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Alloreactive regulatory T cells generated with retinoic acid prevent skin allograft rejection

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CD4$^+$CD25$^+$Foxp3$^+$ regulatory T (Treg) cells mediate immunological self-tolerance and suppress immune responses. Retinoic acid (RA), a natural metabolite of vitamin A, has been reported to enhance the differentiation of Treg cells in the presence of TGF-β. In this study, we show that the co-culture of naive T cells from C57BL/6 mice with allogeneic antigen-presenting cells (APCs) from BALB/c mice in the presence of TGF-β, RA, and IL-2 resulted in a striking enrichment of Foxp3$^+$ T cells. These RA in vitro-induced regulatory T (RA-iTreg) cells did not secrete Th1-, Th2-, or Th17-related cytokines, showed a nonbiased homing potential, and expressed several cell surface molecules related to Treg-cell suppressive potential. Accordingly, these RA-iTreg cells suppressed T-cell proliferation and inhibited cytokine production by T cells in in vitro assays. Moreover, following adoptive transfer, RA-iTreg cells maintained Foxp3 expression and their suppressive capacity. Finally, RA-iTreg cells showed alloantigen-specific immunosuppressive capacity in a skin allograft model in immunodeficient mice. Altogether, these data indicate that functional and stable allogeneic-specific Treg cells may be generated using TGF-β, RA, and IL-2. Thus, RA-iTreg cells may have a potential use in the development of more effective cellular therapies in clinical transplantation.

Keywords: Allogeneic regulatory T cells · Homing · Retinoic acid · Tolerance · Transplantation

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Introduction

Regulatory T (Treg) cells are responsible for inducing and maintaining peripheral tolerance [1]. Treg cells are classified into two major subpopulations: thymus-derived Treg cells, which are generated in the thymus and circulate in the periphery as functional mature Treg cells [2–4], and peripherally derived Treg cells, which are generated in the periphery from CD4$^+$CD25$^-$ naive

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T cells [5–7]. Treg cells target effector T cells and dendritic cells (DCs) by modulating their maturation and function through several mechanisms that suppress immune responses [8–10]; these include secretion of inhibitory cytokines (IL-10, IL-35, and TGF-β), granzyme/perforin-dependent mediated cytolysis, metabolic disruption [11–15], and the expression of LAG3 and CTLA4, which alter DC function [16, 17].

In addition to the aforementioned mechanisms, Treg-cell suppressive capacity is dependent on Treg-cell migration and retention in the microenvironment where regulation is required. Differential expression patterns of chemokine receptors (such as CCR4, CCR5, and CCR9), integrins (α4β7), and selectins (CD62L) contribute to selective retention and trafficking of Treg cells and allow their appropriate localization during an ongoing immune response [18–22].

Transplantation models have suggested that Treg cells not only contribute to the maintenance of tolerance to self-antigens and the regulation of the immune responses against foreign antigens, but also that they may function to limit alloreactive responses [23–26]. Some in vivo models have shown an essential role for donor-specific Treg cells in long-term allograft survival and in the preferential accumulation of donor-reactive Treg cells both in the graft and draining lymph nodes [27, 28].

Among the protocols designed to obtain adequate numbers of Treg cells for use in immunotherapy, the polyclonal expansion of Thymus-derived Treg cells using anti-CD3/anti-CD28 beads plus IL-2 has been shown to be the most efficient method for ex vivo expansion of both mouse and human Treg cells [29–32]. However, the expansion of thymus derived Treg cells and the generation of polyclonal in vitro-induced Treg (iTreg) cells from naive cells have the disadvantage that these cells are expanded/generated from an existing pool, and have a broad rather than a selective reactivity towards alloantigens. Thus, the use of allospecific Treg cells has the advantage of avoiding broad immune suppression as suggested by Anasetti's group [33]. Moreover, the polyclonal expansion obtained by simply using CD3/CD28 beads risks the expansion of other possible auto-reactive cells. Shaping the T-cell response by driving naive $CD4^+$ T cells with donor APCs under Treg polarizing conditions, which include the provision of regulatory cytokines such as TGF-β and IL-10, may avoid this problem by raising the precursor frequency of alloreactive iTreg cells.

Retinoic acid (RA), a natural metabolite of vitamin A, plays an important role in the induction of the gut-homing receptors α4β7 and CCR9 on T cells [34, 35] and has been shown to promote the generation of Treg cells in vitro and in vivo [36–39]. Recently, it has been demonstrated that the presence of RA enhances TGF-β signaling by increasing the expression and phosphorylation of Smad3. This results in increased Foxp3 expression, even in the presence of Th17-inducing cytokines such as IL-6 or IL-21 [40–42].

Several groups have studied the effect of RA on the generation of clinically useful numbers of human Treg cells, in order to use these cells for transplantation therapy. For instance, it has been demonstrated that RA contributes to the expansion of $Foxp3^+$ Treg cells [43, 44] and enhances Treg-cell differentiation from naïve T cells [44–46]. However, the role of RA in inducing allogeneic Treg cells with potent suppressive capacity and presenting an unbiased homing potential has not been studied in detail.

In this study, we demonstrate that the co-culture of naive $CD4^+CD25^-$ T cells with allogeneic APCs in the presence of TGF-β, IL-2, and RA leads to the production of alloantigen-reactive Treg cells (retinoic acid-in vitro induced regulatory T cells (RA-iTreg)) with potent suppressive capacity. RA-iTreg cells express several cell-surface markers with immunosuppressive potential, and homing receptors that allow RA-iTreg cells to migrate to tissues beyond the intestinal mucosa. Moreover, RA-iTreg cells are stable in time when adoptively transferred. Using a sensitive adoptive transfer model, we show here that RA-iTreg cells can control skin allograft rejection in an alloantigen-specific manner, confirming their functional activity in vivo. These results suggest that recipient-derived alloreactive RA-iTreg cells may have potential as cellular therapeutics in clinical transplantation.

