

# Extracellular adenosine regulates naive T cell development and peripheral maintenance

Caglar Cekic,<sup>1</sup> Duygu Sag,<sup>1</sup> Yuan-Ji Day,<sup>2,3</sup> and Joel Linden<sup>1</sup>

<sup>1</sup>Division of Inflammation Biology, La Jolla Institute for Allergy and Immunology, La Jolla, CA 92037

<sup>2</sup>Graduate Institute of Clinical Medical Sciences, Chang Gung Memorial Hospital, and <sup>3</sup>Graduate Institute of Clinical Medical Sciences, Chang Gung University, Kwei-Shan, Tao-Yuan, Taiwan 333, Republic of China

**Adenosine produced as a byproduct of metabolic activity is present in all tissues and produces dose-dependent suppression of TCR signaling. Naive T cell maintenance depends on inhibition of TCR signals by environmental sensors, which are yet to be fully defined. We produced mice with a floxed adenosine A<sub>2A</sub> receptor (A<sub>2A</sub>R) gene, *Adora2a*, and show that either global A<sub>2A</sub>R deletion or cre-mediated T cell deletion elicits a decline in the number of naive but not memory T cells. A<sub>2A</sub>R signaling maintains naive T cells in a quiescent state by inhibiting TCR-induced activation of the phosphatidylinositol 3-kinase (PI3K)–AKT pathway, thereby reducing IL-7R $\alpha$  down-regulation and naive T cell apoptosis. Patterns of IL-7R $\alpha$  expression on T cells in chimeric mice reconstituted with *Adora2a*<sup>+/+</sup> and *Adora2a*<sup>-/-</sup> bone marrow cells suggest that decreased IL-7R $\alpha$  in naive T cells is a cell-intrinsic consequence of *Adora2a* deletion. In addition, A<sub>2A</sub>R expression increases in early thymic T cell development and contributes to progression of double-negative thymic precursors to single-positive thymocytes with increased IL-7R $\alpha$  expression. Therefore, A<sub>2A</sub>R signaling regulates T cell development and maintenance to sustain normal numbers of naive T cells in the periphery.**

## CORRESPONDENCE

Joel Linden:  
jlinden@liai.org

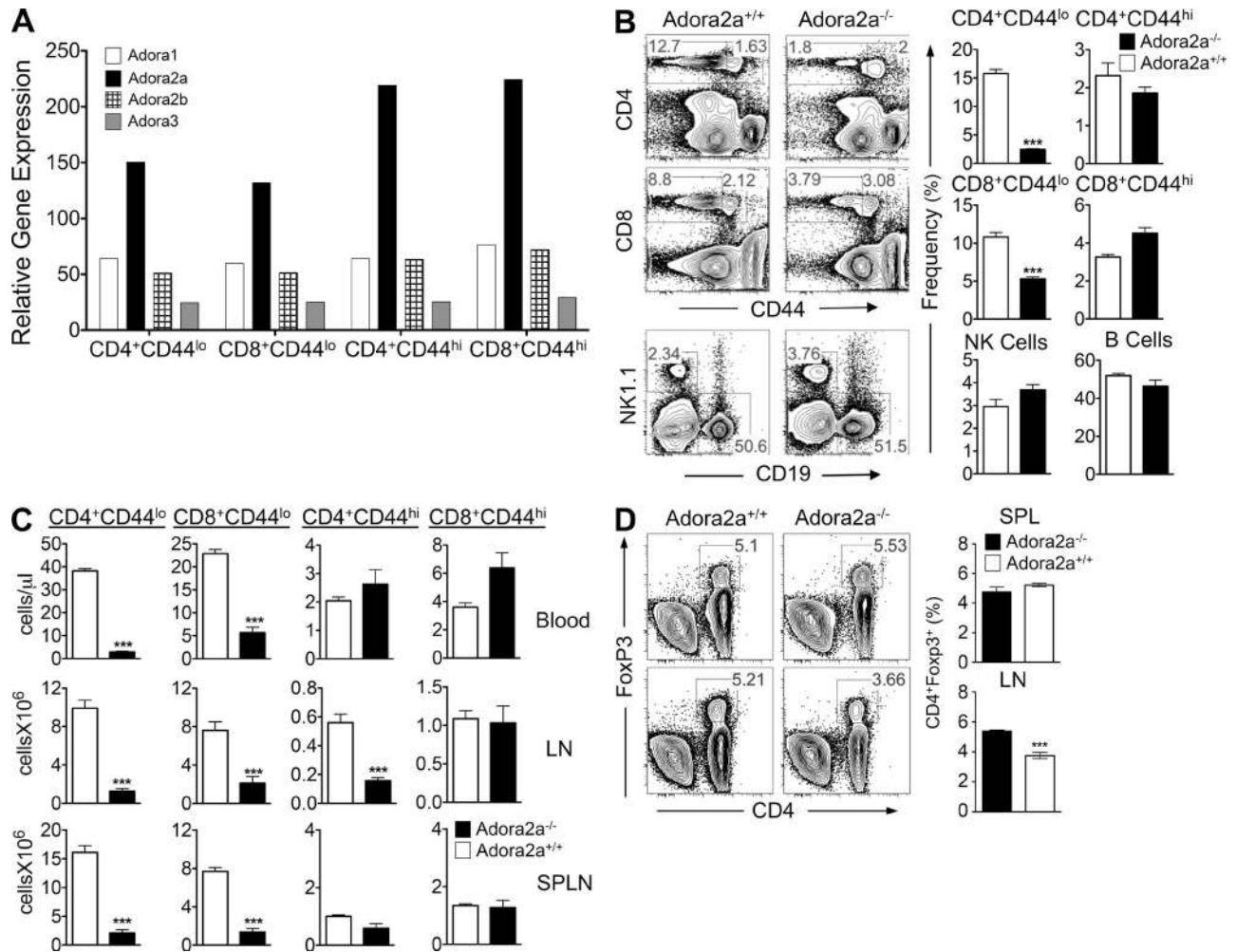
Abbreviations used: DN, double negative; DP, double positive; ES, embryonic stem; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; SP, single positive.

Adenosine is produced as a result of metabolic processes and has cell signaling roles that are mediated by four G-protein-coupled adenosine receptors: A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, and A<sub>3</sub> (Fredholm et al., 2011). These receptors are antagonized by naturally occurring and widely consumed methylxanthines, caffeine and theophylline, as well as by more potent synthetic antagonists (Haskó et al., 2008; Fredholm et al., 2011; Linden and Cekic, 2012). Adenosine is constitutively produced and increases in response to cellular hypoxia and stress. It readily crosses cell membranes via nucleoside transporters to gain access to cell surface receptors (Yao et al., 2011). Extracellular adenosine is also produced from the degradation of adenine nucleotides by exonucleases. ATP and ADP are converted to AMP and adenosine after release to the extracellular space through membrane channels (Huang et al., 2007), from cell death, or as granular components of platelets, mast cells, or neurons. Therefore, adenosine is available in all tissues and organs and changes in concentration depending on the tissue type and physiological conditions.

Naive and memory T cells are maintained in the periphery to provide appropriate antigen-specific recognition to eliminate pathogens and

tumors. IL-7 signaling and TCR engagement by self-peptide–MHC molecules provide signals needed for the development, survival, and homeostatic proliferation of naive T cells. Memory T cells also rely on IL-7 for survival but use IL-15 for homeostatic proliferation (Surh and Sprent, 2000, 2008). Recent evidence suggests that naive T cells are actively maintained in a quiescent state that requires integration of proliferative and survival signals with signals from environmental cues. However, the nature of these environmental cues is not fully understood. Here we identify one such cue as adenosine. The A<sub>2A</sub>R 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). A<sub>2A</sub>R 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 A<sub>2A</sub>Rs (Su et al., 2004). We show that

© 2013 Cekic et al. This article is distributed under the terms of an Attribution–Noncommercial–Share Alike–No Mirror Sites license for the first six months after the publication date (see <http://www.rupress.org/terms>). After six months it is available under a Creative Commons License (Attribution–Noncommercial–Share Alike 3.0 Unported license, as described at <http://creativecommons.org/licenses/by-nc-sa/3.0/>).



**Figure 1. A<sub>2A</sub>R deletion impairs peripheral T cell homeostasis.** (A) Relative expression of adenosine receptor transcripts in naive and memory T cells (derived from the ImmGen database, with permission; Heng and Painter, 2008). (B and C) Percentage of lymphocytes (frequencies) in blood (B) and corresponding cell counts of CD44<sup>lo</sup> (naive) and CD44<sup>hi</sup> (memory phenotype) CD4<sup>+</sup> and CD8<sup>+</sup> T cells in blood, LN, and spleen (SPLN); (C) of 5–7-wk-old *Adora2a*<sup>+/+</sup> and *Adora2a*<sup>-/-</sup> mice ( $n \geq 11$  from four independent experiments; \*\*\*,  $P < 0.001$  by unpaired two-tailed Student's *t* test). (D) Intracellular staining for Foxp3 (from two independent experiments,  $n = 4$ ; \*\*\*,  $P < 0.001$  by unpaired two-tailed Student's *t* test). Error bars are SEM.

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 naive T cell survival.

**RESULTS**

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

An analysis of the ImmGen 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*, have smaller spleens (wild type = 110 ± 6 mg vs. *Adora2a* 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 naive 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 naive CD4<sup>+</sup> T cell population. Overall, these data demonstrate that basal A<sub>2A</sub>R signaling contributes to the maintenance of naive T cell numbers in the periphery.

