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Adenosine A2A Receptor Agonist–Mediated Increase in Donor-Derived Regulatory T Cells Suppresses Development of Graft-versus-Host Disease

Kyu Lee Han,* Stephenie V. M. Thomas,* Sherry M. Koontz,* Cattlena M. Changpriroa,* Seung-Kwon Ha,† Harry L. Malech,* and Elizabeth M. Kang*

Graft-versus-host disease (GVHD) remains a significant complication of allogeneic transplantation. We previously reported that the adenosine A2A receptor (A2AR) specific agonist, ATL146e, decreases the incidence and severity of GVHD in a mouse transplant model. There is increasing interest in treatments that increase CD4+CD25highFoxp3+ regulatory T cells (Tregs) to suppress GVHD. Our current study found in vitro that A2AR selective agonists enhanced TGF-β–induced generation of mouse Tregs 2.3- to 3-fold. We demonstrated in vivo suppression of GVHD with specific A2AR agonists in two different murine GVHD transplant models associated with profound increases in both circulating and target tissue Tregs of donor origin. Three different A2AR agonists of differing potency, ATL146e, ATL370, and ATL1223, all significantly inhibited GVHD-associated weight loss and mortality. At the same time, Tregs shown to be of donor origin increased 5.1- to 7.4-fold in spleen, 2.7- to 4.6-fold in peripheral blood, 2.3- to 4.7-fold in colon, and 3.8- to 4.6-fold in skin. We conclude that specific activation of A2AR inhibits acute GVHD through an increase of donor-derived Tregs. Furthermore, the increased presence of Tregs in target tissues (colon and skin) of A2AR-specific agonist-treated mice is likely the mechanistic basis for the anti-inflammatory effect preventing acute GVHD. The Journal of Immunology, 2013, 190: 458–468.

Graft-versus-host disease (GVHD) continues to be a significant cause of morbidity and mortality after allogeneic hematopoietic stem cell transplantation (1). Targeted methods to prevent or treat GVHD are in high demand. Regulatory T cells (Tregs) are a subset of CD4+CD25high T cells that express the FOXP3 and have been shown to suppress the proliferation of T conventional cells and help promote tolerance (2, 3). There is increasing recognition that Tregs are important in preventing the development, reducing the severity, and/or mediating the resolution of GVHD (4, 5). It has been reported that donor Treg infusions will suppress the development of GVHD in a mouse model (6–8) and that the number of Tregs in the peripheral blood and affected tissues is decreased during the development of acute GVHD in humans (9). It is likely that Tregs act to modulate immune responses at anatomic sites of GVHD inflammation but also may act to modulate immunity in central and peripheral lymphoid organs as well as in peripheral blood.

cAMP-elevating agents are known to induce alloreactive T cell tolerance and prevent GVHD lethality in murine models (10). Because the activation of the Gs-coupled adenosine A2AR receptor (A2AR) appears to terminate inflammation through the regulation of cells that mediate both innate and adaptive immunity, it is a promising pharmacological target for the treatment of GVHD (11). The selective activation of the A2AR has been shown to potentiate limited inflammation and injury in various inflammatory disease models, and the data suggest a central role for this receptor involves a feedback mechanism that inhibits the inflammation associated with activation of either innate or acquired immunity. A2AR agonists have significant protective effects in multiple models of ischemia–reperfusion injury and inhibit the progression of inflammatory bowel disease and reduce joint destruction because of septic arthrosis (12–16). Furthermore, it has been shown that the anti-inflammatory effects of methotrexate are mediated in part via the induction of adenine nucleotide release from injured tissue and the subsequent activation of A2ARs on local immune cells (17, 18). Notably, T cell tolerance by T cell anergy can also be induced by selective A2AR agonist treatment (19).

We have previously shown that treatment with the specific A2AR agonist, ATL146e, decreases the incidence and severity of GVHD as well as improves survival of mice in a GVHD transplant model (20). However, the mechanism of action of ATL146e mediating this reduction of GVHD mortality was not clearly determined. There had been few reports exploring the relationship between Tregs and A2AR (15, 21–23), but there was no prior evidence to show that activation of A2AR could actually induce immunosuppressive Tregs in the setting of GVHD. In our current study, we found that selective A2AR agonists potently enhanced the TGF-β–induced generation of mouse Tregs in vitro. In vivo in two GVHD mouse transplant models, treatment with selective A2AR agonists greatly increased the number of CD4+CD25highFoxp3+ Tregs in peripheral blood and lymphatic tissue such as the spleen, as well as locally in tissues (skin and colon) that are the target of GVHD.
in these models. Our findings in vitro and in vivo strongly link the action of selective A$_2$R agonists to the induction of Tregs that act to reduce the development of GVHD in target tissues.

