that requires integration of proliferative and survival signals with signals from environmental cues. Here, we identify one such cue as extracellular adenosine. The A2AR is the predominant adenosine receptor subtype expressed by T cells (Su et al., 2004) and is induced when these cells are activated (Lappas et al., 2005). A2AR activation increases cAMP to suppress TCR signaling (Ohta and Sitkovsky, 2001; Lappas et al., 2005; Ohta et al., 2009; Linden and Cekic, 2012). In tissues, basal adenosine concentrations are high enough to engage A2ARs (Su et al., 2004). We show that A2AR signaling regulates T cell development and maintenance to sustain normal numbers of naive T cells in the periphery.
Adenosine regulates T cell homeostasis | Cekic et al.

endogenous adenosine is sensed by A<sub>2A</sub>Rs as an environmental cue that prevents IL-7R down-regulation after TCR stimulation. This signaling pathway increases naïve T cell survival.

**RESULTS**

A<sub>2A</sub>R deficiency impairs peripheral T cell homeostasis

An analysis of the ImmunGen database (Heng and Painter, 2008) confirms prior studies showing that A<sub>2A</sub>R mRNA is the predominant adenosine receptor transcript expressed by T cells (Fig. 1 A). Compared with wild-type animals, mice lacking the A<sub>2A</sub>R gene, Adora2a<sup>−/−</sup>, have smaller spleens (wild type = 110 ± 6 mg vs. Adora2a<sup>−/−</sup> deficient = 73.75 ± 6 mg) and LNs (not depicted), suggesting intrinsic signaling by A<sub>2A</sub>Rs even in unstressed mice. We compared the frequencies and numbers of lymphoid cell populations in Adora2a<sup>+/+</sup> and Adora2a<sup>−/−</sup> mice. Global Adora2a deletion significantly reduced the number of naïve T cells (CD44<sup>lo</sup>CD4<sup>+</sup> and CD44<sup>lo</sup>CD8<sup>+</sup> T cells) in blood and peripheral LNs (Fig. 1, B and C) without affecting numbers of B, NK (Fig. 1 B), or myeloid cells (not depicted). To a lesser but still significant extent, numbers of A<sub>2A</sub>R-deficient CD4<sup>+</sup>CD44<sup>hi</sup> and CD4<sup>+</sup>Foxp3<sup>+</sup> T cells were also reduced in LNs but not spleen (Fig. 1 D). This may be because of a reduction in the precursor naïve CD4<sup>+</sup> T cell population. Overall, these data demonstrate that basal A<sub>2A</sub>R signaling contributes to the maintenance of naïve T cell numbers in the periphery.

**Cell-intrinsic A<sub>2A</sub>R signaling regulates IL-7Rα expression**

Maintenance of naïve T cells is mediated by homing signals to peripheral lymphoid organs through CCR7 and CD62L and survival signals from growth factor receptors. Deletion of Adora2a did not cause significant reductions in the expression of homing receptors CCR7 (Fig. 2 A) or CD62L (Fig. 2 B), suggesting that the decrease in naïve T cell numbers in Adora2a<sup>−/−</sup> mice is not caused by a homing defect. IL-7 is
the major survival factor for naive T cells. We observed a substantial reduction in the cell surface expression of IL-7Rα (CD127) on naive T cells from Adora2a−/− mice as compared with wild-type controls (Fig. 2 C). CD127 is not changed on Adora2a+/+ or Adora2a−/− CD44hiCD8+ T cells and only slightly decreased on Adora2a+/+ or Adora2a−/− CD44hiCD8+ T cells. Hence the number of cells with a memory phenotype (CD44hi) is minimally changed in Adora2a−/− mice (Fig. 1 B) despite a substantial decrease in the naive T cell population (Fig. 1 B and C).

Global A2AR deletion in mice produces a constellation of effects mediated by deletion of receptors on multiple cell types. The consequences vary among mouse strains but may include aggressiveness, hypogalgesia, high blood pressure, and increased basal levels of inflammatory mediators derived in part from APCs (Ledent et al., 1997; Ohta and Sitkovsky, 2001). To determine whether Adora2a deletion has cell-intrinsic effects to influence T cell number or IL-7R expression, we performed mixed bone marrow reconstitution experiments. We transferred 1:1 mixtures of bone marrow cells derived from Adora2a+/+ (CD45.1+) and Adora2a−/− (CD45.2+UBC-EGFP) mice to irradiated wild-type (CD45.2) recipients and measured the ratio of transferred T cells after 8 wk. Similar experiments used EGFP+ hosts and bone marrow from Adora2a+/+ (CD45.1+) and Adora2a−/− (CD45.2+) to exclude the possibility that a decrease in T cell numbers might be caused by EGFP expression. Analogous experiments were conducted using mixtures of Adora2b+/+ (CD45.2+) and Adora2b−/− (CD45.1+) bone marrow. Compared with wild-type cells, low numbers of Adora2a-deficient but not Adora2b-deficient T cells were detected in reconstituted recipients, consistent with the conclusion that T cell A2AR (Fig. 2 D) but not A2bR signaling (Fig. 2 E) facilitates T cell homeostasis.