Results

Alloreactive Treg cells are generated in the presence of allogeneic APCs, and TGF-β plus RA

Based on previous studies [47, 48], we designed a protocol to generate donor-reactive iTreg cell ex vivo. Purified C57BL/6 naive T cells ($CD4^+CD25^-$) were cultured with BALB/c APCs in the presence of TGF-β1, IL-2, and RA (referred to as "TILRA"), and analyzed 6 days later for CD25 and Foxp3 expression. As shown in Figure 1A, stimulation of $CD4^+CD25^-$ cells in the absence of the added cytokines (untreated) resulted in the up-regulation of CD25 but not Foxp3 expression. In contrast, stimulation in the presence of TILRA resulted in striking up-regulation of Foxp3 expression such that the resultant population was 65–85% $CD25^+Foxp3^+$. Although the presence of TGF-β plus RA in the absence of IL-2 resulted in a modest induction of Foxp3 that did not exceed approximately 20%, cell recovery in these cultures rarely exceeded 10% compared with the TILRA condition. To study whether the conversion of naive T cells to Treg cells occurs as a consequence of allogeneic stimulation, we co-cultured naïve T cells with autologous (C57BL/6) APCs in the presence of TILRA. In this setting, we obtained only a 10% of $Foxp3^+$ cells, therefore the conversion of naive T cells into $Foxp3^+$ cells occurs mainly during allogeneic stimulation (Supporting Information Fig. 1). In order to confirm that RA-iTreg cells are generated de novo, we sorted naive $CD4^+CD25^-Foxp3^-$ (GFP^-; 99% pure) cells from Foxp3-GFP (C57BL/6) reporter mice and co-cultured these cells with allogeneic APCs (BALB/c) in the presence or absence of TILRA and analyzed GFP expression on day 6. The presence of TILRA in the cultures produced over a 60% conversion of the input $CD4^+Foxp3^-$ T cells to $CD4^+Foxp3^+$ compared with a 1.5% conversion in the absence of TILRA (Fig. 1B).

Naive $CD4^+$ T cells stimulated in vitro can differentiate into different Th lineages such as Th1, Th2, Th17 cells, or

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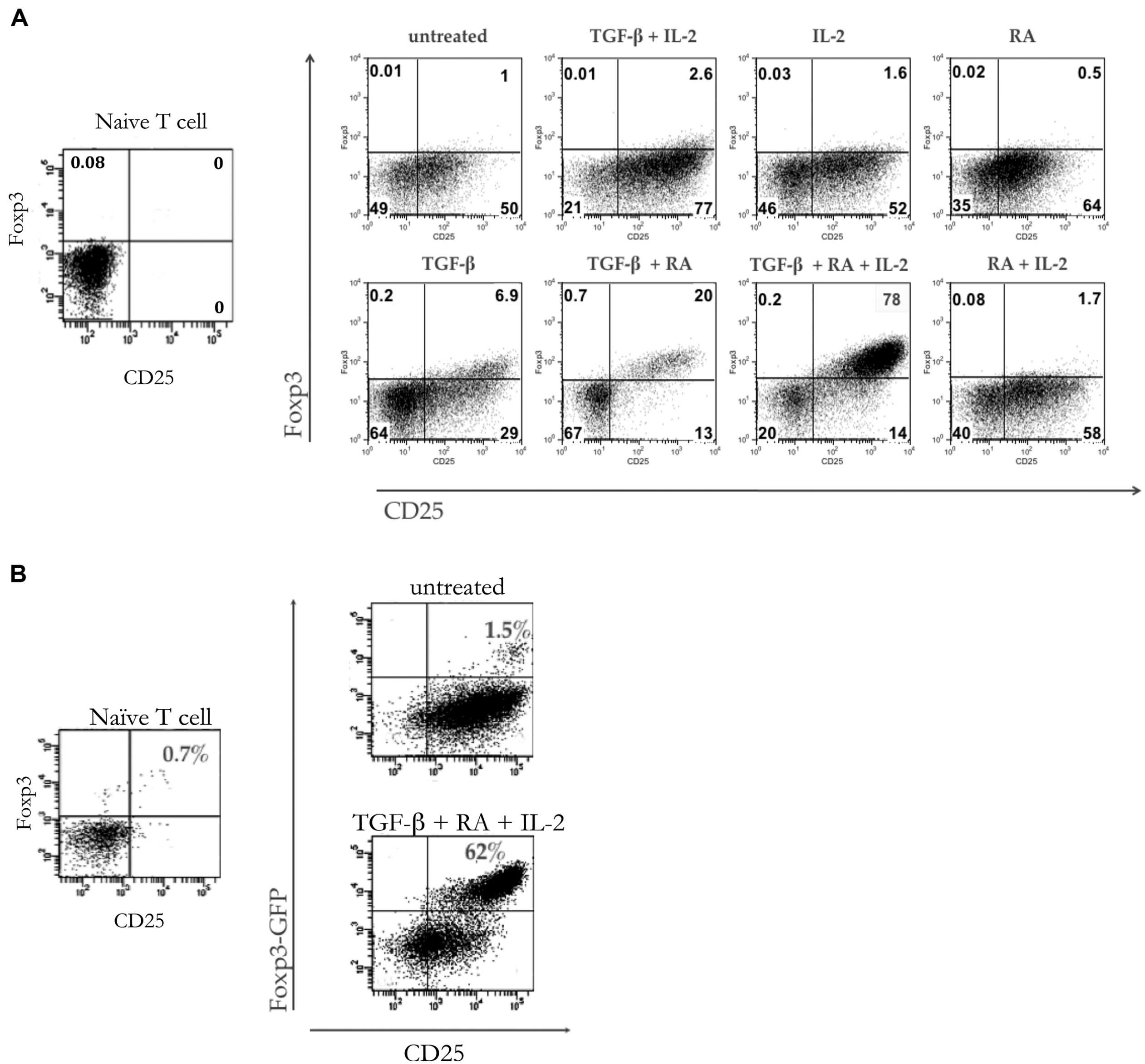