Materials and Methods

Mice

For these studies, five mouse strains (with relevant H2 major histocompatibility type indicated) were purchased from The Jackson Laboratory (Bar Harbor, ME): C57BL/6j (H2-Kb); B6.PL-Thy1.1C57J (congenic to C57BL/ 6j carrying the Thy 1.1 allele [H2-Kb]); BALB/c (H2-Kd); B6D2F1/J (F, hybrid cross between C57BL/6J female × DBA/2J male [H2-Kb]); and B6, Cg-Foxp3$^{+/+}$ (Foxp3 deficient scurfy on C57BL/6J background [H2-Kb]). The National Institute of Allergy and Infectious Disease Animal Care and Use Committee (Bethesda, MD) approved all animal studies (Institutional Animal Care and Use Committee–approved protocol LHD-3E).

Specific A$_2$R agonists

The highly specific A$_2$R agonists ATL146e, ATL370, and ATL1223 were gifts from Forest Laboratories (New York, NY) (or their fully owned subsidiaries) under material transfer agreements. ATL146e (apadenson; Stedivaza), the most potent of the three agonists used in our murine studies, is in clinical development by Forest Laboratories as a coronary vasodilator for use in nuclear stress single-photon emission computed tomography myocardial perfusion imaging.

Murine transplant models used for the study of GVHD induction

Exclusively female mice, 8–12 wk old, were used both as transplant recipients and as sources of donor hematopoietic stem cells in the form of CD3$^+$ T cell–depleted bone marrow (TCD-BM) and donor spleen-derived purified CD3$^+$ T lymphocytes. Bone marrow cells were collected from the femurs and tibia of donors and depleted of T cells as previous described (24) to yield TCD-BM. Purified T cells were obtained from the spleens of donors using mouse CD3$^+$ T cell enrichment columns (R&D Systems, Minneapolis, MN), resulting in a T cell product of 93–97% purity.

Two transplant recipient models of murine GVHD were studied: C57BL/6J (B6) or B6.PL-Thy1.1C57J (B6-Thy1.1) donor cells transplanted into B6D2F1/J (B6-D2) recipients (H2-K$^+$ into H2-K$^-$ model); or B6 donor cells transplanted into BALB/C recipients (H2-K$^+$ into H2-K$^-$ model). For some experiments, B6.Cg-Foxp3$^{+/+}$ (Foxp3 deficient scurfy on C57BL/6J background [H2-Kb]), the most potent of the three agonists used in our murine studies, is in clinical development by Forest Laboratories as a coronary vasodilator for use in nuclear stress single-photon emission computed tomography myocardial perfusion imaging.

Preparation of lymphocytes from colonic lamina propria or skin

To isolate infiltrating lymphocytes in the skin, 100 mg of skin was pooled, minced, and then digested with 2.5 mg/ml type I collagenase (Sigma-Aldrich) and 0.25 mg/ml hyaluronidase (Sigma-Aldrich, St. Louis, MO) for 2 h at 37˚C (28). Colonic lamina propria lymphocytes were isolated as described previously (29). Briefly, 100 mg of colonic tissue was washed in medium (RPMI 1640 supplemented with penicillin and streptomycin), then minced. The specimen was then digested in RPMI 1640 medium containing 2% PBS and 1 mg/ml type IV collagenase (Sigma-Aldrich, St. Louis, MO) for 2 h at 37˚C with 5% CO$_2$. A single-cell suspension was obtained by passing the digested tissue through a cell strainer. Absolute cell number was determined as described previously (29).

RNA extraction and real-time RT-PCR for Foxp3

Total RNA from colon and skin was isolated using TRIzol (Invitrogen, Grand Island, NY). cDNA was synthesized from 500 ng total RNA using Maxima First Strand cDNA synthesis kit (Fermentas, Glen Burnie, MD) according to the manufacturer’s recommendation. For Foxp3 and GAPDH transcripts, real-time PCR was performed with a 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA) based on specific primers and general fluorescence detection with SYBR Green. The following primer combinations were used: mouse Foxp3, forward 5′-AGGAGCCG-CAAGCAAAAAAGC-3′ and reverse 5′-TGGCTTCTCGGCACCTGT-3′; and mouse GAPDH, forward 5′-CTCATGACACAGTCATGC-3′ and reverse 5′-CACAATGGGGGCGAACAGC-3′. All PCRs were performed using Maxima SYBR Green qPCR Master Mix (Fermentas, Glen Burnie, MD). Relative gene expressions of Foxp3 in skin or colonic tissues were normalized to GAPDH.

