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Cutting Edge

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Cutting Edge: Adenosine A2a Receptor Signals Inhibit Germinal Center T Follicular Helper Cell Differentiation during the Primary Response to Vaccination

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Adenosine A2a receptor (A2aR) signaling acts as a barrier to autoimmunity by promoting anergy, inducing regulatory T cells, and inhibiting effector T cells. However, in vivo effects of A2aR signaling on polyclonal CD4 T cells during a primary response to foreign Ag has yet to be determined. To address this problem, we immunized mice with peptide Ag 2W1S coupled to PE in CFA and treated with the selective A2aR agonist CGS-21680 (CGS). 2W1S:I-A^{b}-specific tetramer-binding CD4 T cells did not become anergic or differentiate into Foxp3^{+} regulatory T cells. Additionally, CGS treatment did not inhibit Th1 or Th17 differentiation. However, CGS did abrogate germinal center T follicular helper cells, and blunted PE-specific germinal center B cell responses. The use of A2aR-deficient CD4 T cells established that this CGS effect was T cell intrinsic. Therefore, this study has identified a unique role for A2aRs in regulating CD4 T cell differentiation during vaccination. ***The Journal of Immunology*****, 2017, 198: 623–628.**

Adenosine is a purine nucleoside that regulates a broad spectrum of physiological responses via four different G protein–coupled adenosine receptors (A1, A2a, A2b, and A3). Adenosine A2a receptors (A2aRs) are expressed primarily on cells of hematopoietic origin, particularly on activated cytotoxic CD8 and helper CD4 T cells (1–3). Studies using mouse models of T cell–mediated autoimmune disorders (4, 5) as well as graft-versus-host disease (6) have shown that A2aR signaling can restore immune homeostasis by promoting the induction of T cell anergy and regulatory T cell (Treg) differentiation. Loss of A2aR signaling in *Adora2a*-deficient mice also leads to enhanced control of tumor growth by CD8 T cells (7). In vitro polarizing assays suggest that A2aR signaling alters CD4 T cell differentiation by inhibiting the induction of Th1 (Tbet^{+}), Th2 (GATA3^{+}), and Th17 (RORγt^{+}) effector T cell phenotypes (8, 9). In vivo models of asthma (10), autoimmunity (4, 5), and infection (11) also suggest that A2aR signaling can reduce Th1 and Th17 responses. However, CD4 T cell differentiation during a primary response to foreign Ag in the context of vaccination has not been assessed.

Most investigations of A2aRs to date have correlated changes in disease outcome in vivo with the in vitro modulation of signaling in cloned T cells, TCR-transgenic T cells, or bulk primary T cells. Nonetheless, Zarek et al. (4) took advantage of hemagglutinin and pigeon cytochrome c peptide–specific TCR-transgenic CD4 T cells made deficient for A2aRs (*Adora2a*$^{-/-}$) to show in vivo that both endogenous adenosine as well as an A2aR agonist can act to inhibit dangerous effector responses to an experimental self-antigen and promote the development of anergy and Tregs. More recently, Shehade and colleagues (11) found that activation of A2aRs during acute infection with *Toxoplasma gondii* reduced the number of endogenous bulk IFN-γ–producing CD4 T cells from secondary lymphoid organs. In cancer, an investigation of tumor Ag–specific CD8 polyclonal T cells in *Adora2a*-deficient mice has also indicated that endogenous adenosine limits their clonal expansion in response to tumor Ags (7). However, A2aR-mediated regulation of endogenous polyclonal Ag-specific CD4 T cells remains unknown.