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

Maintenance of naive 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 naive 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-7R $\alpha$  (CD127) on naive T cells from *Adora2a*<sup>-/-</sup> mice as compared with wild-type controls (Fig. 2 C). CD127 is not changed on *Adora2a*<sup>-/-</sup> CD44<sup>hi</sup>CD4<sup>+</sup> T cells and only slightly decreased on *Adora2a*<sup>-/-</sup> CD44<sup>hi</sup>CD8<sup>+</sup> T cells. Hence the number of cells with a memory phenotype (CD44<sup>hi</sup>) is minimally changed in *Adora2a*<sup>-/-</sup> mice (Fig. 1 B) despite a substantial decrease in the naive T cell population (Fig. 1, B and C).

Global A<sub>2A</sub>R 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, hypoalgesia, 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*<sup>+/+</sup> (CD45.1<sup>+</sup>) and *Adora2a*<sup>-/-</sup> (CD45.2<sup>+</sup>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<sup>+</sup> hosts and bone marrow from *Adora2a*<sup>+/+</sup> (CD45.1<sup>+</sup>) and *Adora2a*<sup>-/-</sup> (CD45.2<sup>+</sup>) 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*<sup>+/+</sup> (CD45.2<sup>+</sup>) and *Adora2b*<sup>-/-</sup> (CD45.1<sup>+</sup>) 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 A<sub>2A</sub>R (Fig. 2 D) but not A<sub>2B</sub>R signaling (Fig. 2 E) facilitates T cell homeostasis.

We observed different patterns of IL-7R $\alpha$  expression on T cells in chimeric mice reconstituted with *Adora2a*<sup>+/+</sup> and *Adora2a*<sup>-/-</sup> bone marrow cells, suggesting that decreased IL-7R $\alpha$  in naive T cells is a cell-intrinsic consequence of *Adora2a* deletion (Fig. 2 F). Accordingly, transfer of a 1:1 mixture of *Adora2a*<sup>+/+</sup> (CD45.1<sup>+</sup>) and *Adora2a*<sup>-/-</sup> (CD45.2<sup>+</sup>UBC-EGFP) spleen and LN cells to wild-type recipients (CD45.2<sup>+</sup>) for 0–2 wk resulted in a reduced ratio of *Adora2a*<sup>-/-</sup> to *Adora2a*<sup>+/+</sup> in naive but not memory phenotype T cells over time (Fig. 2 G). To determine whether the decrease in IL-7R $\alpha$  expression in *Adora2a*<sup>-/-</sup> T cells influences their sensitivity to IL-7, we cultured *Adora2a*<sup>-/-</sup> or *Adora2a*<sup>+/+</sup> cells from LNs in the absence or presence of IL-7. A<sub>2A</sub>R 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 A<sub>2A</sub>R signaling favors peripheral T cell accumulation by regulating IL-7R expression.

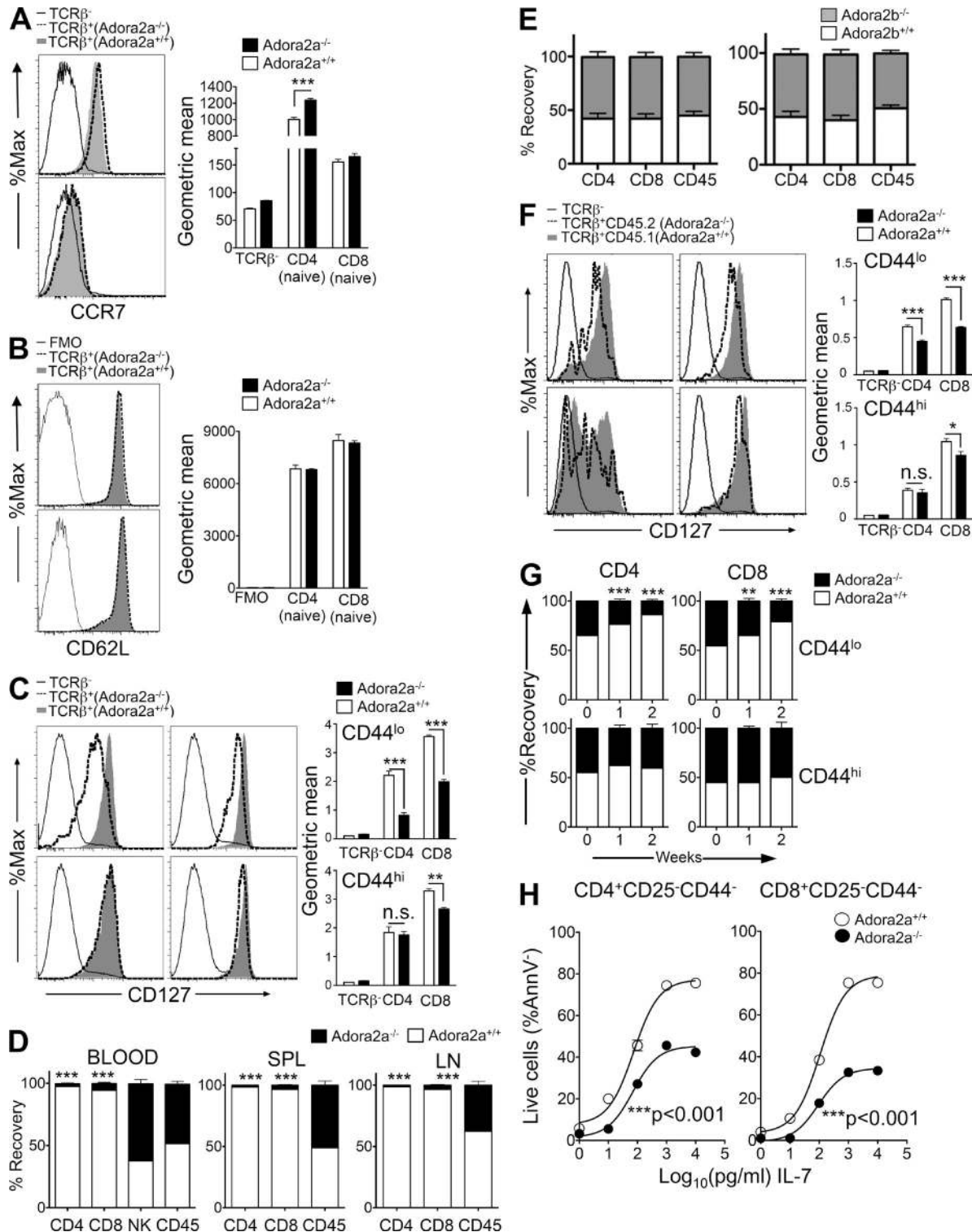
#### Protein kinase A (PKA) stimulation by A<sub>2A</sub>R activation prevents TCR-induced down-regulation of IL-7R $\alpha$

IL-7R and TCR signaling are both known to inhibit IL-7R $\alpha$  expression. Therefore, we measured the effects of A<sub>2A</sub>R activation

on TCR- or IL-7-induced decreases in cell surface IL-7R $\alpha$  expression. Fig. 3 A shows that the addition of the selective A<sub>2A</sub>R agonist CGS 21680 to T cells in vitro significantly inhibits TCR- but not IL-7-induced down-regulation of cell surface IL-7R $\alpha$  (CD127), suggesting that A<sub>2A</sub>R signaling interferes with TCR but not IL-7R signaling. CGS 21680 reduced the potency but not the maximal effect of  $\alpha$ CD3 to down-regulate CD127. These results are consistent with the concept that A<sub>2A</sub>R 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-7R $\alpha$  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 $\alpha$  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 A<sub>2A</sub>R signaling regulates T cell signaling events primarily through PKA, we hypothesized that A<sub>2A</sub>R signaling reduces AKT activation and down-regulates IL-7R $\alpha$  in a PKA-dependent manner. A<sub>2A</sub>R stimulation significantly reduced AKT phosphorylation (Fig. 3 B) after TCR signaling, and this effect was blocked by the competitive A<sub>2A</sub>R antagonist SCH 58621. Accordingly, basal AKT phosphorylation, which was measured by flow cytometry analysis (because immunoblotting was not sufficiently sensitive), was higher in A<sub>2A</sub>R-deficient naive T cells than in wild-type naive T cells (Fig. 3 C). The effect of A<sub>2A</sub>R stimulation to increase IL-7R $\alpha$  expression after TCR stimulation was completely reversed by the highly selective PKA inhibitor KT 5720 (Fig. 3 D). To determine whether PI3K inhibition and A<sub>2A</sub>R stimulation are additive, we examined the effects of strong TCR stimulation in the absence or presence of the PI3K inhibitor LY 294002 and the A<sub>2A</sub>R 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 A<sub>2A</sub>R stimulation may not be observed. LY 294002 alone was sufficient to inhibit TCR-dependent IL-7R $\alpha$  down-regulation, and A<sub>2A</sub>R 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





**Figure 2.** A<sub>2A</sub>R 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 (CD44<sup>lo</sup>) T cells from blood of Adora2a<sup>+/+</sup> and Adora2a<sup>-/-</sup> mice and corresponding geometric means. Data are from two independent experiments. (C) CD127 (IL-7Rα) staining of naive (CD44<sup>lo</sup>) and memory (CD44<sup>hi</sup>) T cells from blood of Adora2a<sup>+/+</sup> and Adora2a<sup>-/-</sup> mice and corresponding geometric means. (D) Recovery of Adora2a<sup>+/+</sup> and Adora2a<sup>-/-</sup> (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<sup>+/+</sup> (CD45.1) and Adora2a<sup>-/-</sup> (CD45.2) bone marrows (results are representative of three independent experiments, n = 5). (E) T cell and CD45<sup>+</sup> cell recoveries in mixed bone marrow chimeras of Adora2b<sup>+/+</sup> (CD45.2) and Adora2b<sup>-/-</sup> (CD45.1) 8 wk after reconstitution (results are representative of two or three independent experiments, n = 5). (F) CD127 (IL-7Rα) staining of Adora2a<sup>+/+</sup> 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 A<sub>2A</sub>R signaling. Therefore, we stimulated naive and memory T cells and tested the effect of A<sub>2A</sub>R stimulation on CD127 expression. A<sub>2A</sub>R 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) A<sub>2A</sub>R 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 A<sub>2A</sub>R activation controls IL-7R $\alpha$  expression by inhibiting TCR–induced AKT activation more in naive than memory T cells.