Generation of Tregs in vitro by culture of CD4$^+$CD25$^-$ T cells with TGF-β

CD4$^+$CD25$^-$ T cells were isolated from single cell suspensions of spleens from naive B6 mice using components of the Miltenyi CD4$^+$CD25$^-$ Regulatory T Cell Isolation Kit (Miltenyi Biotec, Auburn, CA) to first positively select for CD4$^+$ T cells and then negatively select the purified CD4$^+$ T cells to isolate the CD25 negative subset following manufacturer’s instructions. The purified CD4$^+$CD25$^-$ T cells were cultured in a 24-well plate (3 × 10$^5$ cells/well), stimulated with anti-CD3 and anti-CD28 Ab Dynabeads (Invitrogen, Carlsbad, CA) for 3 d in the presence of TGF-β (2 ng/ml) (PeproTech, Rocky Hill, NJ), without or with the addition of rapamycin (10 nM) (LC Laboratories, Woburn, MA), cyclosporine A (CSA, 10 nM) (LC Laboratories, Woburn, MA), the A$_2$A$_2$R antagonist
CD4+CD25− T cells purified from B6 spleens were stimulated in culture with anti-CD3 and anti-CD28 Ab Dynabeads together with TGF-β, in the absence or presence of the selective A2A R agonists ATL370 or ATL1223 for 3 d. Initially, <0.1% of the CD4+CD25− T cells were Foxp3 positive. Controls substituted rapamycin or CsA for the selective A2A R agonists. Other controls omitted TGF-β from the culture. At 3 d with TGF-β alone, the proportion of CD4+Foxp3+ T cells increased to 4.9 ± 0.3% compared with 0.47 ± 0.7% for the control cultures without TGF-β (Fig. 1A, upper group [representative experiment], and Fig. 1C [average of three independent experiments]). The addition of rapamycin (known to enhance generation of Tregs (30)) enhanced the TGF-β-stimulated generation of CD4+Foxp3+ T cells to 9.7 ± 2.5%. In contrast, addition of CsA (known to interfere with generation of Tregs (30)) inhibited the TGF-β-stimulated generation of CD4+Foxp3+ T cells to 0.3 ± 0.2% (Fig. 1C).

The addition of the selective A2A R agonists ATL370 or ATL1223 enhanced the TGF-β-stimulated generation of CD4+Foxp3+ T cells to 14.5 ± 1.0 or 11.5 ± 1.3%, respectively (Fig. 1A, lower group, and Fig. 1C, block bar). Thus, when used at equimolar concentrations (10 nM), the two selective A2A R agonists, ATL370 or ATL1223, were more effective than rapamycin at enhancing TGF-β-stimulated generation of CD4+Foxp3+ T cells in vitro, increasing generation by 2.3- to 3-fold over TGF-β alone (p = 0.0009 and p = 0.0257 comparing TGF-β alone versus with ATL370 or ATL1223, respectively, over three experiments). This observation in vitro suggested that selective A2A R agonists could act in vivo to directly increase Treg generation as a mechanism to reduce development of GVHD.

In addition, use of the A2A R antagonist (ZM 241385) 24 h before the addition of the selective A2A R agonists was used to further confirm that the increase of the T regulatory population was due to the effect of the A2A R agonist. ZM 241385 treatment combined with the A2A R agonist decreased the generation of CD4+Foxp3+ T cells in vitro by 2.9- to 3.5-fold compared with ATL370 or ATL1223 alone (p = 0.0008 and p = 0.0131, respectively, comparing ATL370 or ATL1223 alone versus with the antagonist over three experiments; Fig. 1B). In fact the number of CD4+Foxp3+ T cells with both ZM 241385 and A2A R agonist treatment (4.1 ± 1.5% with ATL370 or 3.9 ± 1.2% with ATL1223; Fig. 1C) was similar to treatment with TGF-β alone (4.9 ± 1.3%; Fig. 1C). Interestingly, ZM 241385 did not affect CD4+Foxp3+ T cell generation in cells treated with either TGF-β or rapamycin (Fig. 1C).

Selective A2A R agonists ameliorates weight loss and mortality due to acute GVHD

We previously reported that the A2A R specific agonist, ATL146e, can decrease the incidence and severity of GVHD-induced weight loss as well as improve survival of mice in a B6 into B6-D2 model of transplant-related GVHD (20) but did not delineate a mechanism for this effect. In the current study, we also studied the effects of additional A2A R-specific agonists, ATL370 and ATL1223, as well as used a second model with a greater degree of mismatch, that is the B6 into BALB/C model.
In Fig. 2A and 2B, we confirmed our previous observation that continuous administration of ATL146e by s.c. osmotic pump for 14 d, beginning 2 d before transplant of B6 TCD-BM plus T cells into B6-D2 mice reduces both GVHD-associated weight loss and mortality compared with mice treated with vehicle control. In addition, the protective effect of ATL146e administered for 12 d posttransplant is observable up to 40 d posttransplant as shown in this experiment. In Fig. 2C and 2D, both ATL370 and ATL1223 similarly delivered for 14 d by osmotic pump beginning 2 d before transplant of B6 TCD-BM plus T cells into B6-D2 mice reduces both GVHD-associated weight loss and mortality compared with mice treated with vehicle control. Furthermore, the effect is again observable out to 30 d posttransplant as shown in this experiment. Finally, using the more severe GVHD model of B6 into BALB/C, we examined the ability of ATL370 or ATL1223 again delivered for 14 d by osmotic pump beginning 2 d before transplant to reduce the development of severe GVHD-associated weight loss and accelerated mortality compared with mice treated with vehicle control (Fig. 2E, 2F).

