

A_{2A} Receptor Signaling Promotes Peripheral Tolerance by Inducing T cell Anergy and the Generation of Adaptive Regulatory T cells.

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Abstract

Tissue-derived adenosine, acting via the adenosine A_{2A} receptor (A_{2A}R), is emerging as an important negative regulator of T cell function. In this report we demonstrate that A_{2A}R stimulation not only inhibits the generation adaptive effector T cells, but also promotes the induction of adaptive regulatory T cells. *In vitro*, antigen recognition in the setting of A_{2A}R engagement induces T cell anergy, even in the presence of costimulation. T cells initially stimulated in the presence of an A_{2A}R agonist fail to proliferate and produce IL-2 and IFN- γ when rechallenged in the *absence* of A_{2A}R stimulation. Likewise, in an *in vivo* model of autoimmunity, tissue-derived adenosine promotes anergy and abrogates tissue destruction. Indeed, A_{2A}R stimulation inhibits IL-6 expression while enhancing the production of TGF- β . Accordingly, treating mice with A_{2A}R agonists not only inhibits Th1 and Th17 effector cell generation but also promotes the generation of Foxp3⁺ and LAG-3⁺ regulatory T cells. In this regard, A_{2A}R agonists fail to prevent autoimmunity by LAG-3^{-/-} clonotypic T cells implicating an important role for LAG-3 in adenosine-mediated peripheral tolerance. Overall, our findings demonstrate that extracellular adenosine stimulates the A_{2A}R to promote long-term T cell anergy and the generation of adaptive regulatory T cells.

Introduction:

Under normal physiologic conditions, the level of adenosine in the tissue microenvironment is relatively low.¹ At the height of inflammation, the destruction of host tissue by a vigorous immune response combined with damaged microcirculation and hypoxia lead to increases in extracellular adenosine. The presence of adenosine in the microenvironment prevents further tissue destruction by inhibiting inflammation.^{2,3} The importance of the adenosine A_{2A} receptor (A_{2A}R) in mediating this negative feedback loop has been dramatically demonstrated in A_{2A}R null mice.⁴ These knockout mice are unable to control inflammation, which leads to extensive, often fatal, tissue destruction. For example, when immune-mediated liver inflammation was induced in mice by injecting Con A, the A_{2A}R null mice died due to massive immune-mediated liver destruction.

The inability of the thymus to eliminate all self-reactive T cells necessitates peripheral mechanisms of T cell tolerance to prevent tissue damage from autoreactive T cells. The role of clonal deletion and T cell anergy in maintaining peripheral tolerance has been demonstrated in a number of *in vivo* models.^{5,6} More recently, the roles of Foxp3⁺ and LAG-3⁺ regulatory T cells in peripheral tolerance has been better defined.^{7,8} Likewise, cell surface receptors such as CTLA-4 and PD-1 and cytokines such as IL-10 and TGF- β are believed to not only inhibit T cell activation but also promote peripheral T cell tolerance.⁹⁻¹² In this report we identify extracellular adenosine as a novel inducer of peripheral T cell tolerance. We propose that tissue-derived adenosine acts through the A_{2A}R to generate peripheral T cell tolerance by inducing T cell anergy and adaptive regulatory T cells.

Material and Methods:

Reagents: Cyclosporine A (Calbiochem, Cambridge, MA) was dissolved in ethanol and used at 1 μ M. CGS-21680 (Sigma, St. Louis, MO) was dissolved in PBS and used at indicated concentrations. Anti-CD3 (2C11, BD PharMingen, San Diego, CA) was diluted in PBS and used at 1 μ g/mL, as indicated. Soluble anti-CD28 (a kind gift from Dr. J. Allison, UC Berkeley, CA) was used at a 1/1000 dilution. Hemagglutinin (HA) class II is an I-E^d specific peptide (SFERFEIFPKE) which was manufactured by The Johns Hopkins School of Medicine Oncology Department Peptide Synthesis Facility. Anti-phospho-ERK (Santa Cruz Biotechnology, Santa Cruz, CA) was used at 1:400 in 5% NFDm/TBS-tween (0.1%). Anti-p42/p44 MAPK (Cell Signaling Technology, Danvers, MA) was used at 1:500 in 5% BSA/TBS-tween (0.1%). Anti-jun B (Santa Cruz) and anti-Actin (Sigma) were both used at 1:1000 in 5% NFDm/TBS-tween (0.1%) mAbs used for staining: biotinylated anti-clonotypic 6.5 TCR (generously provided by H. Von Boehmer); avidin-PE (Caltag, Burlingame, CA); FITC-conjugated anti-CD4 (RM4-4); Cy-Chrome-conjugated anti-CD4 (RM4-5); PE-conjugated anti-Thy-1.1 (OX-7); FITC-conjugated anti-CD44 (1 M7); APC-conjugated anti-IFN- γ (XMG1.2); PE-conjugated anti-IL-17 (TC11-18H10); biotinylated anti-Thy1.2 (53-2.1) (all purchased from BD PharMingen). Anti-IFN- γ and anti-IL-4 (BD PharMingen) were used at 10 μ g/mL. TGF- β (Sigma) was used at 5 ng/mL. IL-6 (Peprotech, Rocky Hill, NJ), was used at 20 ng/mL. FACSCalibur was used for flow cytometry event collection, and events were analyzed using FlowJo software (Ashland, OR). Sorting was done with FACSaria (BD Biosciences, San Jose, CA).

Cell Culture: A.E7 is a clonotypic CD4⁺, Th1 T cell clone specific for pigeon cytochrome c (PCC) and is maintained as previously described.¹³ A.E7s were rendered anergic with plate-bound anti-CD3 (1 µg/mL) or by incubation with APC, PCC (10 µM), and CGS (1 µM).

Splenocytes and lymphocytes from A_{2A}R wt or null 6.5⁺ mice (see below) were harvested and enriched for CD4⁺ T cells via negative selection with the CD4⁺ T cell isolation kit and LS MACs column (all from Miltenyi Biotech, Auburn, CA). The T cells were then cultured in the presence or absence of 10 µg/mL HA and 1 µM CGS. The T cells were rechallenged for 3-4 hours with 100 µg/mL HA, GolgiStop (BD PharMingen), and irradiated APCs.

cAMP Production: Total cAMP of naïve or previously activated cells were assayed with the Biotrak EIA system (Amersham Biosciences, Buckinghamshire, UK).

Microarray: *In vitro* microarray was performed as previously described.¹⁴ *In vivo* microarray was performed as previously described.⁸

Transgenic Mice: The C3HA expressing transgenic (recipient) mice express hemagglutinin (HA) under the rat C3 promoter and has been previously described.¹⁵ The HA-specific TCR-transgenic mouse line 6.5 (donor mice) has been previously described.⁸ A_{2A}R^{-/-} mice were bred to this 6.5 mouse line. LAG-3 knockout mice on a C57/B6 were

a generous gift of Dr. Dario Vignali. The LAG-3 KO genotype was bred onto a B10.D2 6.5⁺ TCR background. All experiments involving the mice were performed in accordance with protocols approved by the Animal Care and Use Committee of The Johns Hopkins University School of Medicine.

5C.C7 TCR-transgenic *Rag2*^{-/-} mice on a B10.A background were purchased from Taconic. T cells from 5C.C7 mice are specific for pigeon cytochrome c.

Adoptive Transfer: Clonotypic CD4⁺ T cells were harvested from 6.5⁺ transgenic mice. The unfractionated population was resuspended to contain 1.2x10⁶ 6.5⁺ T cells in 200 μ L of HBSS for i.v. injection into C3HA mice. Recipient mice were given twice daily i.p. injections of vehicle (PBS alone) or CGS (2.5 mg/kg) on Days 1-4 following the transfer. No CGS was administered after Day 4.

The numbers of IFN- γ and IL-17 cells were determined by multiplying the number of infiltrating cells recovered from the entire lung by the percentage of donor (Thy1.1⁺) cells and the percentage of clonotypic cells that produce the cytokine.

Western Blot: For phospho-ERK and total ERK Western blots, splenocytes from 6.5⁺ mice were stimulated overnight with 10 μ g/mL HA class II peptide then harvested and enriched for CD4⁺ T cells. CD4⁺ purified T cells were then stimulated in 500 μ L with soluble anti-CD3 (10 μ g/mL) and soluble anti-CD28 (1:20 dilution).

For the junB and actin Western blots, CD4⁺ purified, 6.5⁺ T cells were stimulated overnight in the presence or absence of 1 μ M CGS. Nuclear extracts were then probed for junB and actin.