Figure 1. De novo allospecific Treg cells are generated by TGF-β, IL-2, and retinoic acid (RA) in the presence of allogeneic APCs. (A and B) Naive $CD4^+CD25^-$ T cells from (A) C57BL/6 mice and (B) $CD4^+GFP^-$ T cells from Foxp3-GFP knock-in mice were co-cultured with BALB/c APCs for 6 days in the presence of RA and different combinations of cytokines. The expression of Foxp3 and CD25 was analyzed by flow cytometry (gating on $CD4^+$ T cells). (A and B) The results are representative of four independent experiments.

Treg cells depending on the cytokine environment and the nature/maturation status of the APC population [49]. Therefore, we sought to determine whether, in addition to promoting the generation of iTreg cells, our TILRA protocol could also impede the production of other Th cell lineages. Cell supernatants were harvested during RA-iTreg-cell generation and analyzed for signature Th1, Th2, and Th17 cytokines. As shown in Figure 2, when compared with the untreated control, the addition of TILRA resulted in a striking reduction in the production of IFN-γ, TNF-α, IL-4, IL-10, and IL-6. Although there was only a modest reduction in IL-17 production, overall these data indicate that the induction of RA-iTreg cells shifts the entire alloreactive response away from other Th subsets.

Alloreactive Treg cells exhibit suppressive activity

In order to characterize RA-iTreg cells further, we analyzed the expression of additional markers associated with Treg cell function. As shown in Figure 3A, when compared with the untreated control, stimulation in the presence of TILRA resulted in the clear up-regulation of CD101, CD103, CTLA-4, LAG3, and CD39 within the induced $Foxp3^+$ population.

To determine whether the TILRA protocol generates functional iTreg cells, we assessed their in vitro suppressive activity. For this, RA-iTreg cells were generated as indicated above and after 6 days of culture, the resultant population was co-cultured with CFSE-labeled naive T cells in the presence of APCs and anti-CD3, in a

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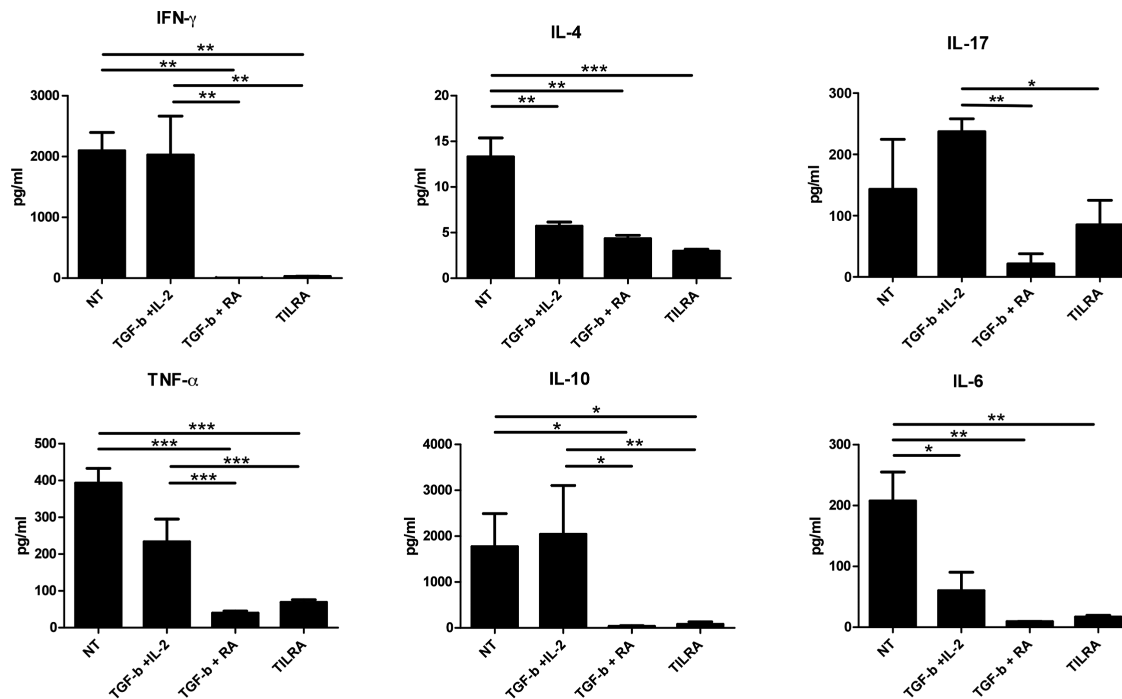