Mice in all groups that received donor TCD-BM only (without additional donor T cells) did not develop acute GVHD-associated weight loss, and they did not have significant mortality over the indicated observation periods (data not shown). All of the A2aR agonist treated mice had reduced GVHD-associated weight loss and mortality as compared with vehicle only-treated mice. Furthermore, when using ATL370 or ATL1223 (Fig. 2D, 2F), >50% of the A2aR agonist-treated animals survived beyond 30 d posttransplant as compared with 0% survival of vehicle-treated controls in either transplant model. This effect was dose dependent because osmotic pump administration of ATL370 at 10 ng/kg/min and ATL1223 at 50 ng/kg/min did not at all inhibit GVHD-associated weight loss in either model (data not shown), but the use of 50 ng/kg/min ATL370 and 200 ng/kg/min ATL1223 as shown in Fig. 2C–F significantly reduced weight loss and mortality.

Inhibitory effect of selective A2aR agonists on proinflammatory cytokine and chemokine production in the acute GVHD mouse model

In the setting of GVHD, G-CSF, IL-6, IFN-γ, and TNF-α appear to be proinflammatory cytokines that correlate with disease severity, whereas increased levels of IL-10 and TGF-β may be protective or...
may correlate with control or resolution of GVHD (31–33). Serum levels of G-CSF, IL-6, IFN-γ, TNF-α, IL-10, and TGF-β were thus analyzed as an indicator of inflammatory activity of acute GVHD at 16–20 d in the B6 into B6-D2 model of TCD-BM plus T cell transplant-related GVHD. We examined the effect of vehicle, ATL146e (10 ng/kg/min), ATL370 (50 ng/kg/min), or ATL1223 (200 ng/kg/min) delivered for 14 d by osmotic pump beginning 2 d prior to transplant. For these studies, we used the levels from B6-D2 mice transplanted with cells from B6-D2 donors (BM only) as our baseline control.

The serum levels of TNF-α, IFN-γ, IL-6, and G-CSF were significantly lower in mice treated with the A2AR agonists compared with the vehicle-treated controls (Fig 3A–D). Furthermore, when we measured serum levels of the anti-inflammatory, tolerance-inducing cytokines IL-10 and TGF-β (Fig 3E, 3F), these were significantly increased by 1.5- to 2.5-fold, respectively, when compared with vehicle only. It should be noted that in the BM only control, baseline levels of proinflammatory cytokines were very low, whereas the levels of the anti-inflammatory cytokines were much higher compared with any of the experimental values. Treatment of the experimental mice with A2AR agonists resulted in all cases in reversion of the cytokine levels toward the values seen in the BM only controls. These data indicate that activation of the A2AR can reduce systemic levels of proinflammatory cytokines and increase immunotolerogenic cytokines in the setting of transplant-related GVHD.

Selective A2AR agonist induces CD4+CD25highFoxp3+ Tregs in acute GVHD mouse model

In this study, we showed in vitro that A2AR activation significantly augments TGF-β–induced generation of mouse CD4+Foxp3+ T cells. Furthermore, our in vivo studies in the transplant-related GVHD mouse model demonstrated that the A2AR activation-associated amelioration of GVHD is associated with significant reduction in serum proinflammatory cytokines and significant increase in serum levels of IL-10 and TGF-β, which are known to be factors in the generation of Tregs. In addition, there is further evidence in vivo from a mouse model of autoimmunity that A2AR-induced amelioration of autoimmunity is associated with generation of Tregs (23).

We therefore next sought to determine whether specific A2AR agonist treatment in the mouse transplant-related GVHD models was associated with changes in Tregs in lymphoid organs, peripheral blood, and at anatomic target sites of GVHD. In the analysis shown in Fig. 4 we used our mouse GVHD models (B6 into B6-D2 shown in Fig. 4A–F; B6 into BALB/c shown in Fig. 4G–I).