EMSA: Nuclear extracts from CD4⁺ purified 6.5⁺ T cells that were prepared. The AP-1 probe was: 5'-CGC TTG ATG ACT CAG CCG GAA-3'. The NF κ B probe was: 5'-AGT TGA GGG GAC TTT CCC AGG C-3'.

RT-PCR: For *in vitro* studies, 5C.C7 splenocytes were stimulated overnight with 10 μ M PCC in the presence or absence of 1 μ M CGS. mRNA was isolated with Trizol and cDNA was generated. RT-PCR was performed as previously described.¹⁴

For *in vivo studies*, recipient C3HA mice were given 1.2×10^6 6.5⁺ donor T cells as described above. On Day 3, donor T cells were sorted and cDNA generated as above. RT-PCR was performed as previously described.¹⁴

LAG-3 primers and probe sets used were: Primer 5'-ACATCAACCAGACAGTGGCCA-3'/Primer 5'-GCATCCCCTGGTGAAGGTC-3'/Probe 5'-6FAM-CCCACTCCCATCCCGGCC-TAMRA-3'

FoxP3 primers and probe sets used were: Primer 5'-GGC CCT TCT CCA GGA CAG A-3'/Primer 5'-GCT GAT CAT GGC TGG GTT GT -3'/Probe 5'-6FAM-ACT TCA TGC ATC AGC TCT CCA CTG TGG ATT AT-TAMRA-3'

IL-6 and TGF- β primers and probe sets were purchased from Applied Biosystems (Foster City, CA).

Th17 Driving Conditions: 4×10^6 5C.C7 splenocytes were stimulated as previously described.¹⁶ Briefly, cells were stimulated in media containing TGF- β (5 ng/mL), IL-6 (20 ng/mL) and anti-IL-4 and anti-IFN- γ blocking antibodies (10 μ g/mL).

Statistics: Quantitative data were expressed as mean \pm standard deviation and compared using paired Student *t* tests. Values of $p < 0.05$ were considered significant and are indicated by an asterisk (*).

Results:

A_{2A}R expression is induced by Signal 1 alone in an NF-AT dependent fashion

Rao and colleagues have proposed that the genes that promote T cell anergy are induced by NF-AT in the absence of AP-1. If Cyclosporine A (CSA), which inhibits the translocation of NF-AT to the nucleus, is added during priming, the induction of anergy is inhibited (Figure S1A). We established a microarray database of T cells stimulated under conditions that promote or inhibit the induction of anergy and devised a novel statistical algorithm (hypothesis-based analysis of microarray or HAM) to analyze this data.^{14,17} The adenosine A_{2A} receptor was among the NF-AT-dependent genes that showed increased expression upon TCR engagement and was inhibited by CSA (Figure S1B). For comparison, the adenosine A₁ receptor expression was not regulated by TCR signaling. The adenosine A_{2B} receptor displayed a similar, although less robust, expression profile to the A_{2A}R.

In order to confirm these microarray results, we assayed the CD4⁺ Th1 T cell clone, A.E7, for their expression of functional A_{2A}R. We took advantage of the fact that the A_{2A}R is a G-Protein Coupled Receptor and directly stimulates adenylyl cyclase. At a given dose of an A_{2A}R-specific agonist, differences in intracellular cAMP levels indicate the relative abundance of the A_{2A}R on the cell surface.¹⁸ We stimulated A.E7s with PBS alone (mock), plate-bound anti-CD3 or anti-CD3 + CSA overnight, rested the cells in fresh media for 24 hours, treated the cells with the A_{2A}R-specific agonist, CGS-21680 (CGS), and measured intracellular cAMP. Consistent with the microarray results, T cells treated with anti-CD3 alone produced more cAMP than mock-stimulated cells, indicating that the A_{2A}R is more highly expressed on activated T cells than resting ones (Figure 1A). Moreover, the presence of CSA during TCR stimulation prevented the cAMP increase, indicating that there is no upregulation of the A_{2A}R when TCR-induced NF-AT activation is inhibited.

Next, we analyzed A_{2A}R expression in primary T cells from Wt and A_{2A}R^{-/-} mice. In the absence of T cell stimulation, CGS-treatment did not increase intracellular cAMP (“Wt Naïve”). Upon activation, however, CGS-treatment dramatically increased cAMP production in Wt T cells, again indicating that A_{2A}R protein expression has increased. Activated T cells from A_{2A}R^{-/-} mice did not produce cAMP in response to CGS treatment, which is expected and confirms the specificity of CGS for the A_{2A}R (Figure 1B). These results directly confirm our findings with the A.E7 T cell clones and show that in primary T cells the A_{2A}R is expressed upon TCR signaling in an NF-AT dependent manner. Previous studies have clearly demonstrated the ability of the A_{2A}R to inhibit T cell activation both *in vitro* and *in vivo*.¹⁹⁻²³ In agreement with these studies, we found that

A_{2A}R engagement during A.E7 stimulation markedly inhibited IL-2 production (Figure 1C). By comparison, the inhibition of IFN- γ by A_{2A}R stimulation was less robust. This finding was not secondary to CGS-induced cell death, as determined by trypan blue exclusion (data not shown). Furthermore, we failed to see any inhibition of T cell proliferation by CGS in this dose response range (Figure 1D). Thus, fitting the Rao paradigm, the A_{2A}R is induced by Signal 1 alone in an NF-AT dependent fashion and plays a role in inhibiting T cell function, most specifically IL-2 expression.

A_{2A}R engagement promotes long-term tolerance

In light of these initial findings we wanted to determine the effect of A_{2A}R engagement on the induction of T cell anergy. We examined whether A_{2A}R engagement during the initial antigen encounter could promote tolerance such that the T cells would be unresponsive upon rechallenge **in the absence of A_{2A}R stimulation**. To this end, A.E7 T cells were stimulated for 4 days with irradiated APCs (signal 1+2) in the presence or absence of Pigeon Cytochrome c (PCC, the cognate antigen for A.E7s) and in the presence or absence of CGS. The cells were then harvested, washed extensively, and rechallenged with irradiated APCs and PCC in the **absence** of CGS. Incubating A.E7s with CGS in the absence of peptide had no consequence upon rechallenge with PCC (Figure 2A,C left bars). These results were consistent with the fact that A_{2A}R expression requires TCR engagement. However, if the T cells were activated by PCC in the presence of CGS they failed to proliferate or produce IFN- γ upon subsequent rechallenge (Figure 2B,C). Exogenous IL-2 can prevent and reverse clonal T cell anergy.^{24,25} Therefore we explored the effect of exogenous IL-2 on A_{2A}R stimulation. The addition of exogenous IL-2 during the induction mitigated the effect of A_{2A}R engagement (Figure

2D,E). When IL-2 was present in the induction cultures, CGS had no effect on proliferation and IFN- γ production upon rechallenge. Overall, our data indicate that A_{2A}R stimulation even in the presence of costimulation can induce a hyporesponsive state upon rechallenge that resembles T cell anergy.

Having determined that A_{2A}R engagement can promote anergy in A.E7 T cell clones, we next wanted to examine whether A_{2A}R signaling can induce tolerance in primary T cells. We isolated CD4⁺ T cells from Wt or A_{2A}R^{-/-} 6.5⁺ mice and cultured them with irradiated APCs and HA peptide in the presence or absence of CGS. The T cells were harvested, extensively washed, and rechallenged with HA peptide in the **absence** of CGS. Upon rechallenge with HA, Wt T cells produced IFN- γ . In contrast, T cells that were initially incubated with HA peptide and CGS were hyporesponsive upon rechallenge. Since no CGS was present during the rechallenge, the decreased cytokine production reflects the induction of anergy. This effect was A_{2A}R specific as T cells from A_{2A}R^{-/-} mice cultured initially in the presence or absence of CGS have equivalent numbers of IFN- γ ⁺ T cells (Figure 3A). A_{2A}R signaling during an initial antigen encounter can induce a state of anergy in primary T cells such that they are hyporesponsive upon rechallenge.