#### A<sub>2A</sub>R 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 A<sub>2A</sub>R signaling on T cell proliferation, we performed BrdU incorporation assays. Fig. 4 A shows that a greater percentage of *Adora2a*<sup>-/-</sup> than *Adora2a*<sup>+/+</sup> 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 A<sub>2A</sub>R signaling. Similar BrdU incorporation in *Adora2a*<sup>-/-</sup> and *Adora2a*<sup>+/+</sup> memory T cells suggests that lymphopenia in *Adora2a*<sup>-/-</sup> mice is not responsible for increased proliferation of naive *Adora2a*<sup>-/-</sup> T cells.

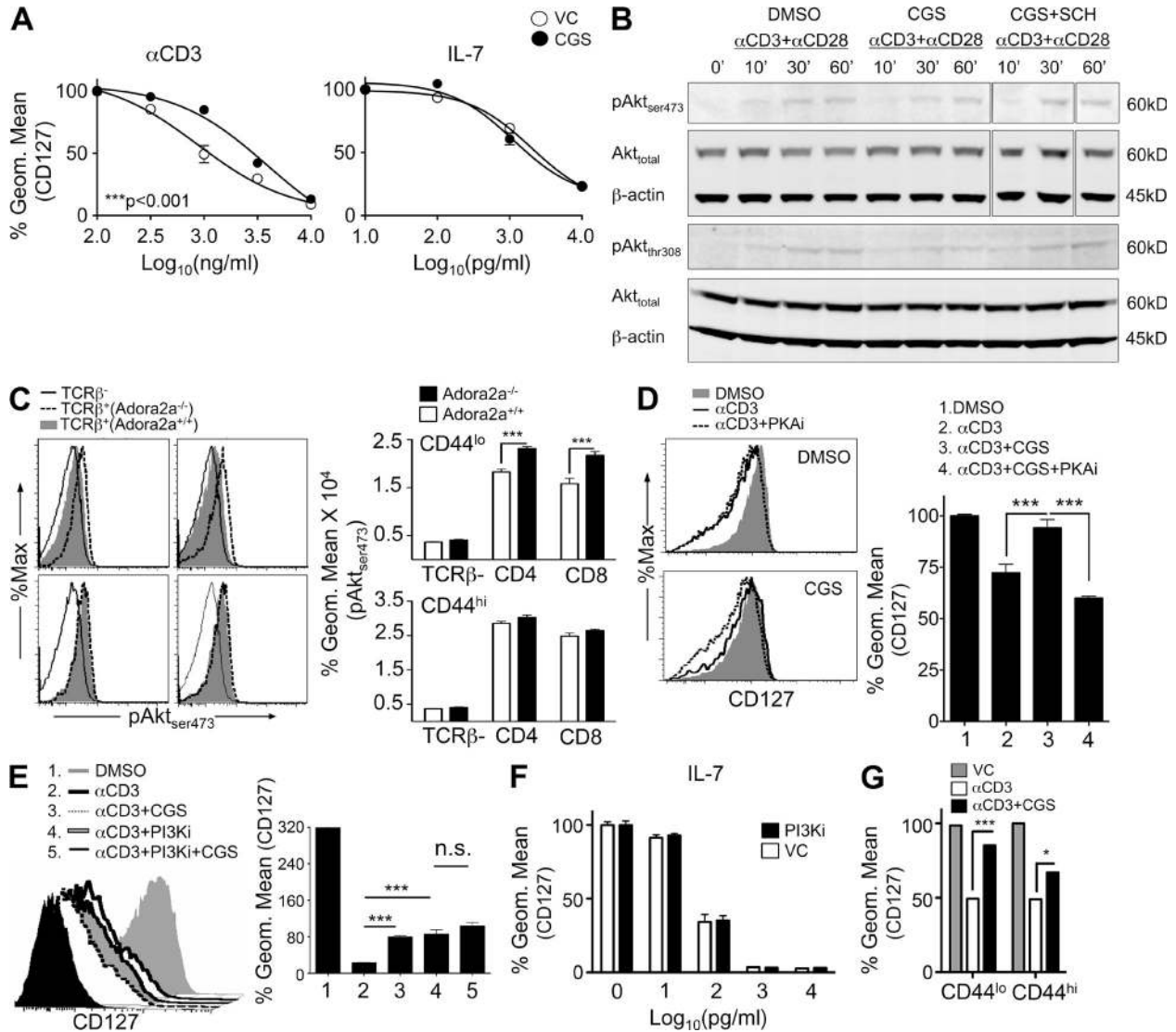
IL-7 signaling prevents apoptosis and keeps naive T cells alive (Surh and Sprent, 2008). We observed that *Adora2a*<sup>-/-</sup> T cells are hyporesponsive to IL-7 (Fig. 2 E). Accordingly, decreased IL-7R $\alpha$  expression was associated with decreased expression of antiapoptotic Bcl-2 (Fig. 5, A 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<sup>+</sup>Live-Dead<sup>-</sup>) in blood and increased the number of dead T cells (Annexin V<sup>+</sup>Live-Dead<sup>+</sup>) in peripheral LNs (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 A<sub>2A</sub>R-deficient T cells proliferate more than wild-type cells but fail to accumulate as a result of increased apoptosis.

#### A<sub>2A</sub>R 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 A<sub>2A</sub>R signaling influences thymic T cell development. In *Adora2a*<sup>-/-</sup> 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*<sup>+/+</sup> (CD45.1) and *Adora2a*<sup>-/-</sup> (CD45.2) mice transferred to recipient mice (CD45.2<sup>+</sup>UBC-EGFP<sup>+</sup>) 8–12 wk after irradiation. Compared with wild-type cells, large reductions in the relative proportions of *Adora2a*<sup>-/-</sup> DP and SP but not DN thymic precursors were detected in reconstituted recipients (Fig. 6 C). To determine whether A<sub>2A</sub>R expression during early thymic T cell development is important for subsequent progression to DP and SP precursors, we produced *Adora2a*<sup>fl/fl</sup> mice and crossed these to syngeneic C57BL/6J mice carrying the Cre recombinase transgene 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). A<sub>2A</sub>R mRNA expression isolated from *Adora2a*<sup>fl/fl</sup>-*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*<sup>fl/fl</sup> littermates not expressing Cre recombinase, indicating efficient deletion of the *Adora2a* gene from T cells (Fig. 6 D, bottom). As can be

*Adora2a*<sup>-/-</sup> T cells (naive [CD44<sup>lo</sup>] and memory [CD44<sup>hi</sup>]) from blood of irradiated mice reconstituted with 1:1 mixtures of bone marrow cells of *Adora2a*<sup>+/+</sup> and *Adora2a*<sup>-/-</sup> mice (results are representative of two independent experiments,  $n \geq 5$ ). (G)  $15 \times 10^6$  cells pooled from spleens and LNs of *Adora2a*<sup>+/+</sup> (CD45.1) and *Adora2a*<sup>-/-</sup> (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 LNs were measured at 0, 1, and 2 wk after transfer (results are from two independent experiments,  $n \geq 4$ ). (H)  $10^6$  LN cells from *Adora2a*<sup>+/+</sup> and *Adora2a*<sup>-/-</sup> 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<sup>-</sup>/Live-Dead aqua<sup>-</sup>) were measured after 72 h of ex vivo culture (representative of two independent experiments,  $n = 4$ ). EC50s for naive *Adora2a*<sup>-/-</sup> CD4 and CD8 T cells are  $1.84 \pm 0.08$  and  $1.97 \pm 0.07$ , respectively, and EC50s for naive *Adora2a*<sup>+/+</sup> CD4 and CD8 T cells are  $1.87 \pm 0.06$  and  $2.03 \pm 0.04$ , 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.