To investigate the in vivo effects of A2aR signaling on naive polyclonal CD4 T cells during a primary response to foreign Ag we used a vaccination approach that allowed us to track Ag-specific CD4 T cells that differentiate into various T cell lineages such as Th1, Th17, Tregs, and T follicular helper (Tfh) cells. No reports to date have indicated a role for A2aR signaling in Tfh differentiation despite the role of other purinergic receptors such as P2X7 in regulating Tfh homeostasis (12). It is possible that A2aR-mediated effects on Tfh cells may have been overlooked due to a requirement of Ag specificity between T cells and B cells during Tfh differentiation (13). To address this we used a vaccine that consists of 2W1S peptide covalently coupled to PE (14). This allowed us to look at the interplay between endogenous

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Abbreviations used in this article: A2aR, adenosine A2a receptor; B6, C57BL/6; CGS, CGS-21680; FR4, folate receptor 4; GC, germinal center; KO, knockout; LN, lymph node; Tfh, T follicular helper; Treg, regulatory T cell; WT, wild-type.

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Ag-specific 2W1S:I-A^b-specific T cells and PE-specific B cells (14). We initially sought to characterize polyclonal Ag-specific CD4 T cell anergy induction and Treg generation during A2aR signaling by tracking of 2W1S:I-A^b tetramer-binding T cells in mice treated with the selective A2aR agonist CGS-21680 (CGS). We discovered that CGS has no impact on the Ag-induced clonal expansion of polyclonal 2W1S-specific CD4 T cells, nor does it promote the induction of anergy or Treg differentiation in our vaccination system. CGS did not appear to reduce Th1 or Th17 differentiation; instead, A2aR signaling directly inhibited 2W1S:I-A^b-driven CD4 T cell differentiation toward the germinal center (GC)-Tfh fate, and reduced cognate GC B cell responses. Therefore, this work identifies a novel A2aR-mediated control mechanism during vaccination that regulates CD4 T cell differentiation and function.

Materials and Methods

Mice

C57BL/6 (B6) wild-type (WT) mice were purchased from Charles River Breeding Laboratories under a contract from the National Cancer Institute (Frederick, MD). *Adora2a*$^{f/f}$ mice containing *loxP* sites on either side of exon 2 of the *Adora2a* gene (a gift from J. Linden, La Jolla Institute for Allergy and Immunology, La Jolla, CA) (3) were crossed with CD4-Cre mice (a gift from M. Farrar, University of Minnesota, Minneapolis, MN) to generate conditional A2aR T cell knockout (KO) mice. Non-Cre littermates were used as WT controls. Mice were bred and housed in specific pathogen-free conditions in animal facilities at the University of Minnesota, Twin Cities. All experimental protocols were performed in accordance with guidelines of the University of Minnesota Institutional Animal Care and Use Committee and the National Institutes of Health.

Immunization and selective A2aR agonist treatment

Mice were given an i.p. vaccine containing 200 μl of 0.6 μg of 2W1S peptide conjugated to 25 μg of PE emulsified in CFA (Sigma-Aldrich) as previously described (14). Mice were then given a 7 d course of twice daily i.p. injection with the selective A2aR agonist, CGS-21680 (CGS; Tocris) 2.5 mg/kg or with vehicle alone (PBS) as previously described (4).