Figure 2. RA-iTreg cells do not secrete Th1-, Th2-, or Th17-related cytokines during their generation. Naive $CD4^+CD25^-$ T cells from C57BL/6 mice were co-cultured with APCs from BALB/c mice for 6 days in the absence or presence of different cytokines and RA. The supernatants of these cultures were analyzed using the Th1, Th2, and Th17 cytometric bead array (CBA) kit to measure the concentration of IFN-γ IL-4, IL-17, TNF-α, IL-10, and IL-6. Data are shown as mean ± SD ($n = 3$) and are pooled from three independent experiments. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ (ANOVA); NT: untreated; TILRA: TGF-β, RA, and IL-2.

conventional T-cell suppression assay. As shown in Figure 3B, the proliferation of CFSE-labeled $CD4^+CD25^-$ T cells was almost completely inhibited by RA-iTreg cells. This suppression was at least partially mediated by the generation of adenosine, since APCP, a CD73-specific inhibitor restored $CD4^+CD25^-$ T-cell proliferation (data not shown). The inhibition of T-cell proliferation was antigen-specific since RA-iTreg cells exhibited almost a 50% less suppressive activity when activated with third party APCs (Supporting Information Fig. 2). Accordingly, the secretion of Th1- and Th2-related cytokines in these assays was abrogated (Fig. 3C). Taken together, these data are consistent with the concept that stimulation in the presence of TGF-β, IL-2, and RA generates a population of functional alloreactive iTreg cells.

RA-iTreg cells present an unbiased homing potential

RA has been shown to induce the expression of the gut-homing receptors α4β7 and CCR9 on CD4+ T cells during in vitro activation [34]. However, Zhang and collaborators have shown that IL-2, in conjunction with IL-4, promotes the internalization of CCR9 [50]. Thus, we next addressed whether RA-iTreg cells generated with APCs in the presence of TILRA up-regulated the expression of α4β7 and CCR9. For this, naive $CD4^+CD25^-$ T cells from C57BL/6 mice (>99.5% pure) were stimulated with allogeneic APCs from BALB/c mice for 6 days in the presence of TGF-β, RA and IL-2. The resulting iTreg-cell population was analyzed for the expression of gut- and skin-homing receptors. As shown in Figure 4, we observed an increase in α4β7 and CCR9 expression as well as an up-regulation in the expression of the skin-homing receptors P-selectin ligands in the presence of TGF-β and RA. Although, the addition of IL-2 strongly down-regulated CCR9 and α4β7 expression, these cells still expressed high levels of these gut-homing receptors. Moreover, RA-iTreg cells also expressed E- and P-selectin ligands, suggesting that iTreg cells generated in the presence of RA could potentially migrate not only to the intestine, but also to skin and other inflamed tissues. Similar results have been shown in a graft-versus-host disease model in work done by Steinman and others [28].

Adoptively transferred RA-iTreg cells are stable in time and maintain their suppressive activity

In order to assess the phenotypic and functional stability of Treg cells generated ex vivo using the TILRA protocol, we moved to a TCR transgenic model in which RA-iTreg cells could be followed in vivo for functional analysis. For this, RA-iTreg cells from OT-II/Foxp3-GFP ($CD45.2^+$) mice were sorted based on GFP expression and intravenously injected into B6SJL-PTPRC ($CD45.1^+$) mice. The following day, mice received a 1 mg dose of OVA protein by intraperitoneal injection. Three days later, recipient mice were sacrificed and GFP expression was analyzed in $CD45.2^+$ splenocytes (Fig. 5A). As shown in Figure 5B, approximately 40% of

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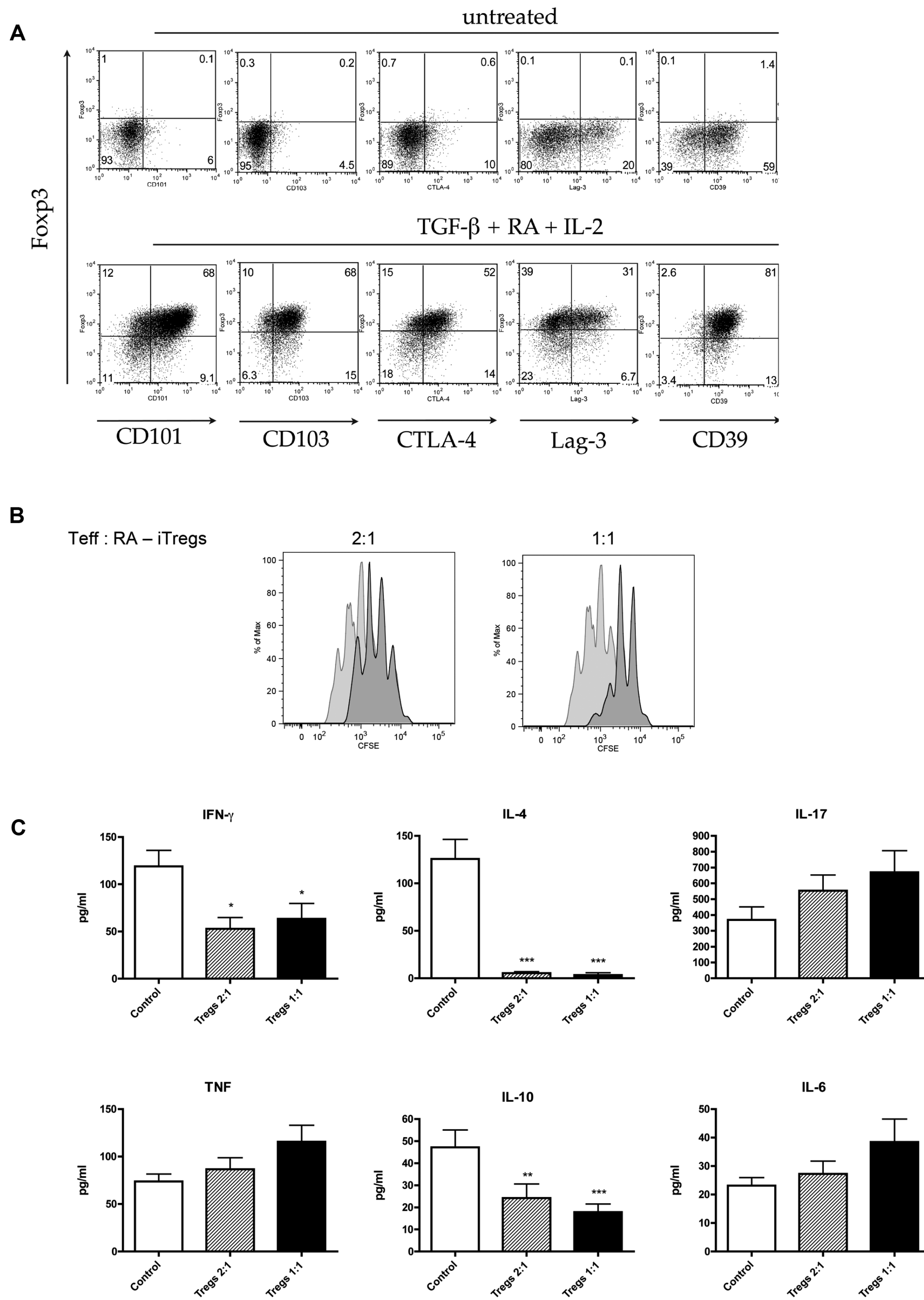