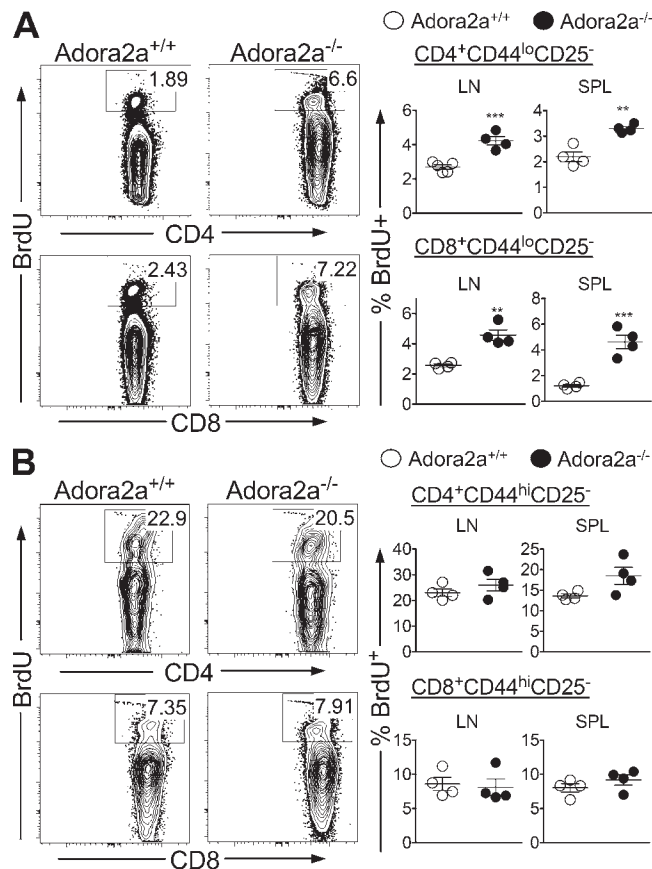


**Figure 3. PKA stimulation by  $A_{2A}R$  activation prevents TCR-induced down-regulation of IL-7 $R\alpha$ .** (A) Wild-type T cells were stimulated with varying concentrations of plate-bound anti-CD3 or recombinant mouse IL-7 in the presence or absence of 1  $\mu$ M CGS 21680 (CGS) or vehicle control (<0.1% DMSO). CD127 (IL-7 $R\alpha$ ) staining was performed after incubation at 37°C (5% CO<sub>2</sub>) 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  $\mu$ g/ml anti-mouse CD3 and 2  $\mu$ g/ml anti-mouse CD28 antibodies in the absence or presence of 1  $\mu$ M 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*<sup>+/-</sup> versus *Adora2a*<sup>-/-</sup> 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  $\mu$ M CGS 21680 or vehicle control (<0.1% DMSO). CD127 staining was performed after incubation at 37°C (5% CO<sub>2</sub>) for 24 h. (E) 2  $\mu$ M of the selective PI3K inhibitor LY 294002 was added to cultures of stimulated T cells in the presence or absence of 1  $\mu$ M CGS 21680 or vehicle control (<0.1% DMSO). CD127 staining was performed after incubation at 37°C (5% CO<sub>2</sub>) for 24 h. Data in C–E are representative of two independent experiments with similar results ( $n = 3$ ). (F) 2  $\mu$ M of the selective PI3K inhibitor LY 294002 was added to cultures of naive T cells stimulated with the indicated concentrations of rml-7. CD127 staining was performed after incubation at 37°C (5% CO<sub>2</sub>) for 24 h ( $n = 5$ , from two independent experiments with similar results). (G) Enriched CD44<sup>lo</sup> and CD44<sup>hi</sup> cells were stimulated by 1  $\mu$ g/ml plate-bound anti-CD3 antibody in the presence or absence of 1  $\mu$ M CGS 21680. CD127 (IL-7 $R\alpha$ ) staining was performed after incubation at 37°C (5% CO<sub>2</sub>) 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  $A_{2A}R$  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  $A_{2A}R$  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  $A_{2A}R$  transcription (Fig. 6, A and D).





**Figure 4. *Adora2a* deletion increases homeostatic proliferation of naive T cells.** (A and B) BrdU incorporation assay as a measure of CD44<sup>lo</sup> (A) and CD44<sup>hi</sup> (B) T cell proliferation. *Adora2a*<sup>+/+</sup> and *Adora2a*<sup>-/-</sup> mice received four i.p. injections of 0.3 mg BrdU 40 h before the assay. BrdU incorporation into the T cell DNA was measured with APC-labeled anti-BrdU antibodies ( $n = 4$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  by unpaired two-tailed Student's  $t$  test). Results are from two independent experiments with  $n \geq 3$ . Error bars are SEM.

### A<sub>2A</sub>R signaling is required for naive T cell maintenance in the periphery

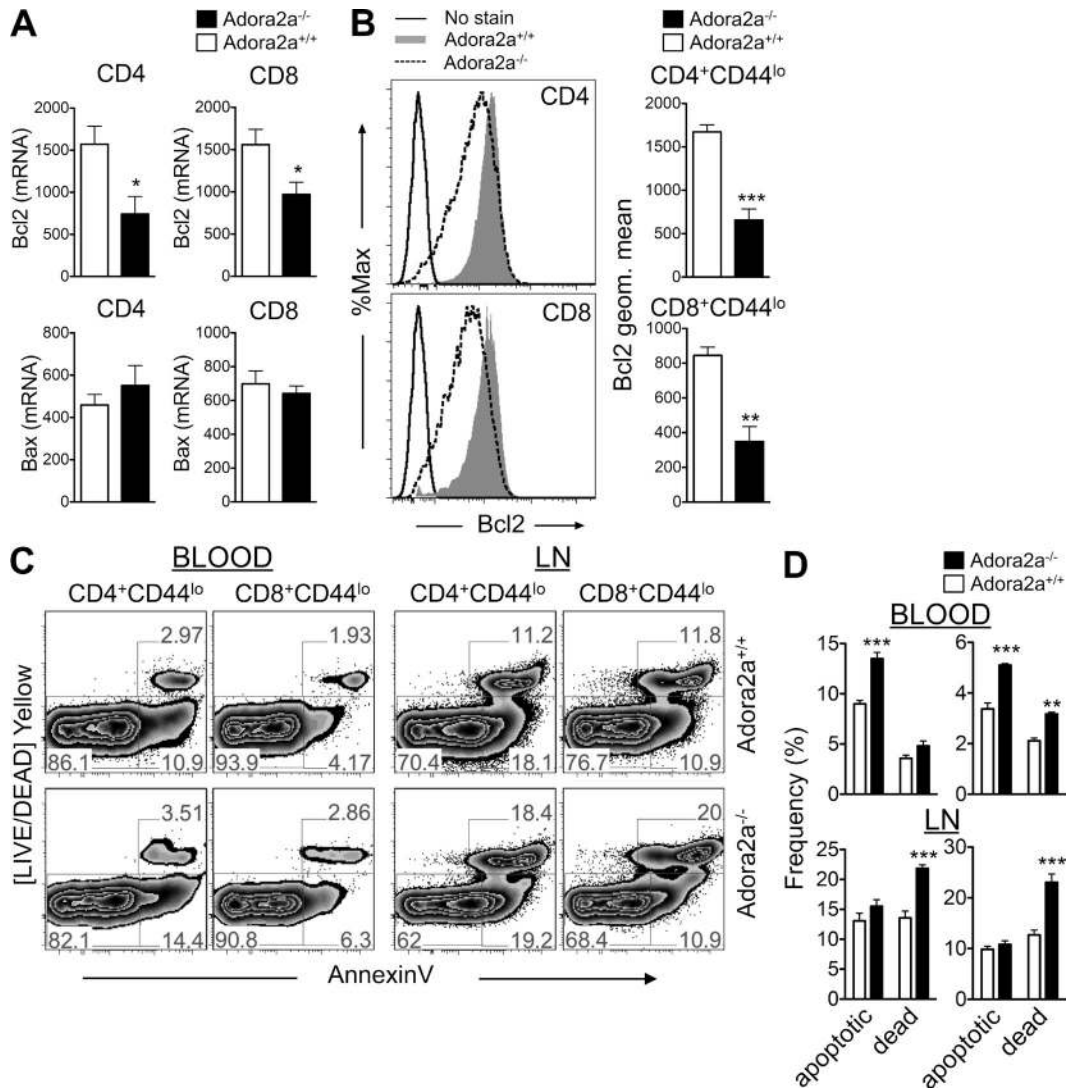
We considered the possibility that naive T cells are reduced in numbers in *Adora2a*<sup>-/-</sup> mice only as a result of decreased thymic output. Because Lck-mediated deletion of *Adora2a* did not affect thymic progression, we compared the numbers of naive T cells in *Cre*<sup>+</sup> mice with *Cre*<sup>-</sup> 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 $\alpha$  expression and IL-7 responsiveness among naive T cells in *Cre*<sup>+</sup> mice (Fig. 7, D and E). Overall, these data suggest that A<sub>2A</sub>R 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*<sup>-/-</sup> 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 A<sub>2A</sub>R 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-7R $\alpha$  (CD127) expression in SP precursors. This effect is cell intrinsic because we observed reduced CD127 expression in *Adora2a*<sup>-/-</sup> thymocytes developing in the same thymus with *Adora2a*<sup>+/+</sup> thymocytes after 1:1 bone marrow reconstitution (Fig. 8 B), suggesting that *Adora2a*<sup>-/-</sup> 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-7R $\alpha$  expression in thymic precursors (Fig. 8 C), suggesting that A<sub>2A</sub>R receptors produced during early thymic development are retained and help to maintain normal numbers of thymic precursors. Decreased IL-7R $\alpha$  expression after DP to SP transition suggests an incomplete maturation of SP *Adora2a*<sup>-/-</sup> thymocytes. The proportions of Qa2<sup>hi</sup>HSA<sup>lo</sup> cells were significantly lower among SP thymocytes isolated from *Adora2a*<sup>-/-</sup> 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*<sup>-/-</sup> 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 A<sub>2A</sub>R signaling that reduces IL-7R $\alpha$  expression, we hypothesized that reduced IL-7R $\alpha$  expression is not a permanent developmental defect; therefore, the cessation of TCR signaling should restore IL-7R expression in *Adora2a*<sup>-/-</sup> T cells. To test this, we measured the expression of IL-7R $\alpha$  on CD4<sup>+</sup> 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-7R $\alpha$  expression in naive *Adora2a*<sup>-/-</sup> CD4<sup>+</sup> 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-4R $\alpha$  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 A<sub>2A</sub>R-deficient and wild-type animals (not depicted). To show that absence of TCR signaling increases CD127 expression in *Adora2a*<sup>-/-</sup> T cells in vivo, we adoptively transferred GFP<sup>+</sup> *Adora2a*<sup>-/-</sup> naive CD4<sup>+</sup> T cells to wild-type or MHCII<sup>-/-</sup> mice. CD127 expression in adoptively transferred naive *Adora2a*<sup>-/-</sup> T cells remained low in wild-type animals but increased to the level in naive host cells in MHCII<sup>-/-</sup> mice (please note CD127 expression in wild-type and MHCII<sup>-/-</sup> T cells is similar). These data suggest that A<sub>2A</sub>R signaling by endogenous adenosine is important for the homeostatic balance of CD127 expression.