Cell enrichment and flow cytometry

Lymph nodes (LNs) and spleens were collected and divided for separate enrichments of 2W1S:I-A^b tetramer-specific CD4 T cells and PE-specific B cells. 2W1S:I-A^b APC-labeled tetramers were used to stain and enrich 2W1S-specific CD4 T cells (14). PE B cell enrichment was performed by mixing cell suspensions with 1 μg of PE (ProZyme) (14). Isolation of PE-specific B cells and 2W1S:I-A^b-specific CD4 T cells was done using magnetic beads (Stemcell Technologies) (14). Enriched 2W1S T cells were first surface stained with CXCR5 (2G8), PD-1 (J43), CD4 (RM4-5), and CD44 (IM7), as well as with the irrelevant cell exclusion Abs CD11c (N418), B220 (RA3-6B2), CD8 (53-6.7), and F4/80(BM8), and then they were fixed/permeabilized using a fixation/permeabilization kit (eBioscience) followed by intracellular staining with Foxp3 (FJK-16s), Tbet (4B10), Bcl6 (K112-91), RORγt (Q31-378), and Ki67 (SoA15). Enriched PE-specific B cells were surface stained with B220 (RA3-6B2), GL7 (GL-7), CD38 (90), IgM (RMM-1), and IgD (11-26c.2a), as well as with the irrelevant cell exclusion Abs CD11c (N418), CD4 (GK1.5), CD8 (53-6.7), and F4/80(BM8), and then they were fixed/permeabilized using a fixation/permeabilization kit (eBioscience) and intracellular stained with goat anti-mouse Ig (H+L) (A11068). Anergy in 2W1S:I-A^b-specific CD4 T cells was assessed by staining with CD73 (TY11.8) and folate receptor 4 (FR4, 12A5) as previously outlined (15, 16). Cells were analyzed on a Fortessa (Becton Dickinson) flow cytometer and analyzed using FlowJo (TreeStar).

Statistical analysis

Statistical tests were performed using Prism (GraphPad) software, and *p* values were obtained using an unpaired one-tailed Student *t* test with a 95% confidence interval.

Results

Activation of A2aRs during the primary response to vaccination fails to induce anergy or promote the differentiation of Foxp3$^+$ Tregs

The activation of A2aRs in many biological systems is associated with the production of intracellular cAMP, a second messenger known for its antiproliferative function (17). To investigate the effects of A2aR signaling during the primary CD4 T cell response to Ag, we used a tetramer of the MHC II I-A^b molecule containing the 2W1S peptide to study the in vivo proliferation and differentiation of polyclonal 2W1S:I-A^b-specific CD4 T cells following immunization with a 2W1S peptide coupled to PE in CFA. This vaccination approach induces 2W1S:I-A^b-specific CD4 T cells to undergo clonal expansion and differentiation to Th1, Th17, Tregs, and Tfh lineages (14). Coupling 2W1S and PE together also allows for the interplay between 2W1S-specific GC-Tfh cells and PE-reactive GC B cells. This occurs when PE-specific B cells internalize 2W1S-PE through BCR recognition of PE resulting in the display of 2W1S:I-A^b complexes, and allows them to exchange helper signals with 2W1S:I-A^b-specific CD4 T cells (14). The effects of A2aR activation were tested by treating immunized mice twice daily with the selective A2aR agonist CGS (2.5 mg/kg i.p.) or vehicle alone as a control (PBS) (4). As shown in Fig. 1A, the clonal expansion of the 2W1S:I-A^b-specific polyclonal CD4 T cell population as a whole was no different in CGS- or PBS-treated mice. Consistent with preserved clonal expansion, we also observed no increase in the number of 2W1S:I-A^b-specific Foxp3$^+$ Tregs during this immunization in the presence of CGS (Fig. 1B). Therefore, A2aR signaling did not inhibit clonal expansion nor did it enhance Treg cell differentiation after vaccination with Ag in CFA.

We next examined the capacity of A2aR signaling to promote anergy in 2W1S:I-A^b-specific polyclonal CD4 T cells during vaccination. To test the effects of A2aR signaling on the development of anergy, we examined two surface molecules whose high gene expression marks functionally unresponsive anergic T cells: CD73 (*Nt5e*) and FR4 (*Izumo1r*) (15, 16). Remarkably, CGS treatment led to a reduction rather than rise in the number of 2W1S-specific CD4 T cells that expressed high levels of these two anergy markers (Fig. 1C). Also consistent with an inability to promote tolerance in this system, CGS treatment led to an increase in the fraction of 2W1S:I-A^b-specific CD4 T cells that continued to express high levels of the proliferative marker Ki67 at day 7 (Fig. 1D). Taken together, these data indicate that A2aR signaling cannot inhibit the proliferation of polyclonal Ag-specific CD4 T cells during Ag priming in the presence of a strong adjuvant, and does not promote Treg generation or anergy induction.