We observed different patterns of IL-7Rα expression on T cells in chimeric mice reconstituted with Adora2a+/+ and Adora2a−/− bone marrow cells, suggesting that decreased IL-7Rα in naive T cells is a cell-intrinsic consequence of Adora2a deletion (Fig. 2 F). Accordingly, transfer of a 1:1 mixture of Adora2a+/+ (CD45.1) and Adora2a−/− (CD45.2+UBC-EGFP) spleen and LN cells to wild-type recipients (CD45.2+) for 0–2 wk resulted in a reduced ratio of Adora2a−/− to Adora2a+/+ in naive but not memory phenotype T cells over time (Fig. 2 G). To determine whether the decrease in IL-7Rα expression in Adora2a−/− T cells influences their sensitivity to IL-7, we cultured Adora2a−/− or Adora2a+/+ cells from LNs in the absence or presence of IL-7. A2aR deficiency significantly decreased the maximum response to IL-7 (Fig. 2 H) without affecting the receptor affinity for IL-7 (see EC50 values for IL-7), suggesting that a decrease in the number of IL-7Rs reduces survival. These data demonstrate that cell-intrinsic A2aR signaling favors peripheral T cell accumulation by regulating IL-7R expression.

**Protein kinase A (PKA) stimulation by A2aR activation prevents TCR-induced down-regulation of IL-7R**

IL-7R and TCR signaling are both known to inhibit IL-7Rα expression. Therefore, we measured the effects of A2aR activation on TCR- or IL-7–induced decreases in cell surface IL-7Rα expression. Fig. 3 A shows that the addition of the selective A2aR agonist CGS 21680 to T cells in vitro significantly inhibits TCR- but not IL-7–induced down-regulation of cell surface IL-7Rα (CD127), suggesting that A2aR signaling interferes with TCR, but not IL-7R signaling. CGS 21680 reduced the potency but not the maximal effect of αCD3 to down-regulate CD127. These results are consistent with the concept that A2aR signaling attenuates submaximal TCR signaling. Unlike naive T cells, maintenance of memory phenotype T cells in general does not require self-peptide–MHC and TCR interactions in vivo (Surh and Sprent, 2008). Therefore, differential TCR stimulation may explain why IL-7Rα expression is strongly reduced in naive but not memory phenotype T cells despite similar levels of Adora2a expression in both T cell types (Fig. 1 A).

TCR stimulation causes activation of the phosphatidylinositol 3-kinase (PI3K)–AKT pathway, which negatively regulates IL-7R expression (Pallard et al., 1999; Barata et al., 2004; Riou et al., 2007; Kerdiles et al., 2009; Hand et al., 2010). Therefore, unrestrained AKT activity reduces naive T cell numbers by decreasing T cell survival and accumulation of memory phenotype T cells. The PI3K–AKT pathway is inhibited by PKA in response to elevated cAMP (Kim et al., 2001; Lou et al., 2002). Because A2aR signaling regulates T cell signaling events primarily through PKA, we hypothesized that A2aR signaling reduces AKT activation and down-regulates IL-7Rα in a PKA-dependent manner. A2aR stimulation significantly reduced AKT phosphorylation (Fig. 3 B) after TCR signaling, and this effect was blocked by the competitive A2aR antagonist SCH 58621. Accordingly, basal AKT phosphorylation, which was measured by flow cytometry analysis (because immunoblotting was not sufficiently sensitive), was higher in A2aR-deficient naive T cells than in wild-type naive T cells (Fig. 3 C). The effect of A2aR stimulation to increase IL-7Rα expression after TCR stimulation was completely reversed by the highly selective PKA inhibitor KT 5720 (Fig. 3 D). To determine whether PI3K inhibition and A2aR stimulation are additive, we examined the effects of strong TCR stimulation in the absence or presence of the PI3K inhibitor LY 294002 and the A2aR agonist CGS 21680. We chose this strategy because IL-7R down-regulation after weak TCR stimulation can be completely reversed by CGS 21680 (Fig. 3 C) and a possible additive effect of PI3K inhibition and A2aR stimulation may not be observed. LY 294002 alone was sufficient to inhibit TCR–dependent IL-7Rα down-regulation, and A2aR stimulation and PI3K inhibition together were not additive (Fig. 3 E). Interestingly, IL-7 is also known to stimulate the PI3K–AKT signaling pathway. However, CGS 21680 had no effect on IL-7–induced down-regulation of the IL-7R. Recent studies have shown that IL-7–induced down-regulation of IL-7R is regulated primarily by the JAK3–STAT5 pathway (Henriques et al., 2010; Ghazawi et al., 2013). In CD8 T cells, inhibition of PI3K did not prevent IL-7–induced IL-7R down-regulation (Ghazawi et al., 2013). Furthermore, addition of the PI3K inhibitor LY 294002 had no effect on IL-7–induced down-regulation of the IL-7R.
Figure 2. A2AR signaling regulates survival of naive T cells by controlling surface expression of IL-7Rα. (A and B) CCR7 (A) and CD62L (B) staining of naive (CD44lo) T cells from blood of Adora2a+/+ and Adora2a−/− mice and corresponding geometric means. Data are from two independent experiments. (C) CD127 (IL-7Rα) staining of naive (CD44lo) and memory (CD44hi) T cells from blood of Adora2a+/+ and Adora2a−/− mice and corresponding geometric means. (D) Recovery of Adora2a+/+ and Adora2a−/− (CD45.2/UBC-EGFP) lymphocyte populations from lethally irradiated wild-type animals (CD45.2) 12 wk after reconstitution with a 1:1 mixture of Adora2a+/+ (CD45.1) and Adora2a−/− (CD45.2) bone marrows (results are representative of three independent experiments, n = 5). (E) CD4+ cell recoveries in mixed bone marrow chimeras of Adora2b+/+ (CD45.2) and Adora2b−/− (CD45.1) 8 wk after reconstitution [results are representative of two or three independent experiments, n = 5]. (F) CD127 (IL-7Rα) staining of Adora2a+/+ or
effect on IL-7–induced IL-7R down-regulation in cultured CD4 T cells (Fig. 3 F), indicating that IL-7 and TCR signaling use different pathways to regulate IL-7 receptor expression. Memory phenotype T cells do not require endogenous TCR signals to be maintained. However, they still may receive these TCR signals, which can be regulated by Adora2a signaling. Therefore, we stimulated naive and memory T cells and tested the effect of Adora2a stimulation on CD127 expression. Adora2a signaling had a larger impact on CD127 expression in naive T cells yet still significantly inhibited CD127 down-regulation in memory phenotype T cells (Fig. 3 G), indicating that (a) Adora2a signaling is more effective at preventing CD127 down-regulation during tonic TCR signaling in naive than in memory T cells, (b) memory phenotype T cells may express different factors that prevent them from down-regulating CD127 expression during tonic TCR signals, or (c) memory phenotype T cells may not respond to tonic TCR signals as naive T cells do. Overall, these data suggest that stimulation of the PKA pathway by Adora2a activation controls IL-7R expression by inhibiting TCR-induced AKT activation more in naive than memory T cells.