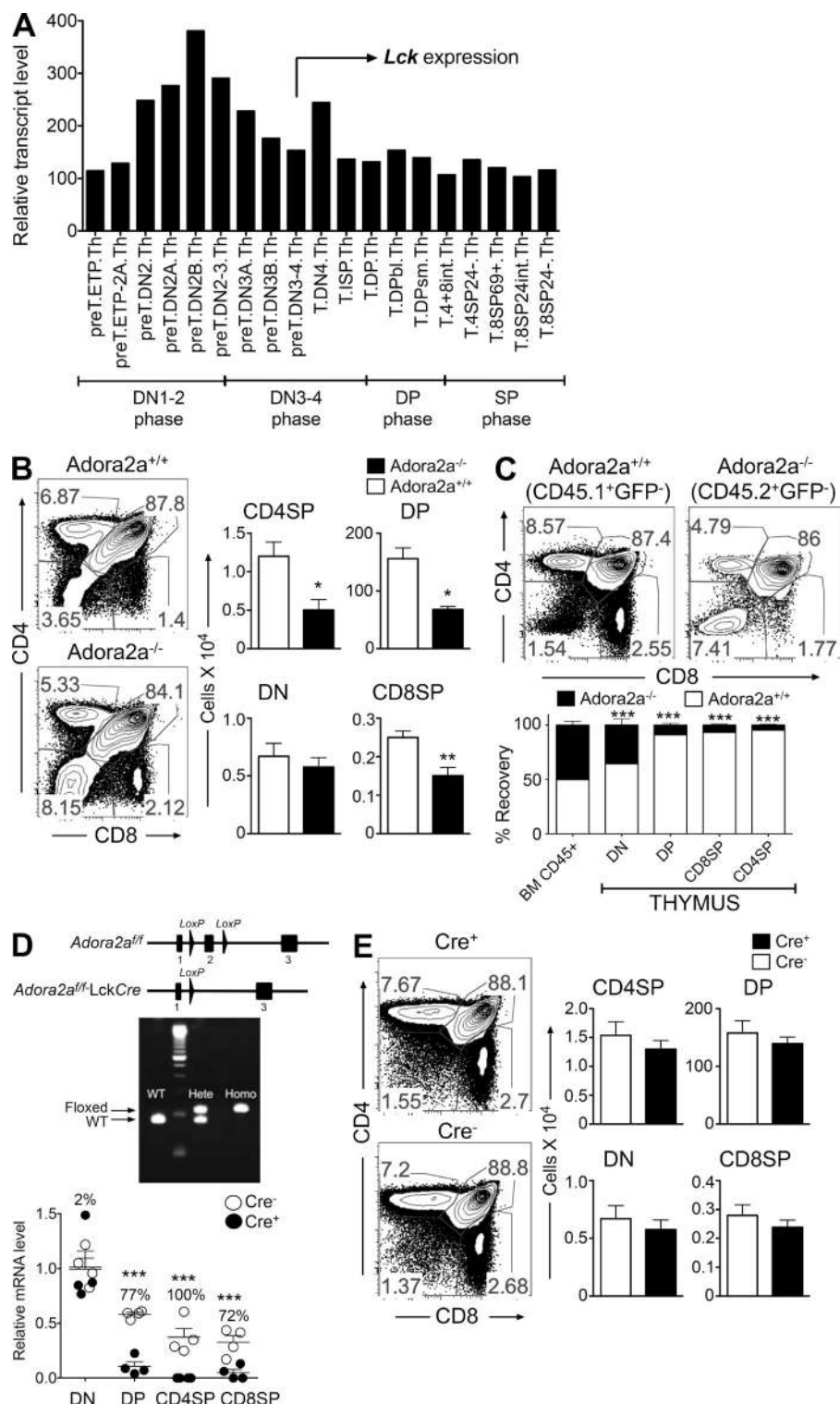
**Figure 5. *Adora2a* deletion increases apoptosis of naive T cells.** (A) Percentages of Bcl2 and Bax expression as compared with the housekeeping gene, RNA polymerase. (B) Intracellular Bcl2 levels in naive T cells isolated from LNs of *Adora2a*<sup>+/+</sup> and *Adora2a*<sup>-/-</sup> mice. (C and D) Flow cytometry analysis (C) and corresponding frequencies of dead (Annexin V<sup>+</sup>/Live-Dead yellow<sup>+</sup>) and apoptotic (Annexin V<sup>+</sup>/Live-Dead yellow<sup>-</sup>) naive T cells (D) from blood and LN of *Adora2a*<sup>+/+</sup> and *Adora2a*<sup>-/-</sup> mice ( $n \geq 3$ ; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  by two-way ANOVA and Bonferroni post-hoc analysis for C and by unpaired two-tailed Student's *t* test for A and B). All experiments were performed twice with  $n \geq 3$ . Error bars are SEM.

**DISCUSSION**

We show here that deletion of *A<sub>2A</sub>R*s strongly impacts naive T cell development and survival in C57BL/6 mice. *A<sub>2A</sub>R*s couple to the heterotrimeric G protein, primarily G<sub>s</sub> 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 *A<sub>2A</sub>R*/PKA activation limits IL-7R down-regulation by reducing

TCR-mediated activation of the PI3K-AKT pathway. The results imply that tissue levels of endogenous adenosine are high enough to activate *A<sub>2A</sub>R*s. 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<sub>2A</sub>R*<sup>-/-</sup> 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