Activation of A2aRs interferes with the differentiation of Tfh and GC-Tfh cells during Ag priming

Paradoxically, primed CD4 T cells in CGS-treated mice expressed even lower levels of the anergy markers CD73 and FR4 than in mice exposed to vehicle alone (Fig. 1C). Previous studies have demonstrated moderate levels of FR4 and CD73 expressed on Tfh cells (18). Although the role of these two molecules in Tfh generation and function remains unknown, this observation led us to the hypothesis that A2aR signaling interferes with Tfh differentiation. Mice primed with 2W1S

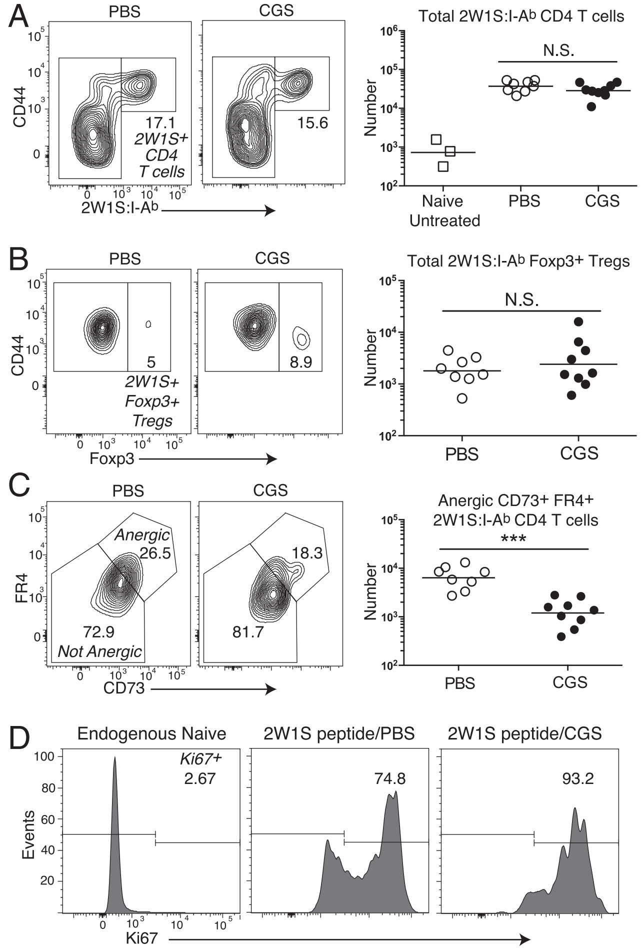

FIGURE 1. A2aR signaling using the selective agonist CGS does not promote anergy or Treg induction during primary immunization. B6 mice were immunized with 2W1S-PE and subsequently given a 7 d treatment course of the selective A2aR agonist CGS 2.5 mg/kg or vehicle alone (PBS). (**A**) Frequency and number of spleen and LN 2W1S:I-A^b-specific CD4 T cells (with naive untreated mice shown for reference). (**B**) $Foxp3^+$ Tregs within the 2W1S:I-A^b-specific CD4 T cell compartment. (**C**) $CD73^+$ $FR4^+$ anergic phenotype cells within the conventional $Foxp3^-$ 2W1S:I-A^b-specific CD4 T cell population. (**D**) Ki67 expression in conventional $Foxp3^-$ 2W1S:I-A^b-specific CD4 T cells 7 d after 2W1S-PE immunization in the presence of CGS (2W1S peptide/CGS) or vehicle alone (2W1S peptide/PBS) (with endogenous naive polyclonal $CD44^{lo}$ CD4 T cells shown as a control). Data are representative from three independent experiments (n = 8–9 mice per group). ***$p < 0.001$.

in CFA did develop a large population of $CXCR5^+$ 2W1S:I-A^b-specific CD4 T cells that expressed moderate levels of FR4 and CD73 (Fig. 2A, 2B). In contrast, treatment of primed mice with CGS led to a loss in the generation of this $FR4^{int}$ $CD73^{int}$ $CXCR5^+$ 2W1S:I-A^b-specific CD4 T cell subset.