A_{2A}R signaling inhibits activation of the Ras-MAP-kinase pathway

Next, we tested the hypothesis that A_{2A}R signaling might promote anergy by inhibiting TCR-induced AP-1 activation. A series of experiments was performed to determine the effect of A_{2A}R signaling on the Ras-MAP-kinase pathway. CD4⁺ 6.5⁺ T cells were stimulated with HA overnight to induce expression of the A_{2A}R. The cells were then harvested and washed before being stimulated with anti-CD3 and anti-CD28 \pm

CGS. In the absence of CGS, ERK was rapidly phosphorylated. However, in the presence of CGS, this phosphorylation was prevented. Total ERK levels remain constant (Figure 3B). We then looked at the effect of A_{2A}R signaling on the expression of junB, a component of AP-1. In the absence of stimulation, CD4⁺ 6.5⁺ T cells had low levels of junB expression. Upon stimulation, junB levels drastically increased. But, if the CD4⁺ 6.5⁺ T cells were stimulated in the presence of the A_{2A}R-agonist, junB levels remained similar to the unstimulated cells (Figure 3C). Finally, EMSA was performed to examine AP-1 activity in the nucleus. In the absence of stimulation, minimal AP-1 binding was observed. Upon stimulation, there was an increase in AP-1 in the vehicle-treated cells; however, in the presence of A_{2A}R signaling AP-1 binding was inhibited (Figure 3D).

Extracellular adenosine promotes long-term tolerance, *in vivo*

Using T cell clones and TCR transgenic primary T cells, we have shown that A_{2A}R signaling promotes a state of hyporesponsiveness similar to T cell anergy, even in the presence of costimulation. We then sought to determine whether endogenous, tissue-derived adenosine, *in vivo*, could promote T cell tolerance. A series of experiments employed a previously established model of T cell tolerance induction and autoimmunity *in vivo*.^{8,15,26,27} C3HA mice express hemagglutinin (HA) as a self antigen, with especially high HA expression in the lung and prostate. When HA-specific 6.5⁺ T cells are adoptively transferred into C3HA mice, the donor 6.5⁺ T cells are initially activated by HA, which is presented as a “self” antigen. But, by Day 4, they have been rendered anergic. If a sufficient number of 6.5⁺ T cells are transferred, the initial activation of the clonotypic cells dominates and the recipient mice succumb to autoimmune-induced pneumonitis. We wanted to determine if the A_{2A}R is upregulated on the HA-specific T

cells, *in vivo*. Clonotypic T cells were adoptively transferred into C3HA mice, isolated 4 days later and assessed by microarray analysis for $A_{2A}R$ expression. When compared to the T cells transferred into non-transgenic mice, or mice infected with vaccinia virus expressing HA, the $A_{2A}R$ was markedly upregulated on the *in vivo* tolerized clonotypic T cells (Figure 4A).

Having determined that the $A_{2A}R$ is upregulated on the adoptively transferred T cells, *in vivo*, we investigated the role that endogenous adenosine might play in promoting tolerance. Wt and $A_{2A}R^{-/-}$ clonotypic T cells were adoptively transferred into C3HA mice. As seen in Figure 4B, the dose of Wt 6.5^+ T cells was insufficient to induce lethal autoimmunity. The clonotypic T cells become tolerant and 100% of the mice survive. On the other hand, transfer of the $A_{2A}R^{-/-}$ 6.5^+ T cells led to 80% death due to autoimmune pneumonitis. Thus, in the absence of $A_{2A}R$ signaling on the adoptively transferred T cells, a tolerizing interaction ($A_{2A}R$ engagement) is removed leading to tissue destruction and death. Since only the adoptively transferred cells are $A_{2A}R^{-/-}$, our observations reflect the direct effect of endogenous tissue-derived adenosine on T cells and not an increase in the general inflammatory milieu (for example, the host APCs are Wt and thus are responsive to the tissue-released adenosine).

We then asked whether we could pharmacologically induce tolerance, *in vivo*, by administering CGS to hosts that receive $A_{2A}R$ competent 6.5^+ T cells. We transferred a lethal dose of T cells followed by a 4-day course of CGS (2.5 mg/kg, ip, twice daily). CGS treatment resulted in long term survival (30+ days) of 76% of the host mice, whereas only 24% of the vehicle treated hosts survived past day 9 (Figure 5A). We wanted to rule out that the ability of CGS to induce tolerance was simply due to the

deletion of the clonotypic cells. To test this, we transferred 6.5^+ T cells into C3HA mice and harvested the donor 6.5^+ T cells 2 or 3 days after transfer. CGS did not affect the frequency of donor 6.5^+ T cells (Figure S2). The relatively equivalent numbers of clonotypic cells is consistent with our *in vitro* finding that CGS is a poor inhibitor of T cell proliferation (Figure 1D). It also suggests that deletion is not the mechanism by which $A_{2A}R$ signaling promotes tolerance, *in vivo*. We next tested the clonotypic T cells from the treated and untreated mice for their function in response to rechallenge with HA antigen, *in vitro*. Donor 6.5^+ T cells harvested from vehicle- or CGS-treated mice on Day 2 showed no differences in proliferation or IFN- γ and IL-2 production (Figure 5B,C,D grey bars). By Day 3, however, donor 6.5^+ T cells from CGS-treated hosts demonstrated greatly reduced proliferation and IFN- γ and IL-2 production when compared to those from vehicle-treated hosts (Figure 5B,C,D open bars). $A_{2A}R$ signaling, *in vivo*, promotes T cell tolerance, resulting in reduced lung damage and increased survival.

Recently, it has been demonstrated that the lethality in the C3HA model is mediated, in part, by lung-infiltrating IL-17 producing cells (Harris and Drake, personal communication). Thus, we tested whether $A_{2A}R$ stimulation was able to affect the generation of Th17 effector cells in this model. Recipient C3HA mice were given 10×10^6 6.5^+ T cells and treated with vehicle or CGS. The lungs were harvested and intraparenchymal clonotypic T cells were stimulated with HA peptide, *in vitro*. As seen in Figures 5E and 5F there was a decrease in the number of not only IFN- γ producing cells but also IL-17-producing lung infiltrating T cells in the mice treated with CGS. Thus, treatment with CGS resulted in a decrease in the tissue infiltration of Th1 and Th17 effector T cells.

The generation of IL-17 producing cells requires both IL-6 and TGF- β . In light of the anti-inflammatory properties of A_{2A}R stimulation we hypothesized that A_{2A}R stimulation might inhibit the generation of IL-17 producing cells by altering the cytokines found in the inflammatory milieu. We tested this hypothesis using a previously defined method of generating Th17 cells *in vitro* from naïve T cells from 5C.C7 TCR-transgenic *Rag2*^{-/-} mice.¹⁶ As seen in Figure 6A, CGS inhibited the expression of IL-6. In the same culture conditions, however, A_{2A}R activation led to a marked upregulation of TGF- β (Figure 6B). These data suggest that CGS limits the generation of IL-17 producing cells, *in vivo*, in part, by inhibiting IL-6 production. Indeed, CGS did not inhibit the induction of IL-17⁺ T cells, *in vitro*, if exogenous IL-6 was added to the culture conditions (Figure 6C).

Inducible Foxp3⁺ regulatory cells are generated when antigen recognition occurs in the presence of TGF- β .²⁸ Since TCR engagement in the presence of CGS led to an increase in TGF- β expression we wanted to know if CGS could promote the expression of Foxp3. Indeed, CGS-induced inhibition of IL-6 and enhancement TGF- β was accompanied by an increase in Foxp3 expression (Figure 6D). This finding, in turn, prompted us to examine Foxp3 expression in the clonotypic cells derived from the CGS treated mice. As seen in Figure 6E there is increased expression of Foxp3 in the clonotypic T cells isolated 3 days after adoptive transfer from the CGS-treated mice when compared with the untreated mice. Thus, under immune activating conditions, A_{2A}R activation can not only inhibit the expression of pro-inflammatory cytokines but also enhance the transcription of inhibitory molecules such as TGF- β and Foxp3.