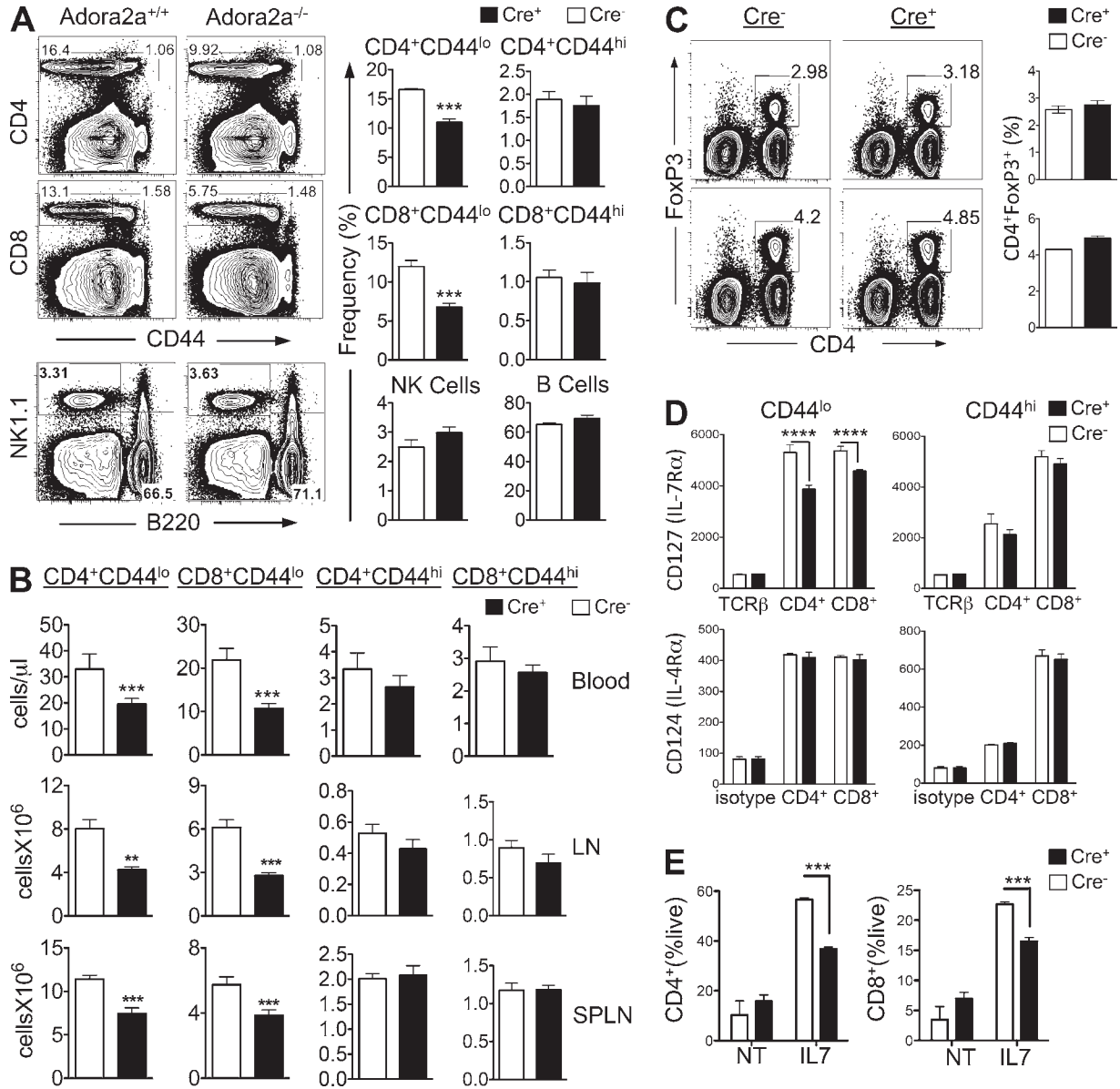


**Figure 6. A<sub>2A</sub>R signaling affects thymic T cell development.** (A) Relative levels of *Adora2a* mRNA in thymic precursors (ImmGen database). (B) Frequencies (left) and numbers (right) of thymic precursors from *Adora2a*<sup>+/+</sup> versus *Adora2a*<sup>-/-</sup> mice (representative of two independent experiments, *n* = 5; \*, *P* < 0.05; \*\*, *P* < 0.01 by Student's *t* test). (C) Recovery of *Adora2a*<sup>+/+</sup> and *Adora2a*<sup>-/-</sup> (CD45.2/UBC-EGFP) thymocytes from lethally irradiated wild-type animals (CD45.2) 12 wk after reconstitution with 1:1 mixture of *Adora2a*<sup>+/+</sup> (CD45.1) and *Adora2a*<sup>-/-</sup> (CD45.2) bone marrows (results are representative of two independent experiments, *n* ≥ 5; \*\*\*, *P* < 0.001 by two-way ANOVA and Bonferroni post-hoc tests). (D) Generation of *Adora2a*<sup>fl/fl</sup> mice. LoxP sites were inserted to flank exon 2, which also contains the ATG transcription start site. Genotyping of mice that are wild-type, heterozygous, or homozygous for the floxed allele are shown at the top. Crossing *Adora2a*<sup>fl/fl</sup> mice with Cre recombinase under control of the *Lck* promoter resulted in progressive reduction in mRNA content in thymocytes starting from DP phase (*n* = 4; \*\*\*, *P* < 0.001 by two-way ANOVA and post-hoc tests). (E) Frequencies of thymic precursors from *Adora2a*<sup>fl/fl</sup>-*LckCre*<sup>-/-</sup> and *Adora2a*<sup>fl/fl</sup>-*LckCre*<sup>-/-</sup> littermates (*n* = 4, results are from two independent experiments). Error bars are SEM.

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,

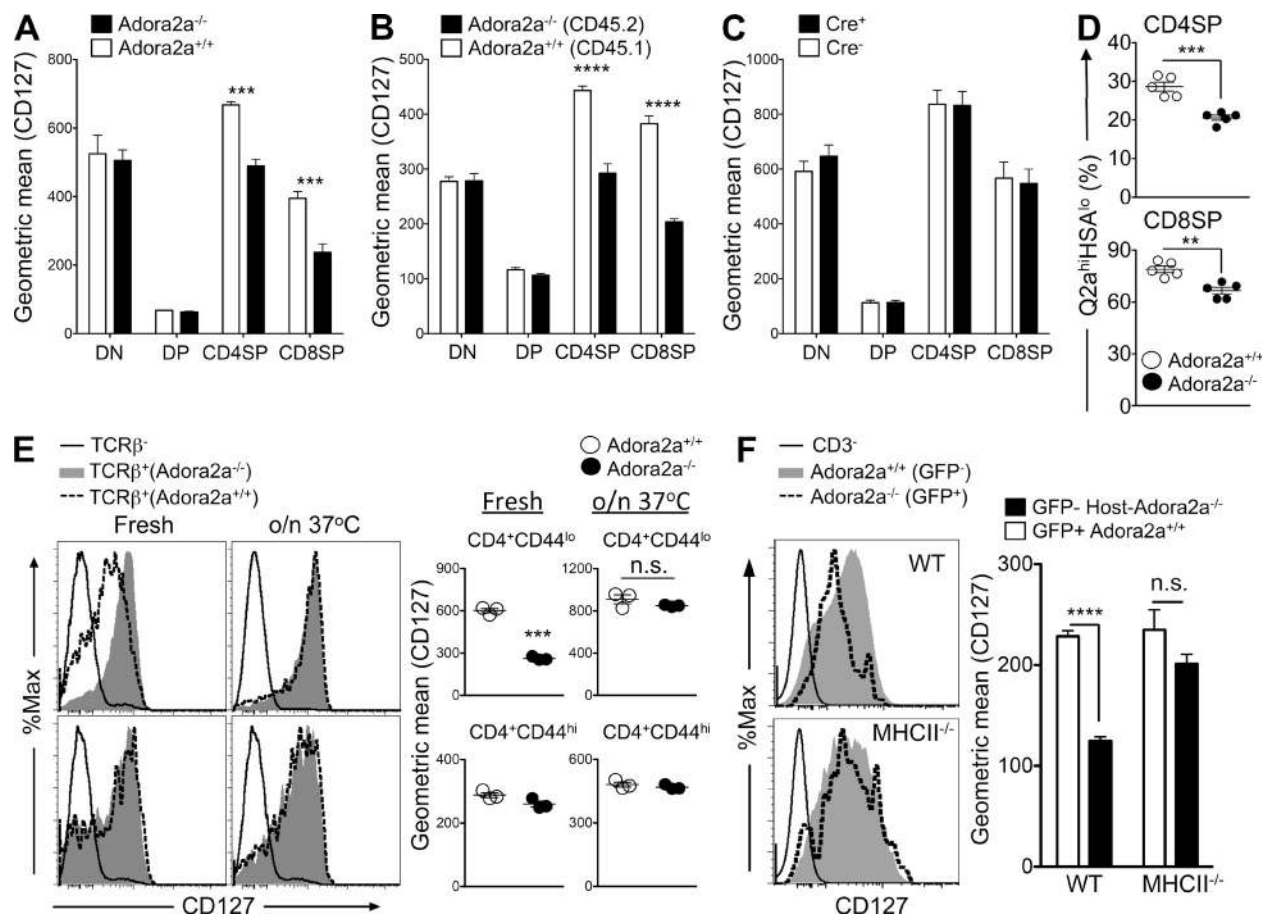
whereas most stay quiescent (Surh and Sprent, 2000). This suggests that survival plays a major role in maintaining naive T cell numbers. This explains why increased T cell proliferation in response to A<sub>2A</sub>R deletion fails to maintain normal T cell numbers. The data implicating the IL-7R as a key target of A<sub>2A</sub>R signaling agree with the proposal by Kerdiles et al.



**Figure 7. Lck deletion of *Adora2a* selectively reduces naive T cell numbers.** (A and B) Percentage of lymphocytes (frequencies) in blood (A) and corresponding cell counts of CD44<sup>lo</sup> (naive) and CD44<sup>hi</sup> CD4<sup>+</sup> and CD8<sup>+</sup> T cells in blood, LN, and spleen (SPLN); (B) of *Adora2a<sup>fl/fl</sup>-LckCre<sup>+/-</sup>* and *Adora2a<sup>fl/fl</sup>-LckCre<sup>-/-</sup>* littermates (pooled data from two different experiments with similar results,  $n \geq 6$ ). (C) Frequencies of Foxp3<sup>+</sup>CD4<sup>+</sup> T cells in LN and spleen of *Adora2a<sup>fl/fl</sup>-LckCre<sup>+/-</sup>* and *Adora2a<sup>fl/fl</sup>-LckCre<sup>-/-</sup>* littermates ( $n = 4$ , results are from two independent experiments). For A–C, \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  by unpaired two-tailed Student's  $t$  test. (D) Geometric means of CD127 staining of naive (CD44<sup>lo</sup>) and memory (CD44<sup>hi</sup>) T cells from blood of *Adora2a<sup>fl/fl</sup>-LckCre<sup>+/-</sup>* and *Adora2a<sup>fl/fl</sup>-LckCre<sup>-/-</sup>* littermates (pooled data from two different experiments with similar results,  $n \geq 6$ ). (E) Equal numbers of T cells from *Adora2a<sup>fl/fl</sup>-LckCre<sup>+/-</sup>* versus *Adora2a<sup>fl/fl</sup>-LckCre<sup>-/-</sup>* mice were cultured in the presence or absence (no treatment [NT]) of 10 ng/ml rIL-7. Frequencies of live T cells (Live-Dead yellow<sup>-</sup>) were measured after 1 wk of ex vivo culture at 37°C and 5% CO<sub>2</sub> ( $n = 4$ , results are from two independent experiments). For D and E, \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$  by two-way ANOVA and Bonferroni post-hoc analysis. Error bars are SEM.

(2009) that IL-7 is a limiting factor for T cell survival. Although PI3K–AKT signaling supports survival and proliferation of T cells, unrestrained AKT activity significantly inhibits IL-7R $\alpha$  expression, Stat5 phosphorylation, and Bcl-2 expression, thereby decreasing the ability of cells to compete for limited IL-7 and to survive, despite increased homeostatic proliferation (Rathmell et al., 2003; Kerdiles et al., 2009; Hand et al., 2010).