To more formally assess A2aR-regulated Tfh differentiation, we investigated the expression of the cell surface marker PD-1 and the transcription factor Bcl6 in primed 2W1S:I-A^b-specific CD4 T cells (19, 20). CD4 T cells that express the highest levels of CXCR5, Bcl6, and PD-1 have been characterized as GC-Tfh cells ($Bcl6^{hi}$ $CXCR5^{hi}$) and are known to provide cognate help to Ag-specific B cells within GCs, whereas CD4 T cells expressing lower levels are characterized as Tfh cells ($Bcl6^{lo}$ $CXCR5^{lo}$) and reside at the T cell/B cell border (21). During primary immunization, CGS treatment reduced both the frequency and number of 2W1S:I-A^b-specific Tfh and GC-Tfh cells (Fig. 2C–E). Consistent with a shift toward alternate differentiation fates, CGS treatment also elicited a small but significant increase in the number of 2W1S:I-A^b-specific non-Tfh cells. Therefore, our experiments revealed a novel role for A2aR pathway activation in the inhibition of Tfh and GC-Tfh cell differentiation.

A2aR inhibition of Tfh differentiation is T cell intrinsic

Although A2aR expression is known to be highly induced on CD4 T cells following TCR ligation (1–3), the expression of this adenosine receptor might also be expected on other cells of hematopoietic origin. Additionally, Tfh cell differentiation is a multifactorial process whose regulation likely involves multiple additional cell types, particularly dendritic cells and B cells (19–21). Therefore, it was important to determine whether the effects of CGS were the result of direct A2aR engagement on the 2W1S:I-A^b-specific CD4 T cells. To address this question, CD4-Cre *Adora2a*$^{f/f}$ conditional KO mice lacking A2aRs only on their T cells were immunized with 2W1S-PE and compared with WT A2aR-expressing littermates following a 7 d course of CGS treatment. In the absence of T cell–expressed A2aRs, CGS treatment lost its capacity to reduce Bcl6 expression and block 2W1S:I-A^b -specific GC-Tfh differentiation, and its inhibitory effects on Tfh cells appeared greatly blunted (Fig. 3A, 3B). Likewise, treatment of CD4-Cre *Adora2a*$^{f/f}$ mice with CGS failed to induce an increase in non-Tfh cells during 2W1S Ag priming. Given that Bcl6 promotes differentiation to the Tfh and GC-Tfh fates in part by repressing other lineage-specific transcription factors such as Tbet and RORγt (19, 20), we further assessed these non-Tfh cells. WT mice significantly increased