A_{2A}R signaling promotes the induction of LAG-3⁺ Tregs, *in vivo*

Recently, it has become clear that the CD4 homologue LAG-3 defines a subset of novel inducible regulatory T cells.^{8,29,30} In the C3HA model, the induction of anergy correlates with increased LAG-3 expression and the generation of these inducible regulatory T cells. In light of the ability of A_{2A}R stimulation to inhibit the generation of Th17 effector T cells in favor of Foxp3⁺ T cells, we inquired whether A_{2A}R engagement would also lead to an increase in LAG-3⁺ regulatory T cells. We stimulated CD4⁺ 6.5⁺ T cells, *in vitro*, with irradiated APCs and HA in the presence or absence of CGS and IL-2. The cells were harvested and the cDNA was assayed for LAG-3 expression by Real Time PCR. T cells stimulated in the presence of CGS showed increased levels of LAG-3 transcript. The presence of exogenous IL-2, however, prevented the CGS-induced upregulation of LAG-3 (Figure 7A). These results are consistent with our *in vitro* tolerance data suggesting that IL-2 can overcome A_{2A}R-induced inhibition (Figure 2D and E). We also analyzed LAG-3 expression from 6.5⁺ T cells that had been stimulated *in vivo* in C3HA host mice. Donor 6.5⁺ T cells were harvested from host mice 3 days after transfer and sorted. Wt T cells that were anergized in C3HA hosts displayed a dramatic increase in LAG-3 expression. In contrast, T cells from A_{2A}R^{-/-} 6.5⁺ mice did not have a robust increase in LAG-3 (Figure 7B). Since only the adoptively transferred cells are A_{2A}R^{-/-} (i.e. host derived APCs are Wt), these data suggest that endogenous adenosine plays a physiological role in the upregulation of LAG-3, *in vivo*. Consistent with this notion is the observation that pharmacologically inducing A_{2A}R signaling by treating hosts with CGS led to enhanced LAG-3 expression on donor T cells when compared to donor T cells from vehicle-treated mice (Figure 7C).

Next we wanted to determine if the increase in LAG-3 corresponded with increased regulatory activity, *in vivo*. First, we transferred LAG-3^{-/-} 6.5⁺ T cells into C3HA hosts and treated them with PBS or CGS. Consistent with our data in Figure 5A, 75% of the vehicle-treated mice receiving Wt clonotypic T cells died, while 100% of the CGS-treated mice survived. On the other hand, eliminating LAG-3 completely abolished the ability of CGS to promote survival, as 100% of the mice that received LAG-3^{-/-} T cells died, regardless of whether they were treated with CGS or not (Figure 7D). These data suggest that the upregulation of LAG-3 by A_{2A}R signaling plays a critical role in the ability of A_{2A}R signaling to promote tolerance and prevent tissue destruction.

Finally, we examined whether A_{2A}R signaling would lead to an increase in regulatory T cells that could mediate dominant-negative suppression upon subsequent rechallenge with naïve clonotypic cells. To this end we designed an *in vivo* suppression assay. In the C3HA model, the mice that survive the initial transfer of autoreactive T cells develop LAG-3⁺ regulatory T cells that can suppress a second challenge of autoreactive 6.5⁺ T cells and prevent lethal tissue destruction.⁸ We injected the surviving mice of Figure 5A with a dose of T cells that would cause 100% death in naïve mice. Indeed, by Day 10, all of the naïve mice receiving this high dose of clonotypic T cells died of lymphocyte-mediated tissue destruction of the lung (Figure 7E). Alternatively, consistent with previously published results, the 20% of the mice that survived the initial adoptive transfer of clonotypic T cells (Figure 5A) had developed Tregs which suppressed the activity of the high dose of clonotypic cells and protected the mice from lung inflammation and death. Likewise, in accordance with our hypothesis that A_{2A}R signaling promotes the generation of Tregs, 100% of the CGS-treated hosts that survived

the initial challenge of autoreactive T cells also survived the subsequent dose of T cells (Figure 7E). That is, 4 days of CGS treatment one month prior led to the generation of regulatory T cells that suppressed the activation of the newly transferred clonotypic T cells, preventing them from inducing tissue destruction and death.

Discussion:

A_{2A}R signaling on macrophages, T cells, and dendritic cells has been shown to directly inhibit effector function.^{19-23,31-34} Our data demonstrate that tissue-derived adenosine acts via the A_{2A}R to promote peripheral T cell tolerance. Specifically, we demonstrate that A_{2A}R engagement can facilitate the induction of T cell anergy, skew T cell differentiation away from adaptive effector cells to adaptive regulatory cells, and promote the generation of LAG-3⁺ regulatory T cells.

T cell anergy occurs when TCR engagement occurs in the absence of costimulation.³⁵ Anergic T cells produce less IL-2 and IFN- γ , and proliferate less upon rechallenge to full stimulation (Signal 1+2). In this report we demonstrate that A_{2A}R stimulation can promote T cell anergy even in the presence of costimulation. These observations are consistent with the ability of A_{2A}R stimulation to profoundly inhibit IL-2 production. However, not all inhibitors of IL-2 promote anergy. CSA for example potently abrogates IL-2 production but actually inhibits anergy induction by blocking NF-AT inducible “anergic factors”. Rao and colleagues have proposed that anergy is induced when TCR engagement results in NF-AT activation in the absence of AP-1 stimulation.³⁶ Along these lines, we are able to demonstrate that A_{2A}R stimulation can inhibit TCR induced Erk phosphorylation and subsequent AP-1 binding (Figure 3B and

D). These findings agree with a recent report demonstrating the ability of an A_{2A}R agonist to inhibit TCR-induced ZAP70 phosphorylation.²¹

Depending on the inflammatory microenvironment, TCR engagement can lead to the development of CD4⁺ adaptive effector cells, such as Th1, Th2 and Th17 cells³⁷. Alternatively, in the setting of TGF-β without IL-6, TCR engagement can lead to the generation of “inducible” Foxp3⁺ regulatory T cells. Our data demonstrate the ability of tissue-derived adenosine, signaling through the A_{2A}R, to drive CD4⁺ T cell away from Th17 differentiation and towards Foxp3⁺ T cell differentiation. Consistent with these observations, A_{2A}R stimulation resulted in increased TGF-β production with a concomitant decrease in the pro-inflammatory cytokine IL-6. Mechanistically, we propose that A_{2A}R stimulation enhances Foxp3 transcription by decreasing IL-6 expression and enhancing TGF-β expression. These observations are consistent with findings suggesting that A_{2A}R stimulation inhibits pro-inflammatory cytokine expression while sparing anti-inflammatory cytokine expression.²³ Interestingly, there have been several recent reports suggesting that adenosine acting, via the A_{2A}R, might mediate, in part, the suppressive function of regulatory T cells by engaging the A_{2A}R on the suppressed cells. It was found that CD39 and CD73 (both of which are ectoenzymes which promote the generation of extracellular adenosine) appear to be a more specific markers for Foxp3⁺ regulatory T cells than CD25.³⁸ The authors suggest that the generation of adenosine by regulatory T cells suppresses effector cells via the A_{2A}R. Likewise in a colitis model it was found that CD45RB^{low} or CD25⁺ T cells from A_{2A}R null mice fail to suppress CD45RB^{high} cells and prevent disease and effector cells from A_{2A}R null mice are not suppressed by suppressor cells derived from Wt mice.²³

In addition to the ability of A_{2A}R stimulation to promote anergy and the expression of Foxp3, we found that tissue-derived adenosine, engaging the A_{2A}R, promoted the generation of LAG-3⁺ T regulatory cells. LAG-3 is a CD4 homologue that is upregulated upon T cell activation and remains elevated on a subset of T cells that possess regulatory cell function.⁸ Our *in vitro* and *in vivo* data demonstrate that A_{2A}R signaling promotes the generation of these regulatory T cells. T cells from LAG-3 null mice have diminished regulatory capacity and the ectopic expression of LAG-3 confers the ability to suppress^{8,29}. Recently it has been shown that upon TCR engagement, LAG-3 is cleaved from the cell surface of activated T cells by metalloproteinases.³⁹ This cleavage is necessary for robust T cell proliferation and cytokine production suggesting that LAG-3 also possesses cell intrinsic regulatory function. In our model, A_{2A}R agonists promote the generation of LAG-3 expressing T cells that have the capability of inducing tolerance and preventing autoimmunity. LAG-3 expression is required for suppressive function, as A_{2A}R agonists were unable to inhibit autoimmunity from the adoptive transfer of autoreactive LAG-3 null T cells. LAG-3 expression has been noted on tumor infiltrating lymphocytes that have the capacity to suppress anti-tumor specific CD8⁺ T cells.³⁰ The fact that the tumor microenvironment contains relatively high levels of adenosine suggests that tumor-derived adenosine may promote the generation of these regulatory cells.^{40 22}

For T cells, the consequences of TCR engagement are determined by the integration of multiple “accessory” signals.⁴¹ Just as IL-10 and TGF-β have been associated with inhibiting immune responses and promoting tolerance induction, we propose that tissue-derived adenosine, acts as a “metabokine,”⁴² to perform a similar

function. Inasmuch as uric acid, HMG1- β , and low molecular weight species of extracellular matrix (i.e. hyaluronic acid) might be considered examples of tissue-derived "danger" signals, we propose that tissue-derived adenosine, acting through the A_{2A}R, is one of many critical accessory signals that promote the induction of peripheral tolerance.⁴³⁻⁴⁵ As such, during "non-infectious" tissue injury, adenosine released into the microenvironment may contribute to the maintenance of peripheral tolerance to *de novo* released self antigens.