Interestingly, IL-7R signaling itself causes the activation of the PI3K–AKT pathway and down-regulation of IL-7R $\alpha$ . However, our findings and others suggest that the PI3K pathway is not the primary regulator of IL-7–induced IL-7R 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



**Figure 8. Recovery of CD127 expression in *Adora2a*<sup>-/-</sup> T cells in the absence of TCR signaling.** (A–C) Expression of CD127 in thymic precursors of *Adora2a*<sup>+/+</sup> and *Adora2a*<sup>-/-</sup> mice (A), recipient mice reconstituted with *Adora2a*<sup>+/+</sup> and *Adora2a*<sup>-/-</sup> bone marrows (B), and *Adora2a*<sup>fl/fl</sup>-LckCre<sup>-/-</sup> and *Adora2a*<sup>fl/fl</sup>-LckCre<sup>-/-</sup> littermates (C;  $n = 4$ , \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$  by two-way ANOVA and Bonferroni post-hoc analysis). (D) Proportions of Qa2<sup>hi</sup>HSA<sup>lo</sup> populations from SP thymocytes isolated from *Adora2a*<sup>+/+</sup> and *Adora2a*<sup>-/-</sup> mice ( $n = 5$  from one of two independent experiments with similar results; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  by unpaired two-tailed Student's  $t$  test). Please see Fig. S1 for gating strategy. (E) CD127 expression on T cells were measured before and after T cells isolated from spleen and LNs of *Adora2a*<sup>+/+</sup> and *Adora2a*<sup>-/-</sup> mice were sorted and cultured ex vivo overnight at 37°C ( $n = 4$  from two independent experiments with similar results; \*\*\*,  $P < 0.001$  after unpaired two-tailed Student's  $t$  test). (F) Enriched GFP<sup>+</sup> *Adora2a*<sup>-/-</sup> naive CD4<sup>+</sup> T cells were transferred to WT or MHCII<sup>-/-</sup> animals. CD127 expression in transferred GFP<sup>+</sup> cells and host GFP<sup>-</sup> naive CD4<sup>+</sup> T cells was measured after 24 h ( $n = 4$ , from two independent experiments with similar results; \*\*\*\*,  $P < 0.0001$  by two-way ANOVA and Bonferroni post-hoc analysis). Error bars are SEM.

regulated by two independent mechanism through Jak3–Stat5 signaling (Henriques et al., 2010; Ghazawi et al., 2013). The fact that A<sub>2A</sub>R activation selectively prevents TCR<sup>-</sup> but not IL-7R<sup>-</sup>–induced down-regulation of IL-7R $\alpha$  suggests that A<sub>2A</sub>R 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-7R $\alpha$  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 A<sub>2A</sub>R signaling selectively prevents TCR-induced IL-7R $\alpha$  down-regulation (Sprent and Surh, 2011). In *Adora2a*<sup>-/-</sup> 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*<sup>-/-</sup> mice and wild-type mice are similar despite the fact that *Adora2a*<sup>-/-</sup> 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%) A<sub>2A</sub>R 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 A<sub>2A</sub>Rs 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 A<sub>2A</sub>R signaling. It will be of interest in future studies to examine the effects of A<sub>2A</sub>R 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 A<sub>2A</sub>R 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 MHCII-deficient mice were purchased from the Jackson Laboratory and used for experiments after being acclimated for 2–6 wk. Generation of the *Adora2a<sup>fl/fl</sup>* mice is described below. LckCre mice (Lee et al., 2001) were obtained from Taconic. *Adora2a<sup>fl/fl</sup>-LckCre<sup>-/-</sup>*, *Adora2a<sup>fl/fl</sup>-LckCre<sup>+/-</sup>*, *Adora2a<sup>-/-</sup>*, *Adora2b<sup>-/-</sup>* (gift from K. Ravid, Boston University, Boston, MA), and RAG1<sup>-/-</sup> 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 A<sub>2A</sub>R mice.** We obtained a targeting construct containing the A<sub>2A</sub>R gene (*Adora2a*) previously used to generate *Adora2a<sup>-/-</sup>* 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 transcription initiation site. Deletion of exon 2 can effectively eliminate A<sub>2A</sub>R 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 clonally isolated 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 enriched 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 chimera 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 x C57BL/6) N1F1 *adora2a<sup>fl/w</sup>* 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<sup>fl/fl</sup>*) by crossing male and female N3F1 heterozygous mice. *Adora2a<sup>fl/fl</sup>* mouse generation formally started from N3F2 and were designated with the official name B6;129P–*adora2a<sup>tm1Ydj</sup>*. We maintained the colony by homozygous inbreeding onto C57BL/6J 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 × 10<sup>6</sup> 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 LSRII 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 (CD44<sup>dim</sup>), and memory T cells (CD44<sup>hi</sup>) 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 resultingly sorted cells were isolated by RNA isolation kit (QIAGEN), and cDNAs from isolated RNAs were synthesized by 4× 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.

**Adoptive cell transfer.** Spleen and peripheral LNs from age-matched *Adora2a<sup>+/+</sup>* and *Adora2a<sup>-/-</sup>* mice were collected, and single cell suspensions

were prepared by passing tissues through 40- $\mu$ M 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 1 or 2 wk after adoptive cell transfer into WT recipients or to measure the CD127 expression 1 d after the transfer into WT or MHCII<sup>-/-</sup> recipients.

**Bone marrow transplantation.** Mice 6–12 wk of age were fasted for 24 h and then lethally irradiated ( $2 \times 450$  Rads for RAG1<sup>-/-</sup> and  $2 \times 500$  Rads for C57BL/6 recipients). After the second radiation exposure, a 1:1 mixture of  $5\text{--}10 \times 10^6$  bone marrow cells from donor mice (WT and A<sub>2A</sub>R<sup>-/-</sup>) were injected i.v. Mice were treated with antibiotics from 3 d before until 2 wk after radiation. Single cell suspensions from spleens and LNs were analyzed by cytofluorometry to determine the proportion of T and B cells 7–8 wk after bone marrow transplantation.

**In vitro test for IL-7 responsiveness and IL-7R $\alpha$  expression.** For TCR stimulation or stimulation with recombinant mouse IL-7 (R&D Systems) T cells were enriched by CD4<sup>+</sup> T cell enrichment kits (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 CD44<sup>dim</sup> (naive) T cell population. To evaluate the effects of A<sub>2A</sub>R signaling on TCR-induced IL-7R $\alpha$  down-regulation, we stimulated isolated T cells with plate-bound anti-CD3 antibody in the presence or absence of 1  $\mu$ M of the selective A<sub>2A</sub>R agonist CGS 21680, 100 nM of the PKA inhibitor KT 5720, or 2  $\mu$ M of the selective PI3K inhibitor LY 294002 (Invitrogen). After 24 h of culture at 37°C (5% CO<sub>2</sub>) in RPMI medium supplied with 5% fetal bovine serum, cell surface staining and flow cytometry were used to detect cell surface expression of IL-7R $\alpha$  (CD127). Doses of KT 5720 and LY 294002 were selected based on their Ki values (50 nM and 1.4  $\mu$ M, respectively), and dose response experiments were performed using CD127 as readout.

10<sup>6</sup> LN cells were isolated from *Adora2a*<sup>+/+</sup> and *Adora2a*<sup>-/-</sup> or *Adora2a*<sup>fl/fl</sup>-LckCre<sup>-/-</sup> and *Adora2a*<sup>fl/fl</sup>-LckCre<sup>+/+</sup> mice cultured in the absence or presence of the indicated concentrations of recombinant mouse IL-7 (BioLegend) for 3 d at 37°C (5% CO<sub>2</sub>) using RPMI medium supplied with 5% fetal bovine serum. Percentages of live T cells were analyzed by flow cytometry using Live-Dead yellow and/or Annexin V staining.

**BrdU incorporation assay for proliferation.** *Adora2a*<sup>+/+</sup> or *Adora2a*<sup>-/-</sup> 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 for 20 min before treatment with wash and permeabilization buffer, and cells were resuspended in FACS buffer.

**Online supplemental material.** Fig. S1 shows the gating strategy used for Fig. 8 D. Table 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*<sup>-/-</sup> mice, Dr. Jiang Fan Chen for his gift of a targeting construct containing the A<sub>2A</sub>R gene (*Adora2a*), Dr. Heba Nowyhed for her help and suggestions for BrdU proliferation assays, Ruy Ken for real-time PCR, Dennis Huynh 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. Cekic).

The authors declare no competing financial interests.

Author contributions: C. Cekic conceived the research, conducted the experiments, analyzed the data, and wrote the manuscript; D. Sag assisted with some experiments; Y.-J. Day generated *Adora2a*<sup>fl/fl</sup> mice; and J. Linden oversaw study design and edited the manuscript.