**Adora2a signaling regulates proliferation and survival of naive T cells**

Homeostatic proliferation of naive T cells is driven by TCR interactions with MHC complexes presenting self-peptides (Ernst et al., 1999; Goldrath and Bevan, 1999; Muranski et al., 2000). Deletion of negative regulators of TCR activation or unrestrained AKT activity leads to loss of quiescence and increased homeostatic proliferation (Yang et al., 2011). To investigate the impact of Adora2a signaling on T cell proliferation, we performed BrdU incorporation assays. Fig. 4 A shows that a greater percentage of Adora2a−/− than Adora2a+/+ naive T cells but not memory T cells (Fig. 4 B) incorporate BrdU, consistent with decreased TCR signaling activity in naive T cells in response to endogenous Adora2a signaling. Similar BrdU incorporation in Adora2a+/− and Adora2a+/+ memory T cells suggests that lymphopenia in Adora2a−/− mice is not responsible for increased proliferation of naive Adora2a−/− T cells.

IL-7 signaling prevents apoptosis and keeps naive T cells alive (Surh and Sprent, 2008). We observed that Adora2a−/− T cells are hyporesponsive to IL-7 (Fig. 2 E). Accordingly, decreased IL-7R signaling expression was associated with decreased expression of antiapoptotic Bcl-2 (Fig. 5, A and B), which is a downstream target of IL-7 signaling, whereas the total cell expression of proapoptotic Bax was not affected (Fig. 5 A). Adora2a deletion increased the number of apoptotic naive T cells (Annexin V+Live-Dead−) in blood and increased the number of dead T cells (Annexin V+Live-Dead+) in peripheral LN (Fig. 5, C and D). (Note that some cell death occurs during tissue processing. Therefore, the actual differences in apoptosis are likely larger than illustrated.) These data indicate that Adora2a−/− T cells proliferate more than wild-type cells but fail to accumulate as a result of increased apoptosis.

**Adora2a signaling affects thymic T cell development**

Adenosine concentrations are higher in the thymus than in other organs, possibly because of rapid cell turnover (Resta et al., 1997; Cekic et al., 2011). Adora2a mRNA transiently increases during early thymic T cell development and peaks during the DN2B phase (ImmGen database consortium [Heng and Painter, 2008]; Fig. 6 A). Therefore, we next sought to determine whether Adora2a signaling influences thymic T cell development. In Adora2a−/− mice, numbers of CD4 and CD8 double-negative (DN phase) populations (early thymic precursors) are unchanged, whereas CD4 or CD8 double-positive (DP) and single-positive (SP) cells decrease significantly (Fig. 6 B). To determine whether this is caused by cell-intrinsic signaling, we measured the ratios of thymic precursors in chimeric mice reconstituted with 1:1 mixtures of bone marrow from Adora2a+/+ (CD45.1) and Adora2a−/− (CD45.2) mice transferred to recipient mice (CD45.2ΔUBC-EGFP+) 8–12 wk after irradiation. Compared with wild-type cells, large reductions in the relative proportions of Adora2a−/− DP and SP but not DN thymic precursors were detected in reconstituted recipients (Fig. 6 C). To determine whether Adora2a expression during early thymic T cell development is important for subsequent progression to DP and SP precursors, we produced Adora2a−/− mice and crossed these to syngeneic C57BL/6j mice carrying the Cre recombinase gene under control of the Lck promoter (Fig. 6 D, top). As illustrated in Fig. 6 A, Lck is first activated toward the end of the DN phase (Sprent and Surh, 2011). Adora2a mRNA expression isolated from Adora2a−/−; LckCre mice was reduced by 77% in DP, 72% in CD8SP, and almost 100% in CD4SP thymocytes, whereas no significant reduction was observed in DN precursors as compared with mRNA expression in thymocytes from Adora2a+/- littermates not expressing Cre recombinase, indicating efficient deletion of the Adora2a gene from T cells (Fig. 6 D, bottom). As can be