**FIGURE 3.** GVHD-induced elevation of proinflammatory cytokine and chemokine levels is reduced by A2AR agonists administration in the B6 into B6-D2 model. Blood was collected 16–20 d after transplant, and serum concentrations of G-CSF (A), IL-6 (B), IFN-γ (C), TNF-α (D), IL-10 (E), and TGF-β (F) were measured using multiplex immunoassay kits with the BioPlex system and ELISA. Data shown are from three independent experiments (n = 9); error bars indicate SEM. The p values were calculated comparing agonist to vehicle control by Student t tests.
to examine the absolute number of CD4+CD25\(^{high}\)Foxp3+ Tregs appearing in the spleen and peripheral blood 16–20 d post-transplant. We compared treatment with vehicle versus specific A2AR agonists delivered by route, dose, and time noted in the previous results and as described in Materials and Methods. Again, “BM only” refers to the B6-D2 into B6-D2 control.

FIGURE 4. A2AR agonists increase Tregs in acute GVHD mouse models. The percentages or number of CD4, CD25, and Foxp3-positive cells in various tissues posttransplant are shown. (A) Data from a representative experiment using the B6-D2 into B6 model. (B and C) Cumulative number of CD4, CD25, and Foxp3-positive cells data from three experiments analyzing 100 mg spleen and 100 μl peripheral blood, respectively. (D) Data from a representative experiment using the same model but treated with either ATL370 or ATL1223. (E and F) Cumulative data from three independent experiments for both agonists. Data from the B6 into BALB/C recipients is shown in (G–I), where (G) is the data from one representative experiment, and (H and I) are the cumulative data for spleen and peripheral blood from three separate experiments. For all experiments, \(p\) values were calculated comparing vehicle control to drug using a Student \(t\) test.
transplanted with unmanipulated marrow and no additional T cells.

As expected, splenic CD4^+CD25^{high}Foxp3^+ Tregs were significantly decreased in both of the vehicle-treated acute GVHD mouse models compared with “BM only” controls. Conversely, the number of Tregs in the “BM only” groups was similar to that measured in the spleens of untransplanted/untreated mice (data not shown). Treatment of mice from both GVHD models with any of the three specific A2A-R agonists resulted in a profound increase in CD4^+CD25^{high}Foxp3^+ Tregs in the spleen compared with the vehicle-treated GVHD mice (Fig. 4A, 4D, 4G). With the B6 into B6-D2 model, this increase in number compared with vehicle treatment averaged ~10-fold with ATL146e (p = 0.0093) treatment and 5- to 7-fold with ATL370 (p = 0.0164) or ATL1223 (p = 0.0258) treatment (Fig. 4B, 4E). In the greater mismatched B6 into BALB/C model, the increase in splenic Tregs averaged ~5- to 6-fold with ATL370 (p = 0.0393) or ATL1223 (p = 0.0616) treatment (Fig. 4H).

In addition to the spleen, we also measured the absolute number of CD4^+CD25^{high}Foxp3^+ Tregs in the peripheral blood finding a 5.4-fold increase with ATL146e (p = 0.0262) treatment and 3.6- to 4.6-fold increase with ATL370 (p = 0.0271) or ATL1223 (p = 0.0200) treatment in the B6 into B6-D2 model (Fig. 4C, 4F). In the B6 into BALB/C model, peripheral blood Tregs were increased 8.1- to 9.4-fold with ATL370 (p = 0.0338; Fig. 4I) or ATL1223 (p < 0.0001; Fig. 4I). These results suggest that specific activation of A2A-R inhibits acute GVHD through decreased proinflammatory cytokine production, induction of IL-10 and TGF-β and an increase in CD4^+CD25^{high}Foxp3^+ immunosuppressive Tregs in the spleen and peripheral blood.

Tregs increased by the selective A2A-R agonist in the GVHD mouse model were of donor origin