The ability of A_{2A}R agonists to promote T cell tolerance suggests that such agents could be employed in the treatment of T cell mediated autoimmune disease and transplantation. In theory A_{2A}R agonists might promote long term, peripheral tolerance in the absence of continued immunosuppression. In support of such a hypothesis are studies showing that A_{2A}R agonists can prevent allograft rejection not only by acutely inhibiting T cell activation but also by promoting the upregulation of the inhibitory molecules PD-1 and CTLA-4.²¹ Alternatively, if tumor-derived adenosine inhibits anti-tumor responses by promoting the generation of anergic and suppressor T cells, A_{2A}R antagonists might prove to be important adjuvants to tumor immunotherapy. Indeed, a role for the A_{2A}R in protecting tumors from anti-tumor T cells has been described.⁴⁶

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Authorship:

P.E.Z. performed the research and wrote the paper. C.T.H. performed research. E.R.L. performed research. J.K. assisted with microarray work. M.R.H. analyzed the data and assisted with research. J.L. provided the A_{2A}R null mice and helped design experiments. C.G.D. provided the C3HA and 6.5 mouse lines and analyzed the data. J.D.P. conceived of the project, designed the research and wrote the paper.

The authors declare no competing financial interests.

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Figure 1: A_{2A}R expression is upregulated upon T cell activation and preferentially inhibits IL-2 production. **a.** cAMP levels following CGS incubation of A.E7s previously mock stimulated (open diamonds) or stimulated with anti-CD3 in the presence or absence of CSA (open triangles or solid circles, respectively). **b.** cAMP levels of naïve or previously activated primary T cells from A_{2A}R Wt or null mice after incubation with 0 or 1 μ M CGS (solid and open bars, respectively, *: p<0.05). **c.** IL-2 (top) and IFN- γ (bottom) production of A.E7 T cells during activation with increasing doses of CGS. ID₅₀ for IL-2 is 8 nM, and for IFN- γ 750 nM. **d.** Proliferation of A.E7s with 0 or 1 μ M of CGS. (All results are representative of 3 separate experiments.)

Figure 2: A_{2A}R engagement during activation promotes T cell tolerance. **a & b.** Proliferation upon rechallenge of A.E7 T cells following 4 day incubation without (**a**) or with (**b**) peptide in the absence (open squares) or presence (solid diamonds) of 1 μ M CGS. **c.** IFN- γ production upon rechallenge of A.E7 T cells following priming without or with peptide (left or right side, respectively) in the absence (grey bars) or presence (open bars) of 1 μ M CGS. **d & e.** Proliferation and IFN- γ production upon rechallenge of A.E7s incubated with peptide and exogenous IL-2 in the absence or presence of 1 μ M CGS. All rechallenges are done in the *absence* of CGS or exogenous IL-2. All data are representative of at least 3 independent experiments. (*: p<0.05)

Figure 3: A_{2A}R signaling promotes decreases signaling of the Ras-MAP-Kinase pathway. **a.** Percentage of A_{2A}R Wt or KO T cells that were IFN- γ positive upon *rechallenge* following incubation with peptide in the absence (grey bars) or presence (open bars) of 1 μ M CGS during induction. (*:p<0.05) **b.** Representative Western blots for phospho-ERK and total ERK (top and bottom respectively). Activated CD4⁺, 6.5⁺ primary T cells were stimulated with anti-CD3+anti-CD28 in the absence or presence of 1 μ M CGS. A vertical line has been inserted to indicate a repositioning of gel lanes from the same experiment. **c.** Representative Western blots for junB, and actin (top and bottom, respectively). CD4⁺, 6.5⁺ primary T cells were stimulated with HA and irradiated APCs overnight in the absence or presence of 1 μ M CGS. **d.** Representative EMSA for AP-1. CD4⁺, 6.5⁺ primary T cells were stimulated with HA and irradiated APCs overnight in the absence or presence of 1 μ M CGS. Data are representative of 3 independent experiments.

Figure 4: Endogenous adenosine is required to prevent death by autoimmunity. **a.** *In vivo* A_{2A}R mRNA expression as determined by Affymetrix microarray analysis for 6.5⁺ TCR transgenic clonotypic T cells specific for hemagglutinin (HA) that had been transferred into B10.D2 mice (NT), vaccinated B10.D2 mice (Vac) or C3HA mice (C3HA). **b.** Survival curve of C3HA mice given Wt (grey diamonds) or A_{2A}R^{-/-} 6.5⁺ T cells (open circles) (n=5 each condition). Data are representative of 2 independent experiments.

Figure 5: A_{2A}R stimulation *in vivo* prevents death by autoimmunity and promotes T cell tolerance. **a.** Survival curve of C3HA mice given 6.5⁺ T cells and 4 days of vehicle (open squares) or CGS (solid diamonds) (n=17 mice, each condition). **b, c, & d.** *In*

vitro proliferation (**b**), IFN- γ production (**c**), and IL-2 (**d**) of T cells harvested from vehicle- or CGS-treated C3HA mice (grey and open bars, respectively). Data are representative of 2 independent experiments, ≥ 3 mice per group. **e & f**. Number of IFN- γ (**e**) and IL-17 (**f**) lung-infiltrating 6.5⁺ T cells from C3HA mice given a lethal dose of autoreactive T cells. Data are a combination of 2 independent experiments, 3 mice per group. (*: p<0.05)

Figure 6: A_{2A}R engagement during inflammation inhibits IL-6 and promotes Foxp3 expression, *in vivo*. **a & b.** Primary T cells from 5C.C7 mice were cultured with PCC \pm 1 μ M CGS for 1 day. Total RNA was harvested and assayed for abundance of IL-6 (**a**), and TGF- β (**b**) transcripts by Real Time PCR. Data are representative of 3 independent experiments. **c.** Splenocytes from 5C.C7 TCR-transgenic *Rag2*^{-/-} mice were cultured with antigen (PCC) \pm 1 μ M CGS for 3 days under Th17-driving conditions (containing TGF- β , IL-6, anti-IFN- γ and anti-IL-4). The cells were then activated with PMA and Ionomycin and assayed for IL-17 by intracellular cytokine staining. Representative FACs plots are shown (left) and bar graph of composite data (right). Data are representative of 3 independent experiments. **d.** Primary T cells from 5C.C7 mice were cultured with PCC \pm 1 μ M CGS for 1 day. Total RNA was harvested and assayed for abundance of Foxp3 transcripts by Real Time PCR. Data are representative of 3 independent experiments. **e.** 6.5⁺ T donor T cells were harvested 3 days after transfer into C3HA hosts. Total RNA was harvested and assayed for abundance of Foxp3 by Real Time PCR. Data are representative of 2 independent experiments, 3 mice per group. All PCR samples were done in triplicate and evaluated for significance. (*:p<0.05)

Figure 7: A_{2A}R signaling promotes LAG-3 expression and regulatory T cells. **a.** CD4⁺, 6.5⁺ primary T cells were cultured with irradiated APCs and HA \pm 1 μ M CGS and IL-2 for 3 days. Total RNA was harvested and assayed for abundance of LAG-3 transcripts by Real Time PCR. **b.** A_{2A}R Wt or KO 6.5⁺ T cells were transferred into C3HA mice, harvested 3 days after adoptive transfer and sorted to >98% purity. LAG-3 expression was determined by Real Time PCR. **c.** Clonotypic 6.5⁺ T cells were transferred into C3HA mice which were treated with vehicle or CGS for 3 days following the adoptive transfer. The donor T cells were harvested and sorted to >98% purity. LAG-3 expression was determined by Real Time PCR. For **a**, **b**, and **c**, data are representative of 3 independent experiments, 3 mice per group. **d.** Survival curve of C3HA mice given Wt or LAG-3 KO T cells and a 4 day treatment with CGS. The “Wt Veh” and “LAG-3 KO CGS” both had 4 mice per group. For The “Wt CGS” and “LAG-3 KO Veh” both had 5 mice per group. Data are representative of 2 independent experiments. **e.** *In vivo* suppression assay in which vehicle- or CGS-treated C3HA mice (the survivors of Figure 5A; open squares [n=4] or grey diamonds [n=13], respectively) are given a higher dose of 6.5⁺ T cells. Naïve mice (open triangles, [n=17]) received only this higher dose of 6.5⁺ T cells. No drug was administered during this phase of the experiment. (*:p<0.05)