---

Adora2a−/− T cells (naive [CD4+] and memory [CD44hi]) from blood of irradiated mice reconstituted with 1:1 mixtures of bone marrow cells of Adora2a+/+ and Adora2a−/− mice (results are representative of two independent experiments, n ≥ 5). (G) 15 × 10⁶ cells pooled from spleens and LN of Adora2a+/+ (CD45.1) and Adora2a−/− (CD45.2; UBC-EGFP) mice were mixed 1:1 and injected into wild-type (CD45.2) recipients. Donor naive and memory T cell percentages in recipient spleens and LN were measured at 0, 1, and 2 wk after transfer (results are from two independent experiments, n ≥ 4). (H) 10⁶ LN cells from Adora2a+/+ and Adora2a−/− mice were cultured in the presence or absence of 0.01–10 ng/ml of recombinant mouse IL-7. Frequencies or numbers of live T cells (Annexin V−Live-Dead−) were measured after 72 h of ex vivo culture (representative of two independent experiments, n = 4). EC50s for naive Adora2a+/+ CD4 and CD8 T cells are 1.87 ± 0.06 and 2.03 ± 0.04, respectively. EC50s were calculated by nonlinear regression analysis using log[agonist] versus response (three parameters: top, bottom, and EC50). *, P < 0.05; **, P < 0.01; ***, P < 0.001 by two-way ANOVA and Bonferroni post-hoc analysis. Error bars are SEM.
Adenosine regulates T cell homeostasis | Cekic et al.

The surge in A2AR transcription that occurs during the DP stage may influence the survival of cells as they enter the SP stage. Thymic progression is not affected by lck-dependent deletion that occurs after the pulse of A2AR transcription (Fig. 6, A and D).

Figure 3. PKA stimulation by A2R activation prevents TCR-induced down-regulation of IL-7Rα. (A) Wild-type T cells were stimulated with varying concentrations of plate-bound anti-CD3 or recombinant mouse IL-7 in the presence or absence of 1 µM CGS 21680 (CGS) or vehicle control (<0.1% DMSO). CD127 (IL-7Rα) staining was performed after incubation at 37°C (5% CO2) for 24 h (n = 4, from three independent experiments). (B) Isolated wild-type T cells were stimulated by transfer to tissue culture plates pretreated with 5 µg/ml anti–mouse CD3 and 2 µg/ml anti–mouse CD28 antibodies in the absence or presence of 1 µM CGS 21680 or CGS 21680 + equimolar SCH 58621. Serine 473 and threonine 308 phosphorylation of AKT was detected by immunoblotting for the indicated times (experiments were repeated three times for serine 473 and twice for threonine 308 detection). (C) Basal serine 473 phosphorylation of Akt in Adora2a+/+ versus Adora2a−/− cells was measured by flow cytometry. (D) 100 nM of the selective PKA inhibitor KT 5720 was added to cultures of T cells stimulated with anti-CD3 in the presence or absence of 1 µM CGS 21680 or vehicle control (<0.1% DMSO). CD127 staining was performed after incubation at 37°C (5% CO2) for 24 h. (E) 2 µM of the selective PI3K inhibitor LY 294002 was added to cultures of stimulated T cells in the presence or absence of 1 µM CGS 21680 or vehicle control (<0.1% DMSO). CD127 staining was performed after incubation at 37°C (5% CO2) for 24 h. Data in C–E are representative of two independent experiments with similar results (n = 3). (F) Enriched CD44lo and CD44hi cells were stimulated by 1 µg/ml plate-bound anti-CD3 antibody in the presence or absence of 1 µM CGS 21680. CD127 (IL-7Rα) staining was performed after incubation at 37°C (5% CO2) for 24 h (n = 4, from two independent experiments with similar results). *, P < 0.05; ***, P < 0.001 by two-way ANOVA and Bonferroni post-hoc analysis. Error bars are SEM.

seen in Fig. 6 E, lck-mediated deletion of the A2R does not change the proportions and numbers of thymic precursors. This suggests that increased Adora2a transcription during early thymic development (Fig. 6 A) has important effects on thymic progression of T cell precursors toward a mature T cell phenotype. The surge in A2R transcription that occurs during the DP stage may influence the survival of cells as they enter the SP stage. Thymic progression is not affected by lck-dependent deletion that occurs after the pulse of A2R transcription (Fig. 6, A and D).
A2AR signaling is required for naive T cell maintenance in the periphery

We considered the possibility that naive T cells are reduced in numbers in Adora2a−/− mice only as a result of decreased thymic output. Because Lck-mediated deletion of Adora2a did not affect thymic progression, we compared the numbers of naive T cells in Cre− mice with Cre− littermates. We still observed a significant reduction in naive T cell numbers in the periphery without changes in B or NK cells or other T cell subtypes (Fig. 7, A–C). We also observed significant reductions in IL-7R expression and IL-7 responsiveness among naive T cells in Cre+ mice (Fig. 7, D and E). Overall, these data suggest that A2AR signaling helps to maintain normal numbers of naive T cells by regulating both thymic output and survival in the periphery.