Figure 3. RA-iTreg cells express immunosuppressive molecules and suppress T-cell responses in vitro. (A–C) Naive $CD4^+CD25^-$ T cells from C57BL/6 mice were co-cultured with APCs from BALB/c mice for 6 days in the absence or presence of TILRA. (A) The expression of CD101, CD103, CTLA-4, Lag-3, and CD39 was analyzed by flow cytometry using a $CD4^+CD25^+$ gate. (B) RA-iTreg cells were co-cultured at the indicated ratios with CFSE-labeled $CD4^+CD25^-$ effector cells from C57BL/6 mice in the presence of APCs from BALB/c mice and soluble anti-CD3. Three days later, CFSE dilution on effector cells was analyzed by flow cytometry. Light gray histogram: $CD25^-$ T cells; Dark gray histogram: $CD25^-$ T cells + RA-iTreg cells. (C) The supernatants of the suppression assays from (B) were analyzed using the Th1, Th2, and Th17 CBA kits. (C) Data are shown as mean + SD ($n = 3$) and (A–C) are pooled from (A and B) four or (C) seven independent experiments. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; (ANOVA).

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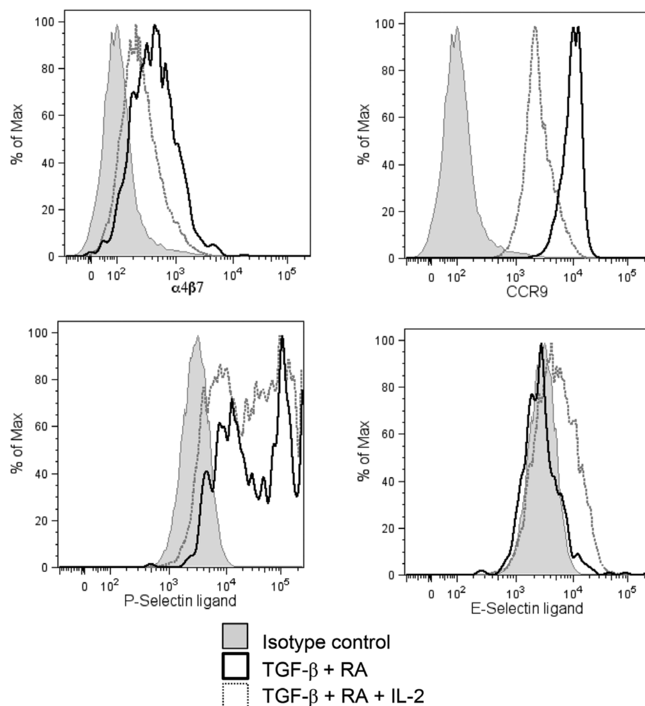

Figure 4. RA-iTreg cells express skin and gut-homing receptors. Naive $CD4^+CD25^-$ T cells from C57BL/6 mice were co-cultured with APCs from BALB/c mice for 6 days in the presence of TGF-β, RA, and IL-2. The expression of CCR9, α4β7, P-selectin, and E-selectin ligands was analyzed by flow cytometry ($CD4^+Foxp3^+$ gate). The results are representative of three independent experiments. Black histogram: TGF-β + RA; gray-dotted histogram: TGF-β + RA + IL-2; light gray shaded histogram: isotype control or secondary antibody control.

RA-iTreg cells maintained GFP expression 4 days after being transferred into $CD45.1^+$ C57BL/6 mice. These cells were still detected 28 days after in vivo transfer and retained GFP expression to a high extent (data not shown). Moreover, when re-stimulated, GFP^+ cells did not produce IL-17 and only 1–2% of the GFP^+ cells produced IFN-γ (Fig. 5C).