Figure 1:

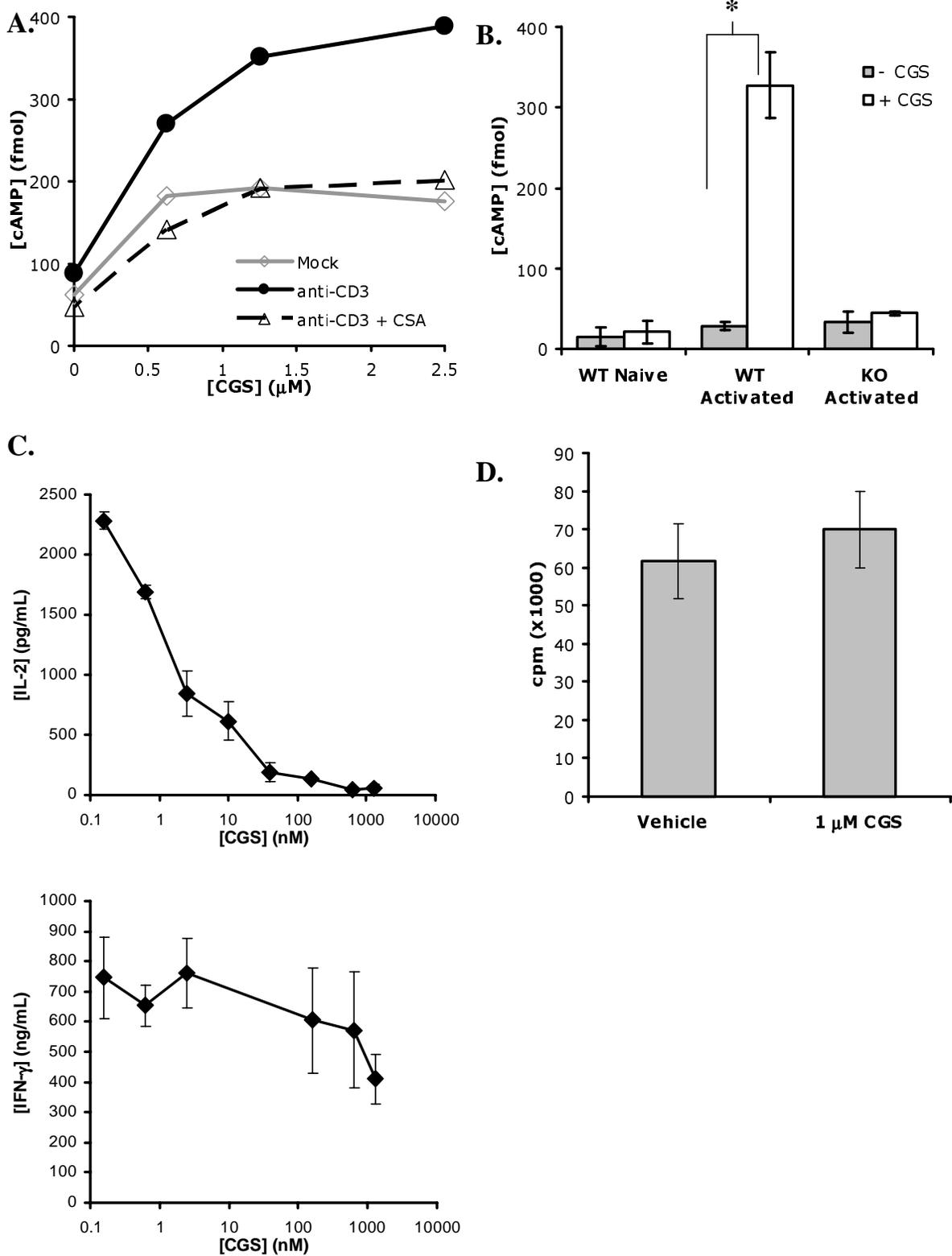


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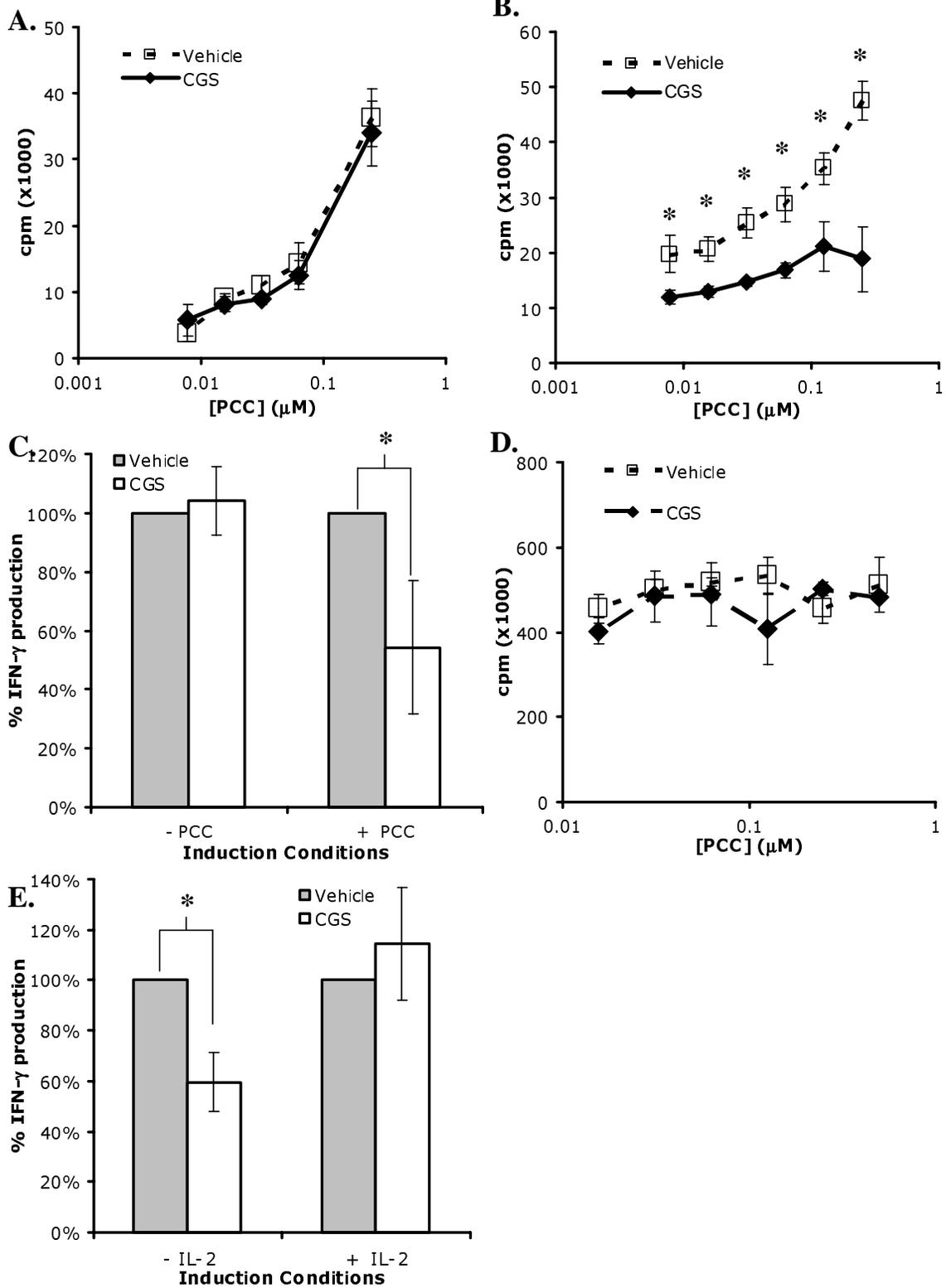