IL-7R expression starts decreasing in Adora2a−/− T cells during thymic development and completely recovers in the absence TCR signaling

Besides engaging self-peptide–MHC complexes in the periphery, T cells also go through a selection process during their development when thymocytes with newly formed rearranged TCRs are selected based on their ability to interact with self-peptide–MHC complexes. IL-7R expression temporarily decreases during this process because of TCR signals and increases when newly formed T cells become SP and mature. DP thymocytes receiving too weak or too strong TCR stimuli are deleted at this stage. Because we hypothesize that A2AR signaling can fine tune TCR signaling to maintain IL-7R expression, we measured IL-7R expression on thymic precursors. Fig. 8 A shows that global deletion of Adora2a reduces IL-7Rα (CD127) expression in SP precursors. This effect is cell intrinsic because we observed reduced CD127 expression in Adora2a−/− thymocytes developing in the same thymus with Adora2a+/+ thymocytes after 1:1 bone marrow reconstitution (Fig. 8 B), suggesting that Adora2a−/− cells may receive stronger TCR signals during positive selection and either die or fail to fully up-regulate their IL-7Rs. Lck-dependent deletion of Adora2a did not affect IL-7Rα expression in thymic precursors (Fig. 8 C), suggesting that A2AR receptors produced during early thymic development are retained and help to maintain normal numbers of thymic precursors. Decreased IL-7Rα expression after DP to SP transition suggests an incomplete maturation of SP Adora2a−/− thymocytes. The proportions of Qa2+/HSA− cells were significantly lower among SP thymocytes isolated from Adora2a−/− mice as compared with wild-type controls (Fig. 8 D and Fig. S1), suggesting incomplete maturation rather than accumulation in thymus is the contributing factor in reduced naive T cell numbers in the periphery of Adora2a−/− mice.

Because our data suggest that it is the lack of regulation of TCR signals during thymic selection or peripheral maintenance in the absence of A2AR signaling that reduces IL-7Rα expression, we hypothesized that reduced IL-7Rα expression is not a permanent developmental defect; therefore, the cessation of TCR signaling should restore IL-7R expression in Adora2a−/− T cells. To test this, we measured the expression of IL-7Rα on CD4+ T cells in freshly isolated single cell suspensions or after sorting and incubation overnight at 37°C in medium supplemented with 5% fetal bovine serum. As can be seen in Fig. 8 E, IL-7Rα expression in naive Adora2a−/− CD4+ T cells increased to the levels of wild-type T cells after overnight incubation in vitro at 37°C. We observed a more modest but similar pattern for IL-4Ra expression (not depicted). Unlike IL-4 and IL-7R, IL-2R expression does not decrease with TCR stimulation. Accordingly, expression of IL-2R was similar between freshly isolated or incubated T cells from both A2AR-deficient and wild-type animals (not depicted). To show that absence of TCR signaling increases CD127 expression in Adora2a−/− T cells in vivo, we adoptively transferred GFP+ Adora2a−/− naive CD4+ T cells to wild-type or MHCII−/− mice. CD127 expression in adoptively transferred naive Adora2a−/− T cells remained low in wild-type animals but increased to the level in naive host cells in MHCII−/− mice (please note CD127 expression in wild-type and MHCII−/− T cells is similar). These data suggest that A2AR signaling by endogenous adenosine is important for the homeostatic balance of CD127 expression.
DISCUSSION

We show here that deletion of A$_{2A}$Rs strongly impacts naive T cell development and survival in C57BL/6 mice. A$_{2A}$Rs couple to the heterotrimeric G protein, primarily Gs and partially to Golf in the central nervous system (Schwindinger et al., 2010). These G proteins activate adenylyl cyclase and increase cAMP and PKA activity, which in turn inhibits AKT (Kim et al., 2001; Lou et al., 2002). AKT is a master regulator that stimulates T cell proliferation and reduces IL-7R$^\alpha$ expression downstream of TCR stimulation (Pallard et al., 1999; Rathmell et al., 2003; Barata et al., 2004; Riou et al., 2007; Kerdiles et al., 2009; Hand et al., 2010). IL-7 signaling is required for naive T cell survival. The current study shows that $Adora2a$ expression, which increases in early thymic precursors, helps these cells to progress through normal thymic development and to up-regulate IL-7R$^\alpha$ along with thymocyte maturation markers Qa2 and HSA after positive selection. The decrease in IL-7R$^\alpha$ in naive A$_{2A}$R$^{-/-}$ cells is completely reversed by culturing cells in the absence of TCR stimulation in vitro, suggesting that reduced IL-7R$^\alpha$ expression after thymic selection or during peripheral maintenance is not a permanent developmental defect. Therefore, although adenosine-mediated activation of the PI3K–AKT pathway. The results imply that tissue levels of endogenous adenosine are high enough to activate A$_2$ARs. Interestingly, adenosine concentrations are higher in the thymus than other organs (Resta et al., 1997; Cekic et al., 2011). The current study also shows that $Adora2a$ expression, which increases in early thymic precursors, helps these cells to progress through normal thymic development and to up-regulate IL-7R$^\alpha$ along with thymocyte maturation markers Qa2 and HSA after positive selection. The decrease in IL-7R$^\alpha$ in naive A$_{2A}$R$^{-/-}$ cells is completely reversed by culturing cells in the absence of TCR stimulation in vitro, suggesting that reduced IL-7R$^\alpha$ expression after thymic selection or during peripheral maintenance is not a permanent developmental defect. Therefore, although adenosine...
limits homeostatic T cell proliferation, it supports naive T cell development and survival.