We determined the origin of A2A-R induced Tregs by substituting donor cells from B6-Thy1.1 instead of B6 for transplant into B6-D2 recipients to allow detection of Thy1.1 on Tregs appearing in the transplanted mice. This B6-Thy1.1 into B6-D2 model also replicated the acute GVHD symptoms similar to the B6 into B6-D2 model, and also responded to treatment with ATL146e, resulting in less weight loss and improved survival (data not shown). As shown in Fig. 5A and 5B, measuring Tregs in the spleen and peripheral blood, we confirmed again the severe depletion of Tregs in the vehicle treated GVHD model (2.91 ± 2.52% of CD4^+ cells in the spleen, 0.88 ± 0.51% of CD4^+ cells in peripheral blood) compared with “BM only” control (12.06 ± 3.43% of CD4^+ cells in the spleen, 15.64 ± 6.55% of CD4^+ cells in peripheral blood), but significant restoration of the level of Tregs almost to the level
of the “BM only” control in the B6-Thy1.1 into B6-D2 GVHD model treated with ATL146e (10.66 ± 5.18% of CD4+ cells in spleen, 12.10 ± 4.28% of CD4+ cells in peripheral blood; Fig. 5C, 5D). Furthermore, gating on Tregs and assessing expression of the Thy1.1 donor marker versus Thy1.2 recipient marker (Fig. 5A, 5B, right panels) demonstrated that in the “BM only” control all Tregs expressed only Thy1.2, as expected, whereas no Thy1.2 Tregs are detected in the vehicle or ATL146e-treated B6-Thy1.1 into B6-D2 GVHD model transplant groups. Virtually all of the Tregs arising in the GVHD model transplant mice were of Thy1.1 donor origin. This was seen in both the vehicle-treated GVHD model, where there is a relative paucity of Tregs compared with the “BM only” control, and in the ATL146e-treated GVHD model, where relative numbers of Tregs are restored closer to levels seen in the “BM only” control. We also observed the same results when we used either ATL370 or ATL1223 (data not shown).

Because the Tregs arising in the GVHD model of transplant are all of donor origin, we next performed experiments to determine whether functional Foxp3 in donor cells was required for A2AR agonist efficacy. B6-Foxp3–deficient mice (scurfy mouse on B6 background) have been shown to be incapable of generating functional Tregs and suffer from a variety of autoimmune problems. When B6-Foxp3–deficient donors were transplanted into B6 recipients, the two groups of A2AR agonist treatment mice (ATL370 and ATL1223) experienced the same accelerated body weight loss and mortality as the vehicle-treated mice (Fig. 5E, 5F; compare with Fig. 2C, 2D). We measured serum levels of G-CSF, IL-6, IFN-γ, TNF-α, IL-10, and TGF-β at 16–20 d in the B6-Foxp3–deficient mice into B6-D2 model. Proinflammatory cytokines (G-CSF, IL-6, IFN-γ, and TNF-α) were not significantly decreased in two groups of A2AR agonist treatment mice (ATL370 and ATL1223; Fig 5G–J). This result can be compared with the serum levels of proinflammatory cytokine in the B6 into B6-D2 model (Fig. 3A–D). Moreover, serum levels of IL-10 and TGF-β were also no different between the vehicle control group and A2AR agonist treatment groups (ATL370 and ATL1223 [Fig 5K, 5L] compared with [Fig. 3E, 3F]). This then confirms the absolute requirement for the donor graft to develop functional Tregs in order for A2AR agonist treatment to provide protection against increased mortality from GVHD in this model.

**Mice treated with the A2AR have increased numbers of Foxp3+ cells in both skin and colon**

As the skin and gastrointestinal tract are target organs of GVHD, we performed histopathological analysis by H&E staining and immunohistochemical staining on liver, ear, and colon biopsies obtained at day 16–20 in our B6 into B6-D2 GVHD mouse model recipients after treatment with either of the two A2AR agonists (ATL370 or ATL1223) or Vehicle only. Pathological scoring for evaluation of GVHD in the target tissues (liver, skin, and colon) was performed by a pathologist blinded to the treatment status of the mouse (Table I). Animals treated with either agonists had significantly lower scores than vehicle-treated mice and in some cases was similar to that of bone marrow only transplanted mice (Fig. 6A). Vehicle-treated mice group demonstrated severe inflammation, necrosis, edema, and amyloid-like material in the lamina propria of the colon (Fig. 6B), and inflammation with thickness, ulceration, and pustule formation in the skin (Fig. 6C). However, ATL370 or ATL1223 mice had minimal focal ulceration, a less-pronounced submucosal edema in the colon (Fig. 6B) and mostly normal-appearing skin (Fig. 6D).