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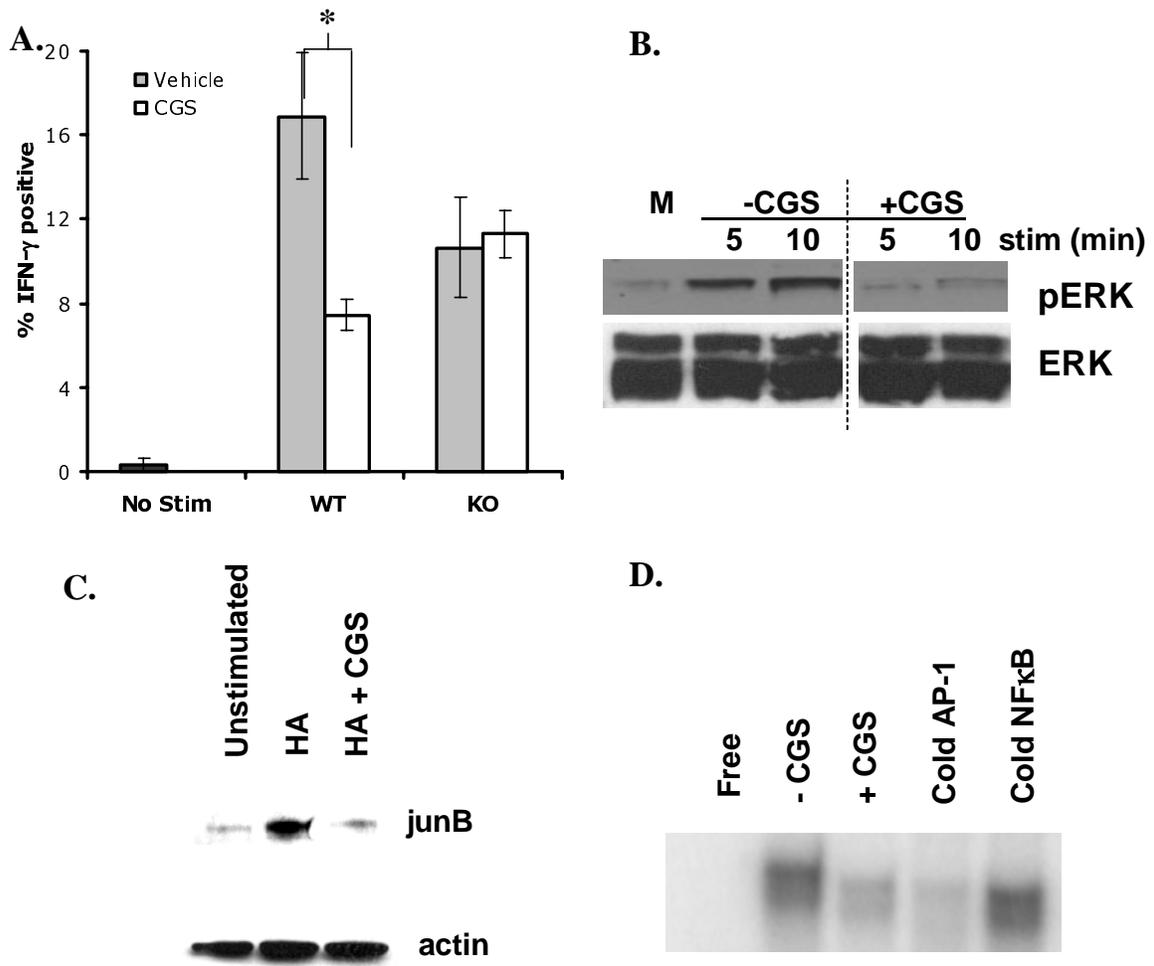


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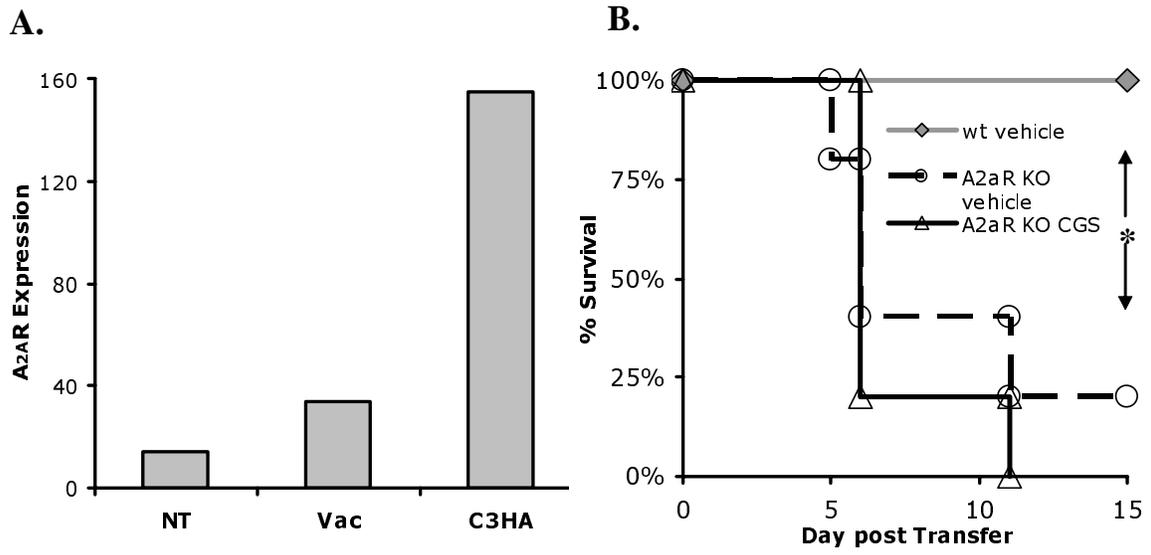


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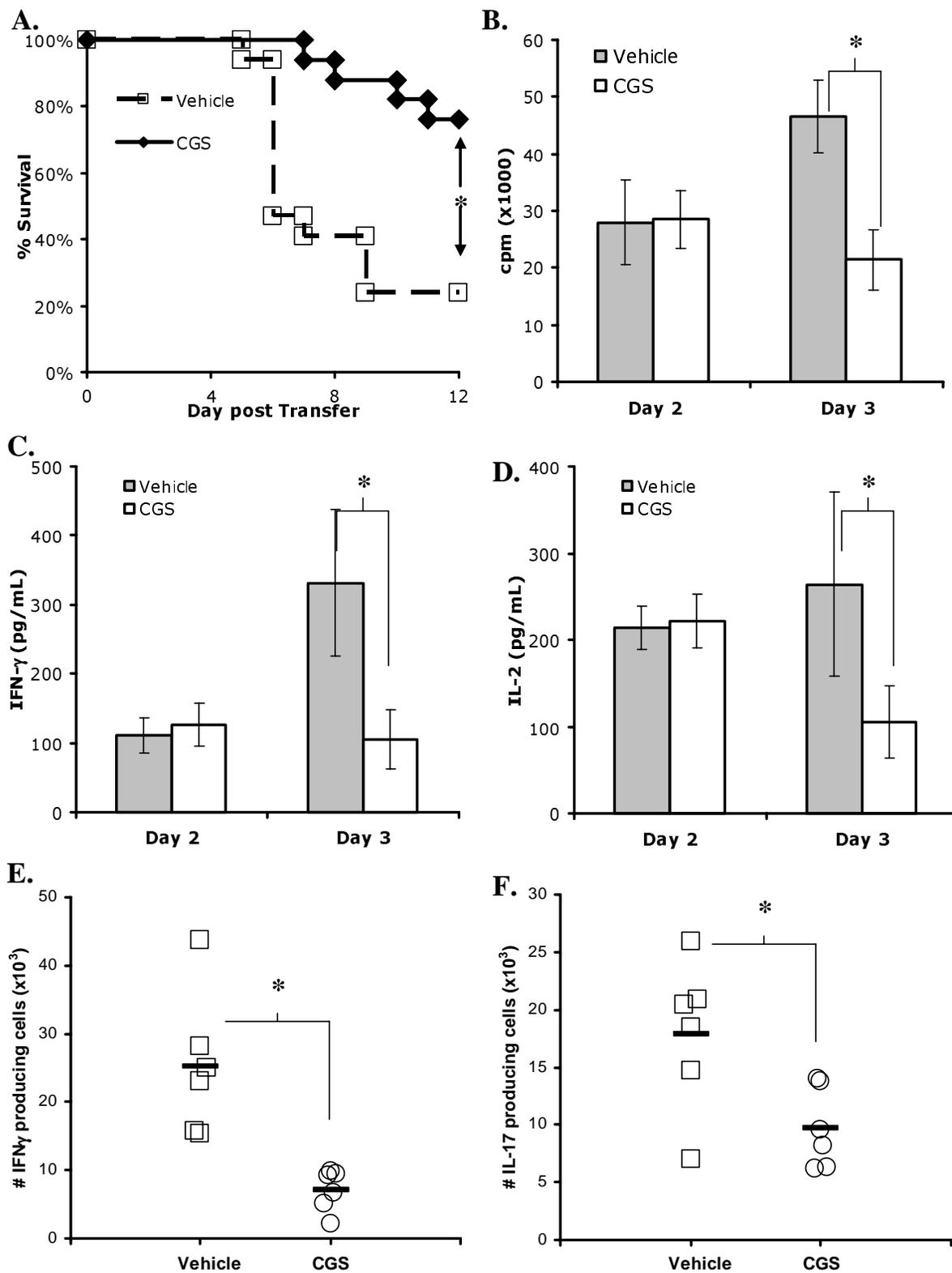