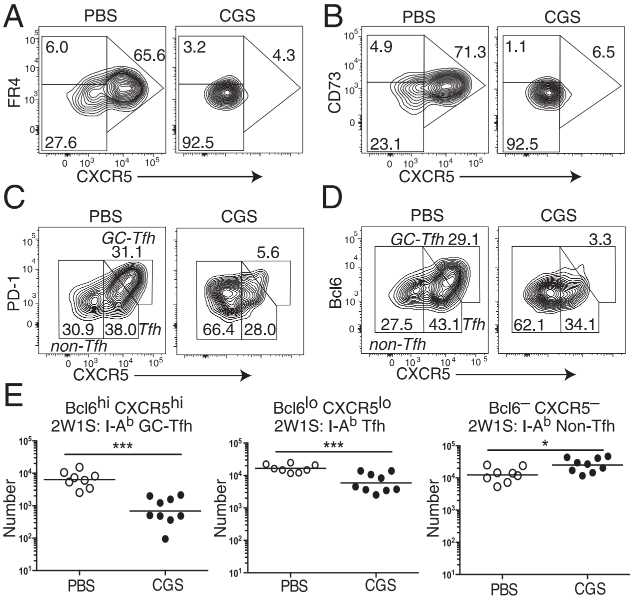

FIGURE 2. A2aR activation reduces Tfh and GC-Tfh cell differentiation. 2W1S:I-A^b tetramer-binding T cells were recovered from the spleen and LNs of 2W1S-PE immunized WT B6 mice after 7 d of treatment with either CGS or vehicle alone (PBS). (**A**) FR4 and CXCR5, (**B**) CD73 and CXCR5, (**C**) PD-1 and CXCR5, and (**D**) Bcl6 and CXCR5 staining in 2W1S:I-A^b-specific CD4 T cells from CGS- or PBS-treated immunized mice. (**E**) Aggregate numbers of $Bcl6^{hi}$ $CXCR5^{hi}$ GC-Tfh, $Bcl6^{lo}$ $CXCR5^{lo}$ Tfh, and $Bcl6^-$ $CXCR5^-$ non-Tfh cells that bind the 2W1S:I-A^b tetramer. Data are representative of three independent experiments (n = 8–9 mice). *$p < 0.05$, ***$p < 0.001$.

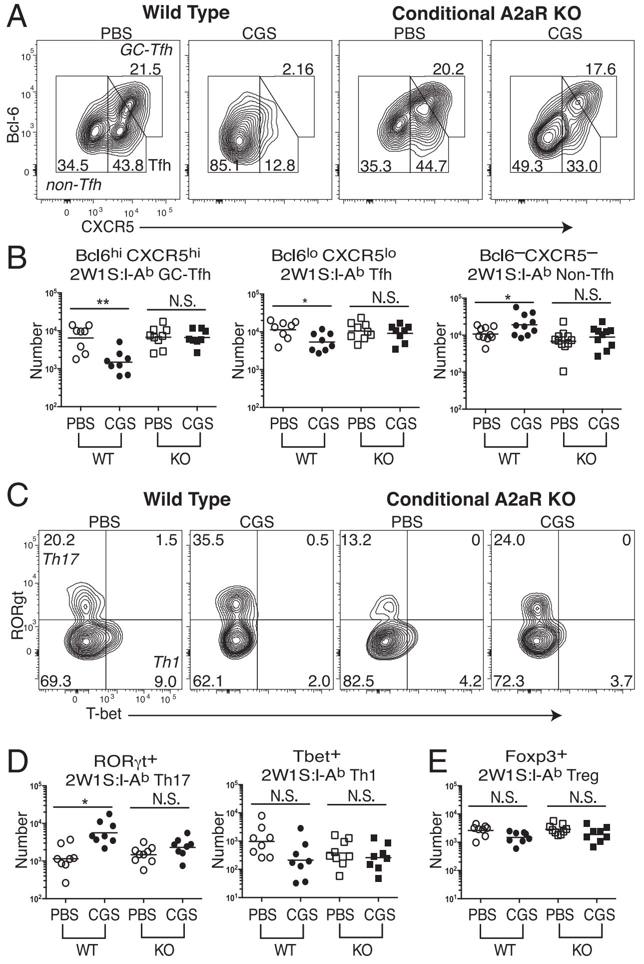

FIGURE 3. A2aR inhibition of GC-Tfh cell differentiation is T cell intrinsic. 2W1S:I-A^b tetramer-bound CD4 T cells were enriched from spleen and LNs of CD4-Cre *Adora2a*$^{f/f}$ conditional KO mice as well as non-Cre expressing WT littermates after 2W1S-PE immunization and a 7 d course of either CGS or PBS treatment. (**A** and **B**) Frequency (A) and number (B) of 2W1S-specific CD44^{hi} Foxp3$^-$ CD4 T cell subsets: Bcl6^{hi} CXCR5^{hi} GC-Tfh, Bcl6^{lo} CXCR5^{lo} Tfh, and Bcl6$^-$ CXCR5$^-$ non-Tfh cells. (**C** and **D**) Frequency (C) and number (D) of Th17 (RORγt$^+$ Tbet$^-$) and Th1 (RORγt$^-$ Tbet$^+$) lineage cells within the non-Tfh fraction of 2W1S:I-A^b tetramer-binding CD4 T cells. (**E**) 2W1S-specific Foxp3$^+$ Treg numbers. Data are representative of three independent experiments (n = 8–9). *$p < 0.05$, **$p < 0.01$.