As shown in Fig. 6D (colon) or Fig. 6E (skin), immunohistochemical staining for Foxp3 showed that transplanted mice receiving the A2AR agonists appeared to have more Foxp3+ cells than those treated with vehicle. We quantified this observation by having a blinded observer count the number of Foxp3+ cells in a random sampling of high power fields as noted in Materials and Methods. The results of this analysis for colon and for skin are shown in Fig. 6F and 6G, respectively. In mice treated with vehicle control and with active GVHD, the number of Foxp3+ cells detectable in colon was 14 ± 6 per high-power field. However, in the ATL370-treated mice, there were 64 ± 14 Foxp3+ cells per high-power field in colon, a difference that was statistically significant compared with the vehicle control group (p < 0.0001; Fig. 6F, middle bar). The trend was similar in the colon of ATL1223-treated mice, although the increase in Foxp3+ cells also reached statistical significance (p = 0.0078; Fig. 6F, right bar). In contrast, the number of Foxp3+ T cells per high-power field in skin was only 8 ± 6 in the vehicle control but increased to a highly significant 30 ± 7 in the ATL370-treated mice (p = 0.0013; Fig. 6G, middle bar) and to 36 ± 14 in the ATL1223-treated mice (p = 0.0030; Fig. 6G, right bar). We also determined the number of infiltrating Tregs in colon and skin by flow cytometry analysis after extraction of lymphocytes from the tissues. In mice treated with vehicle control and with active GVHD, the number of CD4+ Foxp3+ cells in 100 mg colon was 432 ± 101. However, we measured 1256 ± 365 of CD4+Foxp3+ cells in the ATL370-treated mice and 732 ± 110 of CD4+Foxp3+ cells in ATL1223-treated mice, which is statistically significantly higher than the vehicle control group (p = 0.0001, p = 0.0154; Fig. 6H). As well, the number of Tregs in the skin was 287 ± 97 in the vehicle control mouse but increased to 786 ± 132 in the ATL370-treated mice (p = 0.0055) and to 865 ± 115 in the ATL1223-treated mice (p = 0.0004). Therefore, the number of infiltrating Tregs in skin or colonic tissues was increased 1.7- to 3-fold after treatment with an A2AR agonist for acute GVHD. Furthermore, we observed Foxp3 gene expression increased 3.21-fold (p = 0.0069; Fig. 6I) in the colonic tissues of ATL370-treated mice as measured by increased mRNA levels, although we did not see a difference when using ATL1223. In the skin, Foxp3 gene expression also increased 2.5-fold (p = 0.0078 for ATL370, p = 0.0099 for ATL123 compare with vehicle control; Fig. 6I) for each of the A2AR agonist-treated groups.

**Discussion**

Allogeneic transplantation is being increasingly used to cure a wide variety of cancers and monogenetic disorders of blood and other organs. However, acute and chronic GVHD remain as some of

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<tr>
<td>Colon</td>
<td>0.600 ± 0.548</td>
<td>2.286 ± 0.756</td>
<td>0.600 ± 0.894</td>
<td>1.600 ± 0.548</td>
</tr>
</tbody>
</table>

The pathologist was blinded to the samples and graded for GVHD in multiple samples from animals treated with bone marrow transplant only (a non-GVHD control), vehicle, or either A2AR agonist. The mean score (±SEM) is given for each tissue type.
the most significant adverse effects of this potentially life-saving procedure. Current treatment for GVHD involves the use of high-dose immunosuppressants including corticosteroids, various inhibitors of T cell function, and mAbs directed at T and B cell Ags or proinflammatory signaling pathways, which in themselves impair innate and acquired immunity, may impair engraftment and impede antitumor effects and lead to a significantly increased risk of infection. There is a need for new agents with alternate mechanisms of action to prevent GVHD, particularly those that act to enhance tolerance rather than merely suppress T cell function.

More targeted prevention and treatment of GVHD is therefore a goal of many transplanters. Our study demonstrates that specific activation of A<sub>2A</sub>R mediates reduction of acute GVHD pathology and symptoms and improves survival rate in mouse mismatch transplant models in large part by enhancing development of Tregs that act to increase graft tolerance to host Ags.

In addition, A<sub>2A</sub>R blocks the function of effector T lymphocytes by suppressing proinflammatory cytokine production and decreasing expression of activation markers and proliferation (34–36). It has been reported that activated conventional T cells in the presence of an A<sub>2A</sub>R agonist in vitro are anergic (23), and it has been shown that activation of the A<sub>2A</sub>R can prevent T cell proliferation or production of IL-2 or IFN-γ upon restimulation. Our study suggests that in vivo these effects of A<sub>2A</sub>R agonist treatment are likely augmented or mediated by an increased production of Tregs. In this study, we examined the selective A<sub>2A</sub>R agonists of differing potency (ATL146e, ATL370, and ATL1223) in the reduction of GVHD pathology in two models of transplant related GVHD in mice. We showed a profound protection against weight loss and mortality, which was associated with a decrease in proinflammatory cytokines, an increase in protective anti-inflammatory cytokines, and enhanced production of protective Tregs in spleen, peripheral blood, and target tissues (colon and skin). We demonstrated that these A<sub>2A</sub>R agonist induced protective Tregs were of donor origin and that failure to produce Tregs (using Foxp3-deficient donor cells) resulted in failure to provide protection against GVHD-mediated mortality. Tregs unequivocally have been shown to play an important role in development of donor tolerance to host, with protection against development of GVHD (37–41). In a recent clinical study, it was shown that infusion of
Tregs derived in vitro from umbilical cord blood can safely reduce the incidence of GVHD (42, 43). These reports provided an important basis for initiating our assessment of the effect of A2AR activation on generation of Tregs in our acute GVHD mouse models. We showed that CD4+CD25(high)Foxp3+ Tregs are increased by all three of the A2AR agonists that we studied, although in general, the most potent and specific agonist, ATL146e, had the most profound effect. We also documented significant reduction of peripheral blood proinflammatory cytokine levels, including IL-6, IFN-γ, IL-2, and TNF-α and increased levels of the immunosuppressive cytokine IL-10 and TGF-β from administration of A2AR agonist relative to vehicle controls.