Figure 6:

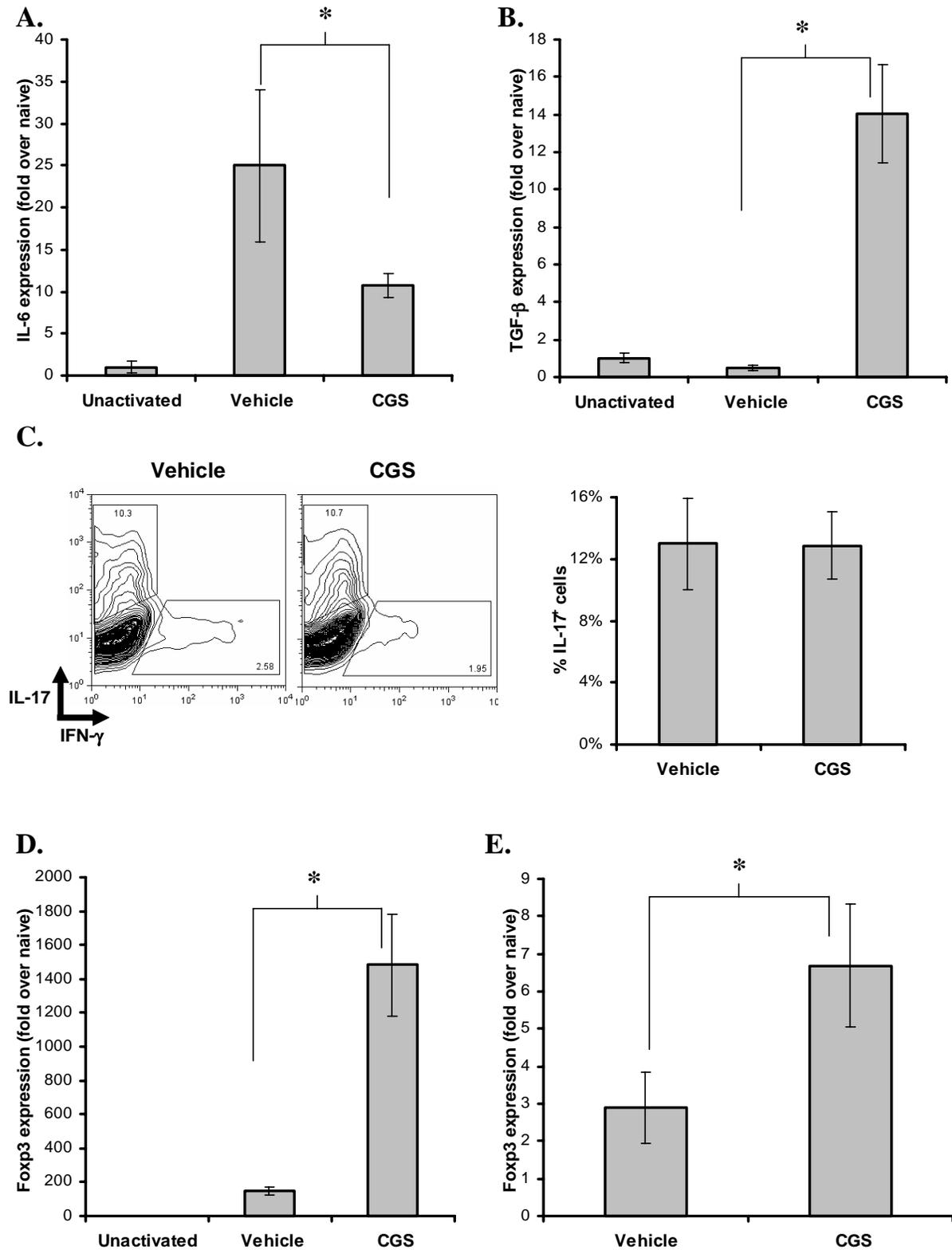
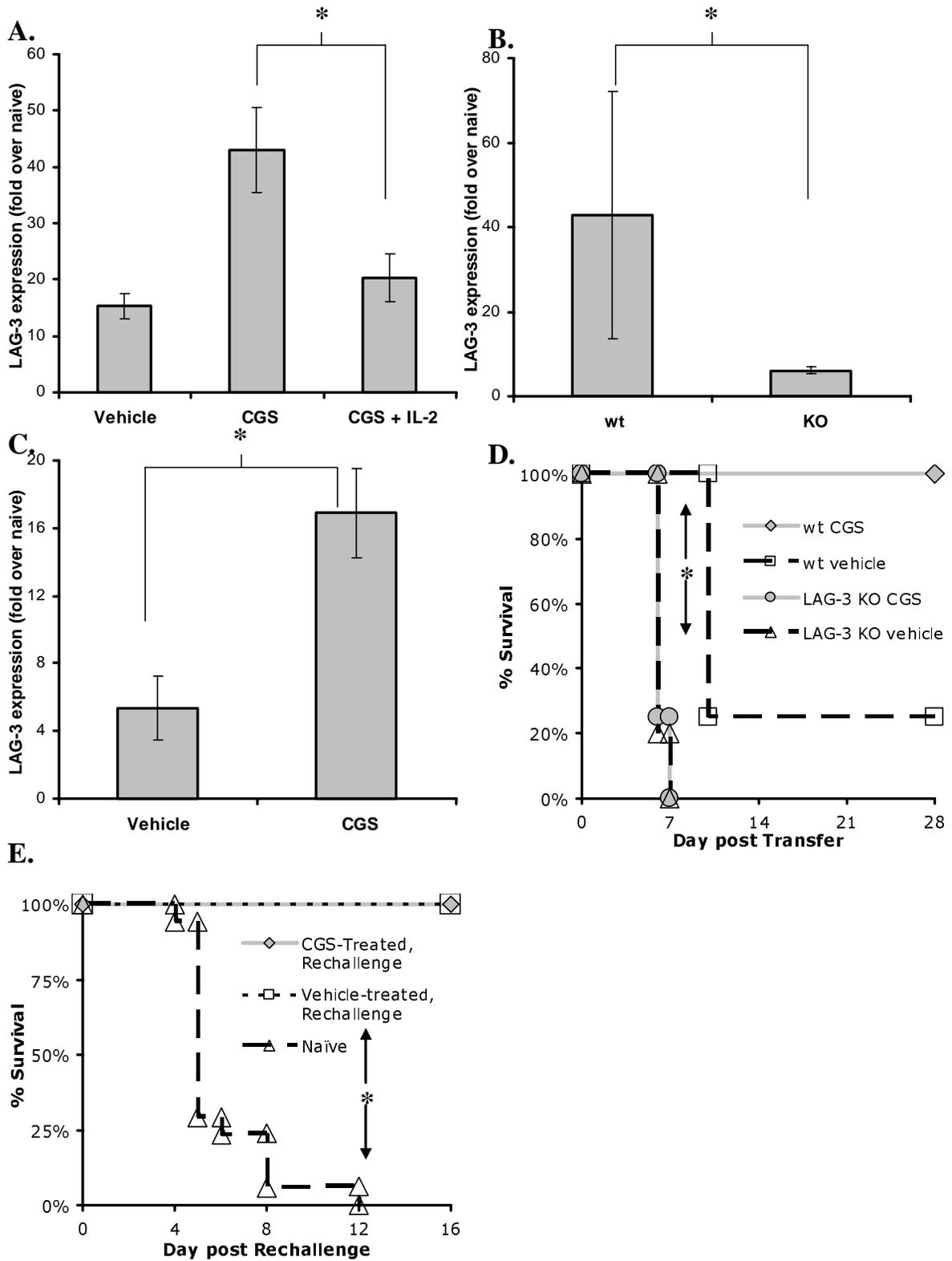


Figure 7:





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A_{2A} receptor signaling promotes peripheral tolerance by inducing T cell anergy and the generation of adaptive regulatory T cells

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