the frequency and number of 2W1S:I-A^b-specific RORγt$^+$ Th17 cells when their A2aRs were directly bound by the adenosine agonist, whereas Tbet$^+$ Th1 and Foxp3$^+$ Treg differentiation appeared not to be regulated by A2aR signaling (Fig. 3C–E). A small but statistically insignificant increase in RORγt$^+$ Th17 cells was also observed in KO mice treated with CGS (Fig. 3C–E). Thus, direct CD4 T cell A2aR signaling shifted the balance of differentiation away from the GC-Tfh fate toward Th17 effector cell generation during the primary response to Ag.

T cell–intrinsic A2aR activation reduces T cell–dependent B cell immunity

GC-Tfh cells promote the survival, differentiation, isotype class switch, and affinity maturation of Ag-specific B cells in GCs (21). Therefore, we hypothesized that A2aR signaling in T cells during primary immunization would interfere with the T cell–dependent B cell response to vaccination. To test this, WT and CD4-Cre *Adora2a*$^{f/f}$ KO mice were immunized with a protein complex containing 2W1S coupled to PE (2W1S-PE) either with or without CGS treatment, and then PE-specific B cells were enriched using magnetic beads and characterized by flow cytometry. An ∼50-fold expansion of PE-specific B cells was seen in WT and KO PBS-treated mice following immunization, as compared with naive mice (Fig. 4A–C). Consistent with the hypothesis, the number of PE-specific B cells found after vaccination was significantly reduced in CGS-treated WT hosts, but not in mice whose CD4 T cells lacked A2aRs. CGS treatment appeared to have its greatest inhibitory effect on the frequency and number of GC phenotype CD38$^-$ GL7$^+$ PE-binding B cells in the WT mice, although all B cell subpopulations were affected (Fig. 4). Importantly, these inhibitory effects of CGS on PE-specific B cells during immunization were blunted in mice that lacked *Adora2a* gene expression specifically within the T cell compartment. Therefore, these data suggest that the loss of Ag-specific GC-Tfh differentiation that occurs during strong A2aR signaling on CD4 T cells is sufficient to abrogate the provision of cognate T cell help to GC B cells during their primary response to Ag.

Discussion

The identification of novel signaling elements that fine-tune CD4 T cell lineage differentiation during primary immunization offers new opportunities to improve the efficacy of vaccines and targeted immunotherapies (22–24). Our data