Since Treg cells from Foxp3-GFP mice have been described as less stable than WT Treg cells [51], we repeated this experiment with the difference that RA-iTreg cells were generated from OT-II mice. RA-iTreg cells were then sorted as $CD4^+CD25^{high}$ cells and intravenously injected into $CD45.1^+$ B6SJL-PTPRC mice. Figure 5B shows that approximately 93% of sorted OT-II T cells were $Foxp3^+$ previous to the adoptive transfer to congenic mice. Following immunization with OVA via intraperitoneal injection, we analyzed the percentage of $Foxp3^+$ T cells within $CD45.2^+$ total cells. As shown in Figure 5B, approximately 70% of the transferred RA-iTreg cells maintained Foxp3 expression.

Furthermore, to test whether these RA-iTreg cells maintained their suppressive potential after in vivo administration, $CD45.2^+$ cells (OT-II RA-iTreg cells) or $CD45.2^+GFP^+$ cells (OT-II/Foxp3-GFP RA-iTreg cells) were sorted from the B6SJL-PTPRC recipient mice and tested for their suppressive potential in in vitro suppression assays. As shown in Figure 5D and Supporting Information Figure 3, RA-iTreg cells were able to completely suppress T-cell proliferation, and show suppressive activity even in a 1:8 ratio. Taken together, these results indicate that Treg cells generated with TILRA are phenotypically and functionally stable.

Donor-specific RA-iTreg cells reduce allograft rejection

Next, to examine the suppressive function of alloreactive RA-iTreg cells in vivo, we used a model of adoptive transfer of skin allografts in immunodeficient mice. For this, C57BL/6-driven RA-iTreg cells were generated by the co-culture of naive T cells from CBA mice with APCs from C57BL/6 mice in the presence of TILRA. Next, CBA $Rag1^{-/-}$ mice were reconstituted intravenously with naive CBA $CD25^-CD4^+$ cells as an effector population plus RA-iTreg cells in a 1:2 ratio. Control mice were reconstituted with naive CBA $CD25^-CD4^+$ cells alone. Importantly, the RA-iTreg cells used in this assay were harvested from 6-day-old cultures, as indicated above, and used as a bulk source population without further enrichment. Twenty-four hours after reconstitution, full-thickness C57BL/6 tail skin allografts were transplanted into graft beds prepared on the left flank. Mice reconstituted with only effector cells acutely rejected C57BL/6 skin grafts (mean survival times [MST] 11 days, $n = 3$). However, reconstitution with effector cells plus 2×10^5 C57BL/6-driven RA-iTreg cells resulted in 60% C57BL/6 skin graft survival with normal hair growth and normal macroscopic appearance (MST > 50 days, $n = 5$). The effect on allograft survival was donor specific since mice reconstituted with the same effector and Treg-cell populations rejected third party BALB/c grafts at control rates (MST 11 days, $n = 4$; Fig. 6).

Discussion

Treg cells are of great interest for immunotherapy since they could be used to prevent transplant rejection and in the treatment of autoimmune diseases. Adoptive transfer of $CD4^+CD25^+$ Treg cells has been successfully used in experimental immunosuppression in several animal models [52–54]. A previous report from our group demonstrated that allogeneic in vitro-induced Treg cells can be produced by stimulating naive T cells with allogeneic APCs in the presence of exogenous TGF-β1, IL-2, and RA [48]. In the present study, we investigated the role of RA in the generation, stability and function of allospecific Treg cells (RA-iTreg). Our results show that allogeneic stimulation of naive T cells in the presence of RA consistently induced the de novo differentiation of Treg cells with an unbiased homing potential, presenting effective in vivo suppressive abilities in a skin allograft model.

Several protocols have been developed to generate Treg cells; some are designed to produce polyclonal Treg cells by stimulating naive T cells with anti-CD3 in anti-inflammatory conditions [55, 56], while others have expanded selected thymus-derived Treg cells [29, 30]. Although these Treg cells can be used to produce tolerance to a transplanted tissue, they cannot be used for cellular therapy in humans due to the lack of specificity. Kuchroo and colleagues have demonstrated that RA synergizes with TGF-β in order to generate iTreg cells, and that RA plays an essential