TCR stimulation with self-peptide–MHC complexes drives homeostatic proliferation of naive T cells (Surh and Sprent, 2000, 2008). However, unlike memory T cells, only a small fraction of naive T cells proliferate during their lifetime, whereas most stay quiescent (Surh and Sprent, 2000). This suggests that survival plays a major role in maintaining naive T cell numbers. This explains why increased T cell proliferation in response to A2AR deletion fails to maintain normal T cell numbers. The data implicating the IL-7R as a key target of A2AR signaling agree with the proposal by Kerdiles et al.
Interestingly, IL-7R signaling itself causes the activation of the PI3K–AKT pathway and down-regulation of IL-7Rα. However, our findings and others suggest that the PI3K pathway is not the primary regulator of IL-7Rα down-regulation (Henriques et al., 2010; Ghazawi et al., 2013). In addition to activating the PI3K–AKT pathway, IL-7R signaling also activates the Jak3–Stat5 pathway. It has recently been shown that IL-7–induced down-regulation of IL-7R is
Our study demonstrates a greater effect of Adora2a deletion on naive T cells than memory T cells. Unlike naive T cells, most memory T cells lose the need for TCR interactions and rely on IL-7 and IL-15 for survival and homeostatic proliferation (Surh and Sprent, 2000, 2008). Accordingly, our study shows that A2AR signaling selectively prevents TCR-induced IL-7Rα down-regulation (Sprent and Surh, 2011). In Adora2a−/− mice, naive T cells have increased basal AKT activation and homeostatic proliferation. These events not only evoke loss of quiescence and reduced naive T cell survival, but also enhance accumulation of memory phenotype T cells. This may explain why the numbers of T cells with a memory phenotype in Adora2a−/− mice and wild-type mice are similar despite the fact that Adora2a−/− mice have lower numbers of precursor naive T cells.

The decrease in the numbers of naive T cells and CD127 expression were less dramatic after Lck-mediated deletion as regulated by two independent mechanism through Jak3–Stat5 signaling (Henriques et al., 2010; Ghazawi et al., 2013). The fact that A2αR activation selectively prevents TCR- but not IL-7Rα–induced down-regulation of IL-7Rx suggests that A2αR signaling selectively interferes with TCR–associated signaling events. Consistent with our observations, constitutive activation of AKT or removal of FoxO1 and Tsc1 (which are negatively regulated by AKT) also decreases naive T cell numbers without affecting numbers of memory T cells (Rathmell et al., 2003; Kerdiles et al., 2009; Yang et al., 2011). Recent studies show that unlike unrestrained AKT activation, constitutive activation of Stat5 significantly increases T cell survival without causing a large decrease in cell surface IL-7Rα expression (Hand et al., 2010). Therefore, it appears that naive T cell quiescence and survival can be achieved through a balanced activation of AKT and Stat5 signaling.

Our study demonstrates a greater effect of Adora2a deletion on naive T cells than memory T cells. Unlike naive T cells, most memory T cells lose the need for TCR interactions and rely on IL-7 and IL-15 for survival and homeostatic proliferation (Surh and Sprent, 2000, 2008). Accordingly, our study shows that A2AR signaling selectively prevents TCR-induced IL-7Rα down-regulation (Sprent and Surh, 2011). In Adora2a−/− mice, naive T cells have increased basal AKT activation and homeostatic proliferation. These events not only evoke loss of quiescence and reduced naive T cell survival, but also enhance accumulation of memory phenotype T cells. This may explain why the numbers of T cells with a memory phenotype in Adora2a−/− mice and wild-type mice are similar despite the fact that Adora2a−/− mice have lower numbers of precursor naive T cells.

The decrease in the numbers of naive T cells and CD127 expression were less dramatic after Lck-mediated deletion as
compared with global deletion of Adora2a, suggesting that reduction in thymic precursors contributes to decreased numbers of naive T cells in the periphery. It is also possible that the remaining (23%) A2AR transcript expression in DP thymocytes after cre-mediated deletion is sufficient to partially rescue T cells from losing CD127 expression. Therefore, our data suggest that early expression of A2ARs in the thymus contributes to the up-regulation of CD127 during positive selection that may contribute to the differential effects of global versus Lck-mediated Adora2a deletion.