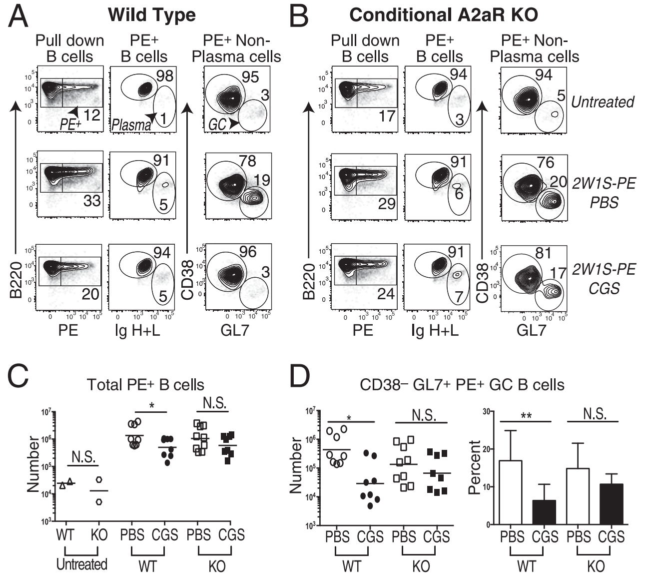

FIGURE 4. T cell A2aR activation reduces GC B cell immunity. PE-specific B cells were enriched from the spleen and LNs of 2W1S-PE primed WT or CD4-Cre *Adora2a*$^{f/f}$ conditional KO mice given a 7 d course of CGS or the PBS vehicle alone. (**A**) Gating strategy to identify PE-specific B220$^+$ total B cells (left column), B220^{intermediate} intracellular Ig (H+L)^{hi} plasma cells (middle column), as well as intracellular Ig (H+L)^{intermediate} CD38$^-$ GL7$^+$ GC B cells (right column) in control untreated (upper row), 2W1S-PE immunized and PBS-treated (middle row), and 2W1S-PE immunized and CGS-treated (lower row) WT mice. (**B**) Representative KO mice treated as in (A). (**C**) Absolute numbers of total PE-specific B cells in WT and KO mice treated as in (A) and (B), with untreated mice shown as a control. (**D**) Absolute numbers and frequency of PE-specific polyclonal CD38$^-$ GL7$^+$ GC B cells in immunized WT and KO mice treated as in (A) and (B). Data are representative of three independent experiments (n = 8–9 mice). *$p < 0.05$, **$p < 0.01$.

suggest that in vivo A2aR signaling during the primary response to Ag plus a strong adjuvant diverts CD4 T helper cells away from the Tfh and GC-Tfh lineages. Given the key role of alternative T cell differentiation fates such as Th17 in protection against mucosal barrier infections (22), it is conceivable that selective A2aR agonists could be useful during vaccination to shape an optimal T cell differentiation response against pathogens.

A2aRs also appear to be a particularly attractive therapeutic target for the treatment of B cell–dependent autoimmune disorders such as systemic lupus erythematosus (25) and rheumatoid arthritis (26). Indeed, previous studies have shown a positive correlation between the number of Tfh cells and disease burden in patients with rheumatoid arthritis (26). Perhaps consistent with this, A2aR agonists effectively suppress animal models of inflammatory arthritis (27). The fact that the first-line antirheumatic drugs methotrexate and sulfasalazine act, in part, through the generation of extracellular adenosine and A2aR signaling (28, 29) lends further support to the notion that A2aR signaling can ameliorate T cell–dependent B cell autoreactivity.

It should be noted that the ablation of A2aRs in these vaccination experiments using conditional KO mice failed to significantly enhance Ag-specific Tfh or GC-Tfh differentiation. Similarly, use of a selective antagonist of A2aRs during immunization did not reliably alter CD4 T cell differentiation (data not shown). Although strong A2aR signaling with an agonist can be inhibitory for Tfh and GC-Tfh differentiation in normal CD4 T cells, under normal circumstances endogenous extracellular adenosine may play no role in CD4 T cell fate selection within secondary lymphoid organs following i.p. immunization in adjuvant. Alternatively, unspecified factors (e.g., increased A2b receptors, decreased adenosine kinase) may compensate for the loss of A2aRs in KO mice. Activated T cells are known to upregulate adenosine deaminase, an enzyme capable of metabolizing adenosine to inosine (30). Therefore, strong continuous A2aR signaling from endogenous adenosine sources may only occur under special circumstances such as hypoxia where CD73 is upregulated to facilitate increased extracellular adenosine production in close proximity to A2aRs (31, 32). Going forward, it will be important to identify the immunological context (spatial and temporal) whereby extracellular adenosine counter-regulates Tfh and GC-Tfh cell differentiation.

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Disclosures

The authors have no financial conflicts of interest.

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