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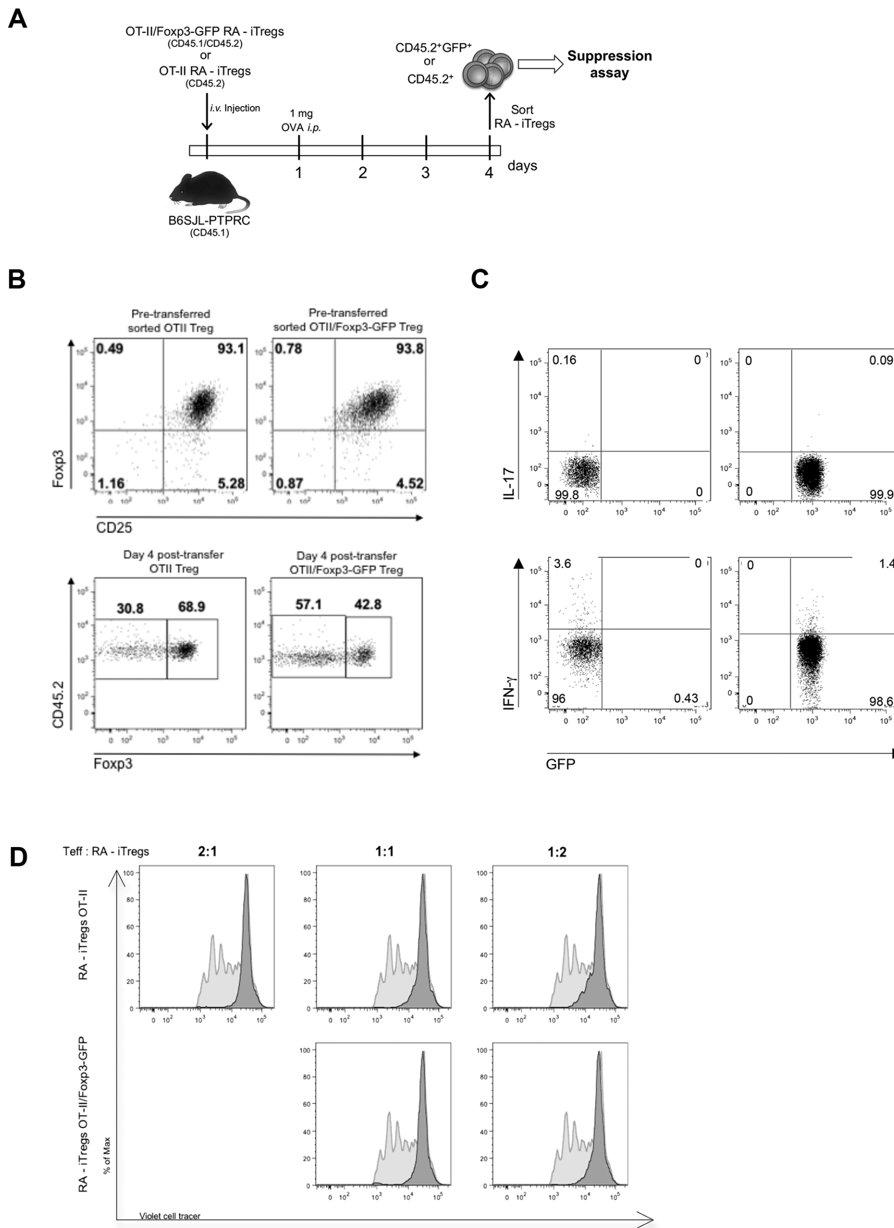

Figure 5. RA-iTreg cells present a stable phenotype and suppressive function after adoptive transfer. (A) Schema of the adoptive transfer of RA-iTreg cells into congenic mice. (B) RA-iTreg cells from OT-II/Foxp3 GFP ($CD45.1^+CD45.2^+$) or OT-II ($CD45.2^+$) mice were transferred intravenously to $CD45.1^+$ congenic mice. One day after adoptive transfer, the recipient mice received 1 mg of OVA protein. At day 4, splenocytes were harvested and $CD45.2^+$ cells were analyzed for GFP or Foxp3 expression by flow cytometry. (C) Splenocytes were harvested and activated for 4 h with PMA plus ionomycin in the presence of BFA in order to assess IL-17 and IFN-γ production on GFP^+ and GFP^- cells. (D) $CD45.2^+$ cells from recipient mice were sorted and co-cultured at different ratios with CellTrace Violet-labeled $CD4^+CD25^-$ effector cells from C57BL/6 mice in the presence of APCs from C57BL/6 mice and soluble anti-CD3. After 3 days, the CellTrace Violet dilution was analyzed by flow cytometry (light gray histograms). Proliferation of effector cells without RA-iTreg cells (proliferation control) is shown in dark gray histograms. (B–D) Data are representative of three independent experiments.

role in the stability of these cells by preventing the development of Th17 cells [41]. Consistent with this observation, the addition of RA to our cultures induced high numbers of iTreg cells and simultaneously abrogated Th1- and Th2-cell differentiation. Moreover, we demonstrated that the RA-iTreg cells generated were de novo Treg cells and not a result of the expansion of already existing Treg cells because a highly purified population of naive T cells from the Foxp3-GFP knock-in mice was able to generate Treg cells in the presence of TILRA.

A previous report by Sawitzki and colleagues showed that alloreactive Treg cells generated in vitro by combined anti-CD4 mAb, TGF-β, and RA treatment significantly prolonged graft survival due to the stabilization of Foxp3 expression [57]. In contrast to our setting, they generated alloreactive Treg cells by the addition of an anti-CD4 antibody, which has been described to perturb T-cell receptor signaling. Although we observed variable conversion rates of naive T cells to iTreg cells in the cultures containing TGF-β1 and RA, the addition of IL-2 was important since it consistently increased cell recovery. This is in agreement with previous reports that show that IL-2, together with TGF-β1, increases the stability of Treg cells [58].

A critical issue for the therapeutic use of allogeneic Treg cells is to obtain sufficient numbers of cells presenting an unbiased homing potential. Although it has been demonstrated that RA contributes to the expansion and generation of Tregs [43–46], RA has also been shown to be responsible for the induction of the gut-homing receptors α4β7 and CCR9 on T cells [34, 35]. In our study, we observed that physiological concentrations of RA and IL-2 did induce the expression of gut-homing receptors. However, RA-iTreg cells also expressed the skin homing receptors

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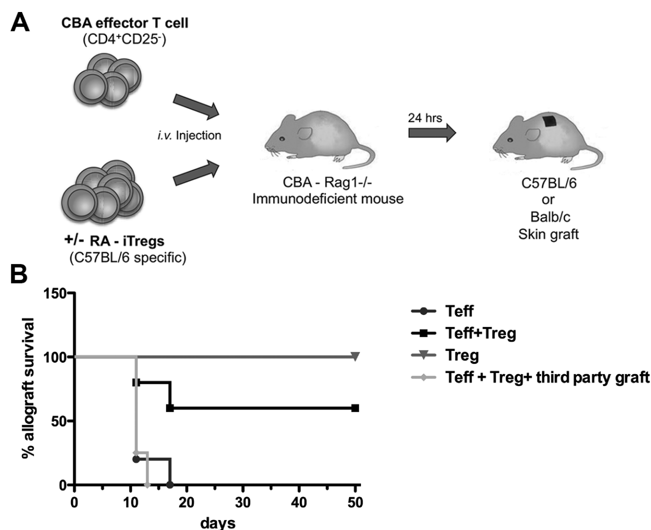