Although adenosine accumulates in stressed or inflamed tissues, it also contributes to baseline homeostasis of several physiological processes, including cardiovascular (tissue oxygen delivery), neuronal (sleep cycle), and renal (glomerular filtration). In this study, we propose a new role for adenosine in the regulation of T cell homeostasis through A2AR signaling. It will be of interest in future studies to examine the effects of A2AR deletion during stressful conditions when adenosine is elevated. In the immune system, adenosine dampens excessive inflammation after tissue injury and activates tissue remodeling responses such as angiogenesis and fibrosis, thereby helping to establish long-term homeostasis after local or systemic disturbances. Ongoing studies indicate that A2AR signaling helps to maintain T cells in adenosine-rich hypoxic solid tumor microenvironments (unpublished data) parallel to our observation that adenosine may regulate CD127 expression even in effector/memory phenotype T cells. Therefore, determining how to extract therapeutic benefits by targeting adenosine receptors without disrupting homeostatic activities will be an important goal of future research.

MATERIALS AND METHODS

Cell lines, animals, and reagents. Animal experiments were approved by the Animal Care and Use Committee of the La Jolla Institute for Allergy and Immunology. 6-wk-old C57BL/6 and MHCIIdeficient mice were purchased from the Jackson Laboratory and used for experiments after being acclimated for 2–6 wk. Generation of the Adora2af/f mice is described below. LckCre mice (Lee et al., 2001) were obtained from Taconic. Adora2af/f-LckCre−/−, Adora2af/f-LckCre+/−, Adora2af/f−/−, Adora2af/f−/− (gift from K. Ravid, Boston University, Boston, MA), and RAG1−/− mice were bred in the La Jolla Institute for Allergy and Immunology. Yellow fluorescent reactive dye was purchased from Invitrogen. Antibodies recognizing total Akt, phosphorylated Akt (Ser473), and β-actin for immunoblotting were obtained from Cell Signaling Technology. Fluorescent antibodies used in this study, their sources, and dilutions used are listed in Table S1.

Generation of floxed A2AR mice. We obtained a targeting construct containing the A2AR gene (Adora2a) previously used to generate Adora2a−/− mice from J.F. Chen as a gift (Boston University). LoxP sites were inserted 300 bp upstream and 1.4 kb downstream 3′ of exon 2, which contains the ATG initiation site. Deletion of exon 2, which contains the ATG initiation site, can effectively eliminate A2AR expression (Ledent et al., 1997). Diphtheria toxin A was placed downstream of the targeting construct to eliminate random integration. We also included positive and negative selection markers (loxP-neo-tk-loxP) to facilitate monitoring homologous recombination and subsequent marker deletion. To achieve this, we designed a targeting vector with some rare enzyme sites in the MCS (multiple cloning site) and three loxP sites in the order: MCS1–loxP–MCS2–loxP–neo-tk-loxP–MCS3–DTA. Exon 2 was inserted into MCS2, the 5′ homologous genome (4.3 kb) into MCS1, and the 3′ homologous genome (4.2 kb) into MCS3. The ultimate goal was to produce embryonic stem (ES) cells such that the only modification to the adora2a locus is the insertion of the two 34-bp loxP recognition sequences surrounding exon 2. After transfection of a targeting vector by electroporation, colonies that survived positive and negative selection were cloned and screened by both PCR and Southern blot analysis for specific adora2a recombination. We used a PCR primer set to identify clones with complete homologous recombination. We deleted the selection marker cassette by transient transfection of Cre into the homologous recombination–positive ES cells in vitro. Correctly modified ES cells were injected and injected into C57BL/6 blastocysts, and then implanted into pseudo-pregnant foster mothers. ES cells were injected into C57BL/6 blastocysts. 64 blastocysts were each injected with 10–15 ES cells. Injected blastocysts were implanted into six pseudo-pregnant foster mothers. 32 pups were born and 13 chimeric mice were identified by coat color. The gender distribution of these chimeras was eight male and five female. Male chimeric mice were test-bred to ascertain the contribution of the injected 129sv ES cells to the germline. We crossed each male chimeric mouse with two C57BL/6 female mice. Only two agouti mice were found in the first test breed, and one of them was an (129 × C57BL/6) F1 (N1F1) adora2a−/− mouse as confirmed by PCR and Southern blotting. Many N1F1 floxed adora2a heterozygous mice of both genders were found in the later litters. To get more heterozygous floxed females to mate with tissue-specific Cre mice, we first used all the N1F1 mice for backcrosses with C57BL/6 mice until we had enough N2F1 and N3F1 mice to generate tissue-specific knockouts. We successfully crossed heterozygous floxed adora2a mice to generate homozygous floxed adora2a mice (Adora2a−/−) by crossing male and female N3F1 heterozygous mice. Adora2a−/− mouse generation formally started from N3F2 and were designated with the official name B6;129P–adora2a−/−. We maintained the colony by homozygous inbreeding onto C57BL/6 and BALB/c backgrounds. For the current study, floxed mice were crossed with mice expressing Cre recombinase gene under Lck promoter as described in Lee et al. (2001) to obtain lymphoid-specific deletion of Adora2a.