Submitted: 4 February 2013

Accepted: 24 September 2013

## REFERENCES

- Barata, J.T., A. Silva, J.G. Brandao, L.M. Nadler, A.A. Cardoso, and V.A. Boussiotis. 2004. Activation of PI3K is indispensable for interleukin 7-mediated viability, proliferation, glucose use, and growth of T cell acute lymphoblastic leukemia cells. *J. Exp. Med.* 200:659–669. <http://dx.doi.org/10.1084/jem.20040789>
- Cekic, C., C.R. Casella, D. Sag, F. Antignano, J. Kolb, J. Suttles, M.R. Hughes, G. Krystal, and T.C. Mitchell. 2011. MyD88-dependent SHIP1 regulates proinflammatory signaling pathways in dendritic cells after monophosphoryl lipid A stimulation of TLR4. *J. Immunol.* 186:3858–3865. <http://dx.doi.org/10.4049/jimmunol.1001034>
- Ernst, B., D.S. Lee, J.M. Chang, J. Sprent, and C.D. Surh. 1999. The peptide ligands mediating positive selection in the thymus control T cell survival and homeostatic proliferation in the periphery. *Immunity.* 11:173–181. [http://dx.doi.org/10.1016/S1074-7613\(00\)80092-8](http://dx.doi.org/10.1016/S1074-7613(00)80092-8)
- Fredholm, B.B., A.P. IJzerman, K.A. Jacobson, J. Linden, and C.E. Müller. 2011. International Union of Basic and Clinical Pharmacology. LXXXI. Nomenclature and classification of adenosine receptors—an update. *Pharmacol. Rev.* 63:1–34. <http://dx.doi.org/10.1124/pr.110.003285>
- Ghazawi, F.M., E.M. Faller, S.M. Sugden, J.A. Kakal, and P.A. MacPherson. 2013. IL-7 downregulates IL-7R $\alpha$  expression in human CD8 T cells by two independent mechanisms. *Immunol. Cell Biol.* 91:149–158. <http://dx.doi.org/10.1038/icb.2012.69>
- Goldrath, A.W., and M.J. Bevan. 1999. Low-affinity ligands for the TCR drive proliferation of mature CD8+ T cells in lymphopenic hosts. *Immunity.* 11:183–190. [http://dx.doi.org/10.1016/S1074-7613\(00\)80093-X](http://dx.doi.org/10.1016/S1074-7613(00)80093-X)
- Hand, T.W., W. Cui, Y.W. Jung, E. Sefik, N.S. Joshi, A. Chande, Y. Liu, and S.M. Kaech. 2010. Differential effects of STAT5 and PI3K/AKT signaling on effector and memory CD8 T-cell survival. *Proc. Natl. Acad. Sci. USA.* 107:16601–16606. <http://dx.doi.org/10.1073/pnas.1003457107>
- Haskó, G., J. Linden, B. Cronstein, and P. Pacher. 2008. Adenosine receptors: therapeutic aspects for inflammatory and immune diseases. *Nat. Rev. Drug Discov.* 7:759–770. <http://dx.doi.org/10.1038/nrd2638>
- Heng, T.S., and M.W. Painter; Immunological Genome Project Consortium. 2008. The Immunological Genome Project: networks of gene expression in immune cells. *Nat. Immunol.* 9:1091–1094. <http://dx.doi.org/10.1038/ni1008-1091>
- Henriques, C.M., J. Rino, R.J. Nibbs, G.J. Graham, and J.T. Barata. 2010. IL-7 induces rapid clathrin-mediated internalization and JAK3-dependent degradation of IL-7R $\alpha$  in T cells. *Blood.* 115:3269–3277. <http://dx.doi.org/10.1182/blood-2009-10-246876>
- Huang, Y.J., Y. Maruyama, G. Dvoryanchikov, E. Pereira, N. Chaudhari, and S.D. Roper. 2007. The role of pannexin 1 hemichannels in ATP release and cell-cell communication in mouse taste buds. *Proc. Natl. Acad. Sci. USA.* 104:6436–6441. <http://dx.doi.org/10.1073/pnas.0611280104>
- Kerdiles, Y.M., D.R. Beisner, R. Tinoco, A.S. Dejean, D.H. Castrillon, R.A. DePinho, and S.M. Hedrick. 2009. Foxo1 links homing and survival of naive T cells by regulating L-selectin, CCR7 and interleukin 7 receptor. *Nat. Immunol.* 10:176–184. <http://dx.doi.org/10.1038/ni.1689>
- Kim, S., K. Jee, D. Kim, H. Koh, and J. Chung. 2001. Cyclic AMP inhibits Akt activity by blocking the membrane localization of PDK1. *J. Biol. Chem.* 276:12864–12870. <http://dx.doi.org/10.1074/jbc.M001492200>
- Lappas, C.M., J.M. Rieger, and J. Linden. 2005. A<sub>2A</sub> adenosine receptor induction inhibits IFN- $\gamma$  production in murine CD4+ T cells. *J. Immunol.* 174:1073–1080.
- Ledent, C., J.M. Vaugeois, S.N. Schiffmann, T. Pedrazzini, M. El Yacoubi, J.J. Vanderhaeghen, J. Costentin, J.K. Heath, G. Vassart, and M. Parmentier. 1997. Aggressiveness, hypoalgesia and high blood pressure in mice lacking the adenosine A<sub>2A</sub> receptor. *Nature.* 388:674–678. <http://dx.doi.org/10.1038/41771>
- Lee, P.P., D.R. Fitzpatrick, C. Beard, H.K. Jessup, S. Lehar, K.W. Makar, M. Pérez-Melgosa, M.T. Sweetser, M.S. Schlissel, S. Nguyen, et al. 2001. A critical role for Dnmt1 and DNA methylation in T cell development, function, and survival. *Immunity.* 15:763–774. [http://dx.doi.org/10.1016/S1074-7613\(01\)00227-8](http://dx.doi.org/10.1016/S1074-7613(01)00227-8)

- Linden, J., and C. Cekic. 2012. Regulation of lymphocyte function by adenosine. *Arterioscler. Thromb. Vasc. Biol.* 32:2097–2103. <http://dx.doi.org/10.1161/ATVBAHA.111.226837>
- Lou, L., J. Urbani, F. Ribeiro-Neto, and D.L. Altschuler. 2002. cAMP inhibition of Akt is mediated by activated and phosphorylated Rap1b. *J. Biol. Chem.* 277:32799–32806. <http://dx.doi.org/10.1074/jbc.M201491200>
- Muranski, P., B. Chmielowski, and L. Ignatowicz. 2000. Mature CD4+ T cells perceive a positively selecting class II MHC/peptide complex in the periphery. *J. Immunol.* 164:3087–3094.
- Ohta, A., and M. Sitkovsky. 2001. Role of G-protein-coupled adenosine receptors in downregulation of inflammation and protection from tissue damage. *Nature.* 414:916–920. <http://dx.doi.org/10.1038/414916a>
- Ohta, A., A. Ohta, M. Madasu, R. Kini, M. Subramanian, N. Goel, and M. Sitkovsky. 2009. A2A adenosine receptor may allow expansion of T cells lacking effector functions in extracellular adenosine-rich microenvironments. *J. Immunol.* 183:5487–5493. <http://dx.doi.org/10.4049/jimmunol.0901247>
- Pallard, C., A.P. Stegmann, T. van Kleffens, F. Smart, A. Venkitaraman, and H. Spits. 1999. Distinct roles of the phosphatidylinositol 3-kinase and STAT5 pathways in IL-7-mediated development of human thymocyte precursors. *Immunity.* 10:525–535. [http://dx.doi.org/10.1016/S1074-7613\(00\)80052-7](http://dx.doi.org/10.1016/S1074-7613(00)80052-7)
- Rathmell, J.C., R.L. Elstrom, R.M. Cinalli, and C.B. Thompson. 2003. Activated Akt promotes increased resting T cell size, CD28-independent T cell growth, and development of autoimmunity and lymphoma. *Eur. J. Immunol.* 33:2223–2232. <http://dx.doi.org/10.1002/eji.200324048>
- Resta, R., S.W. Hooker, A.B. Laurent, S.M. Jamshedur Rahman, M. Franklin, T.B. Knudsen, N.L. Nadon, and L.F. Thompson. 1997. Insights into thymic purine metabolism and adenosine deaminase deficiency revealed by transgenic mice overexpressing ecto-5'-nucleotidase (CD73). *J. Clin. Invest.* 99:676–683. <http://dx.doi.org/10.1172/JCI119211>
- Riou, C., B. Yassine-Diab, J. Van grevenynghe, R. Somogyi, L.D. Greller, D. Gagnon, S. Gimmig, P. Wilkinson, Y. Shi, M.J. Cameron, et al. 2007. Convergence of TCR and cytokine signaling leads to FOXO3a phosphorylation and drives the survival of CD4+ central memory T cells. *J. Exp. Med.* 204:79–91. <http://dx.doi.org/10.1084/jem.20061681>
- Schwindinger, W.F., L.J. Mihalcik, K.E. Giger, K.S. Betz, A.M. Stauffer, J. Linden, D. Herve, and J.D. Robishaw. 2010. Adenosine A2A receptor signaling and golf assembly show a specific requirement for the gamma7 subtype in the striatum. *J. Biol. Chem.* 285:29787–29796. <http://dx.doi.org/10.1074/jbc.M110.142620>
- Sprent, J., and C.D. Surh. 2011. Normal T cell homeostasis: the conversion of naive cells into memory-phenotype cells. *Nat. Immunol.* 12:478–484. <http://dx.doi.org/10.1038/ni.2018>
- Su, A.I., T. Wiltshire, S. Batalov, H. Lapp, K.A. Ching, D. Block, J. Zhang, R. Soden, M. Hayakawa, G. Kreiman, et al. 2004. A gene atlas of the mouse and human protein-encoding transcriptomes. *Proc. Natl. Acad. Sci. USA.* 101:6062–6067. <http://dx.doi.org/10.1073/pnas.0400782101>
- Surh, C.D., and J. Sprent. 2000. Homeostatic T cell proliferation: How far can T cells be activated to self-ligands? *J. Exp. Med.* 192:F9–F14. <http://dx.doi.org/10.1084/jem.192.4.F9>
- Surh, C.D., and J. Sprent. 2008. Homeostasis of naive and memory T cells. *Immunity.* 29:848–862. <http://dx.doi.org/10.1016/j.immuni.2008.11.002>
- Yang, K., G. Neale, D.R. Green, W. He, and H. Chi. 2011. The tumor suppressor Tsc1 enforces quiescence of naive T cells to promote immune homeostasis and function. *Nat. Immunol.* 12:888–897. <http://dx.doi.org/10.1038/ni.2068>
- Yao, S.Y., A.M. Ng, C.E. Cass, S.A. Baldwin, and J.D. Young. 2011. Nucleobase transport by human equilibrative nucleoside transporter 1 (hENT1). *J. Biol. Chem.* 286:32552–32562. <http://dx.doi.org/10.1074/jbc.M111.236117>