There have also been some reports suggesting that adenosine activation, via the A2AR, not only can enhance generation of Tregs, but that the effectiveness of the tolerogenic action of those Tregs can be increased by engaging the A2AR on the target effecter T cells (21). In fact, some have suggested that generation of adenosine by Tregs suppresses effector T cells via the A2AR. In an immune-mediated colitis model, it was found that CD45RBlow or CD25+ Tregs from A2AR−/− mice failed to suppress CD45RBhigh cells and prevent disease induced by pathogenic CD45RBhigh T cells, and that CD45RBhigh effector T cells from A2AR−/− mice are not suppressed by suppressor Treg cells derived from wild-type mice (15). Furthermore, the Powell laboratory group has reported that extracellular adenosine stimulates the A2AR to promote T cell energy and the generation of Foxp3+ and LAG3+ Tregs by reducing the production of IL-6 and enhancing the production of TGF-β after Ag stimulation (23). It has also been shown that mice lacking the CD73/ecto-5′-nucleotidase appear to have increased GVHD due to the inability to convert AMP to adenosine (44). Furthermore, autoimmunity in ADA-SCID patients has been linked to a dysregulation of the CD39/73 adenosinergic pathways resulting in aberrant T regulatory function (45). Finally, methotrexate, a commonly used drug in the management and treatment of GVHD as well as autoimmune disorders such as rheumatoid arthritis has been shown to increase levels of AMP and extracellular adenosine and administration of adenosine A2AR receptor agonists reversed its anti-inflammatory effects in a mouse model of inflammation (46, 47). However binding of adenosine to other receptors can also trigger proinflammatory effects perhaps explaining some of the failures seen with methotrexate use (48).

In our current study, we not only observed that A2AR specific agonists improved survival rate and inhibited weight loss, but we also observed a profound reduction in clinical manifestations of skin sores and diarrhea in the treated mice. Although these manifestations of clinical improvement in the treated mice were difficult to quantify compared with measures of weight loss and survival, the amelioration of skin sores and diarrhea was clearly a manifestation treatment studies in our GVHD mouse models in vivo are ongoing.

Finally, we observed that A2AR agonists, but not cyclosporine A, promoted TGF-β-mediated differentiation of Tregs in vitro (Fig. 1). Treg development and homeostasis are controlled by TCR engagement, cytokines such as IL-2, IL-10, and TGF-β, and costimulatory receptors such as CD28 and CTLA-4 (51). It has also been reported that TCR signaling through PI3K, protein kinase B (Akt), and mammalian target of rapamycin (mTOR) regulates Foxp3 expression in activated CD4 lineage thymocytes and peripheral T cells. Conversely, constitutive activation of the PI3K/AKT/mTOR signaling pathway in chromosome 10 (PTEN, major negative regulator of PI3K/Akt signaling)–deficient T cells reduces Foxp3 induction, which can be restored by PI3K inhibitor (52). The fact that we do not see a synergistic effect in combination with rapamycin suggests that there is some overlap through the mTOR pathway as a mechanism for Treg development; however, this needs to be explored further. There is also data suggesting that A2AR stimulation enhances Foxp3 transcription by decreasing IL-6 expression and enhancing TGF-β expression (23). As seen in the scurfy model, the level of proinflammatory cytokines remained high, and there was no impact by the agonists in the severity of GVHD, insinuating that the generation of Tregs may be dampened by the high cytokine levels and lack of IL-6 production. However, we still have questions as to whether activation of the A2AR is directly involved in TGF-β–induced Foxp3 expression on GVHD mouse model or if this is an indirect effect. Additional studies are needed to further elucidate the mechanism by which A2AR-specific agonist increases Tregs.

In summary, we have demonstrated that specific activation of A2AR enhances development of Tregs in vitro, and that treatment with specific agonist of A2AR inhibits the development of acute GVHD in two types of HLA mismatched mouse transplant models through the increase of donor-derived Tregs in the spleen, blood, and target tissues of GVHD (colon and skin). Our preclinical studies in these mouse models, along with clinical studies using A2AR agonists in normal volunteers documenting their low toxicity profile, provide strong support for bringing highly specific agonists of A2AR to the clinic for the prevention and treatment of GVHD.

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Disclosures

The authors have no financial conflicts of interest.

References