Flow cytometry and cell sorting. Single cell suspensions from the indicated tissues were prepared by sequential pressing through 100- and 40-µm cell strainers. After RBC lysis (BioLegend), cells were washed and resuspended in RPMI medium supplied with 10% fetal bovine serum and counted in a Z2-Coulter particle counter (Beckman Coulter). 3–5 × 106 cells were resuspended in RPMI medium supplied with 10% fetal bovine serum and counted in a Z2-Coulter particle counter (Beckman Coulter). 3–5 × 106 cells were preincubated for 10 min in 100 µl FACS buffer with antibody to block Fc receptors. Each sample tube received 100 µl of fluorescently labeled antibody cocktail and was incubated for 30 min at 4°C in the dark. Cells were analyzed using an LSR II equipped with four lasers and FACS Diva software (BD). CD4 and CD8 T cells were enriched by negative selection (STEMCELL Technologies) or for some experiments positively selected by magnetic cell sorting (Miltenyi Biotech) and then stained for CD44. Thymocytes, naive T cells (CD44dim), and memory T cells (CD44hi) were sorted by FACS Aria sorting (Miltenyi Biotech) and then stained for CD44. Thymocytes, naive T cells (CD44dim), and memory T cells (CD44hi) were sorted by FACS Aria (BD). Live-Dead fixable yellow (Invitrogen) was used to exclude dead cells before analysis or during cell sorting. Flow cytometry data were analyzed using FlowJo software (version 9.0.1; Tree Star).

Quantitative real-time PCR and immunoblotting. RNAs from resulting sorted cells were isolated by RNA isolation kit (Qiagen), and cDNAs from isolated RNAs were synthesized by qScript cDNA Super Mix (Quanta Biosciences). Quantitative real-time PCR was performed by using TaqMan primers and TaqMan PCR master mix obtained from Applied Biosystems. All isolation procedures and reaction assays followed recommended manufacturer’s instructions. For immunoblotting experiments, a negative T cell enrichment kit was used (STEMCELL Technologies). Treated T cells were lysed in RIPA and sample buffer, and immunoblotting was performed as described previously (Cekic et al., 2011), modified to use the semidry iBlot transfer system from Invitrogen. Fluorescently labeled anti–rabbit secondary antibodies and the Odyssey imaging system from LI-COR Biosciences were used for the detection of proteins.

Adaptive cell transfer. Spleen and peripheral LNs from age-matched Adora2a−/− and Adora2a−/− mice were collected, and single cell suspensions

Adenosine regulates T cell homeostasis | Cekic et al.
were prepared by passing tissues through 40-µM mesh filters. Cells were counted, and mixtures of cell suspensions were injected into WT or MHCII mice i.v. by the retroorbital route. Single cell suspensions from spleens and LNs of recipient mice were analyzed by flow cytometry to determine proportions of T cells or BrdU incorporation assay for proliferation. Adora2a+/− or Adora2a−/− mice received 0.3 mg/mouse BrdU 40, 37, 34, and 24 h before the harvest of tissues. Therefore, each mouse received a total of 1.2 mg BrdU. Cells were surface stained and fixed according to the manufacturer’s instructions with a BrdU staining kit (BD). APC-conjugated anti-BrdU antibody was added to the cell suspensions to detect BrdU incorporation and to determine the proportion of T and B cells.

In vitro test for IL-7 responsiveness and IL-7Rα expression. For TCR stimulation or stimulation with recombinant mouse IL-7 (R&D Systems) T cells were enriched by CD4+ T cell enrichment kit (STEMCELL Technologies) and stimulated with different concentrations of IL-7 or carrier-free plate-bound anti-CD3 antibody (clone 145-2C11; BioLegend). Biotinylated CD44 was used during enrichment to specifically isolate the CD44dim (naive) T cell population. To evaluate the effects of A2AR signaling on TCR-induced IL-7Rα down-regulation, we stimulated isolated T cells with plate-bound anti-CD3 antibody in the presence or absence of 1 µM of the selective A2AR agonist CGS 21680, 100 nM of the PKA inhibitor KT 5720, or 2 µM of the MHCII antagonist lipid A stimulation of TLR4.

Fig. S1 shows the gating strategy used for Online supplemental material. Fig. S1 lists antibody sources, clones, and dilutions. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20130249/DC1.

We gratefully acknowledge Dr. Katya Ravid for her gift of Adora2b−/− mice, Dr. Jiang Fan Chen for his gift of a targeting construct containing the A2AR gene (Adora2a), Dr. Heba Nowhyd for her help and suggestions for BrdU proliferation assays, Ruen Ken for real-time PCR, Dennis Huyhn for mouse husbandry and genotyping, and the Immunological Genome Project.

This work was supported by National Institutes of Health grant P01 HL073361 and by an American Heart Association postdoctoral fellowship (to C. Cecić).

The authors declare no competing financial interests.

Author contributions: C. Cecić conceived the research, conducted the experiments, analyzed the data, and wrote the manuscript; D. Sag assisted with some experiments; Y.-J. Day generated Adora2a+/− mice; and J. Linden oversaw study design and edited the manuscript.

REFERENCES


© The American Society for Investigative Pathology. Published October 21, 2013