Figure 6. RA-iTreg cells present suppressive activity in vivo and prolong allograft survival. (A) Schema of the allogeneic skin graft survival in CBA-Rag1$^{-/-}$ mice receiving RA-iTreg cells (B) CBA-Rag1$^{-/-}$ mice were reconstituted with 1×10^5 CD4$^+$CD25$^-$ effector T cells, and in some cases co-injected with 2×10^5 RA-iTreg cells. After 24 h, mice received skin allografts from C57BL/6 or BALB/c (third party) mice. The allograft survival was evaluated visually at day 60. When ≥70% of the graft surface was necrotic, it was considered rejection. Data are pooled from one experiment, each group containing three to five mice.

P- and E-selectin ligands, thus imprinting the RA-iTreg cells with the potential capacity to migrate to different tissues, as suggested by the acceptance of a skin allograft.

One of the suppressive mechanisms attributed to Treg cells is metabolic disruption, ascribed in part to the expression of CD39 and CD73 [11, 59, 60]. These ectonucleotidases act in concert to generate to hydrolyze ATP and ADP to adenosine. The role of CD39$^+$ Treg cells in immunoregulation is supported by the occurrence of alopecia areata and experimental autoimmune encephalomyelitis in CD39-deficient mice, and by the failure of CD39$^-$ Treg cells to suppress contact hypersensitivity [61]. Our results show that more than 80% of the allospecific RA-iTreg cells generated expressed CD39. These results support the idea that these cells could be capable of generating pericellular adenosine, a metabolite directly involved in suppressive activity. This idea is reinforced by the observation that in vitro suppression was abrogated in the presence of APCP, a CD73-specific inhibitor (data not shown). Another well-known suppressive function of Treg cells includes the control of DC maturation and activation by CTLA-4 and LAG-3 expression [62–66]. The high expression of these molecules by RA-iTreg cells supports the notion that these cells may regulate the immune response by also suppressing DC function.

Our results indicate that RA-iTreg cells are capable of almost completely suppressing the proliferation of effector cells in vitro. We have demonstrated that these cells not only present selected markers for highly suppressive activity, but are also capable of inhibiting the proliferation of T lymphocytes activated via allogeneic stimulation in mixed leucocyte reactions. In this scenario, RA-iTreg cells were able of suppressing antigen-specific targets and in a less extent, third party cells. However, functional in vitro assays do not reflect the complexity of the lymphoid environment in vivo due to the absence of other cell types, including subpopulations of DCs, as well as stromal elements and soluble factors present in the tissue microenvironment. We therefore decided to evaluate the microenvironment generated during the suppression assay, and showed that RA-iTreg cells suppressed the secretion of Th1- and Th2-related cytokines by T cells.

A previous report in mice has shown that Treg cells can persist in vivo for as long as 60 days after adoptive transfer [28]. This raises the question as to whether Treg cells are able to maintain their phenotype and function after in vivo transfer. Therefore, we investigated whether RA-iTreg cells maintained Foxp3 expression and suppressed T-cell responses in vitro after adoptive transfer. Our results demonstrate that RA-iTreg cells recovered 4 days after in vivo transfer not only did not produce proinflammatory cytokines such as IL-17 and IFN-γ but also were very efficient at inhibiting the proliferation of effector cells even in a 1:8 (Treg:Teffector) ratio. These results support the notion that RA-iTreg cells generated in this work present a stable phenotype and have potent suppressive properties. Although our data demonstrate that RA-iTreg cells maintain their suppressive capacity up to 28 days after in vivo transfer, additional studies are required in order to demonstrate the long-term stability of RA-iTreg cells. However, in a clinical setting, where the allograft is constantly providing alloantigens, one might expect that there will be a long-term pool of memory/effector Treg cells.

To demonstrate the in vivo suppressive capacity of RA-iTreg cells, we performed skin transplantation in an immune-deficient host. Animals reconstituted with RA-iTreg cells and effector T cells kept the skin graft for over 60 days. The effect of RA-iTreg cells in controlling allograft rejection was antigen-specific since transplanted skin grafts from a third party strain were rejected at a rate similar to the controls. Notably, in these in vivo experiments, RA-iTreg cells were not separated from the small population of activated effector T cells present in the cultures during their generation. These results further support the hypothesis that RA-iTreg cells hold potent suppressive capacity in vivo.

The future translation of our protocol using TGF-β, IL-2, and RA for use in cellular therapy approaches is supported by the observations that RA is able to expand/generate human Foxp3$^+$ Treg cells with potent suppressive ability from Treg cells [43, 44] and naïve T cells [44–46]. Moreover, the generation of alloreactive Treg cells from non-Treg precursors by stimulation with alloantigens may offer immunological advantages over polyclonal expansion of naturally occurring Treg cells, as indicated by other authors [67, 68]. We have shown that the presence of RA, TGF-β1, and IL-2 consistently induces the de novo generation of regulatory RA-iTreg cells. These cells possess a strong and stable suppressor function and are capable of improving the acceptance of an allogeneic transplant. Furthermore, these cells retain their suppressive capacities after in vivo administration. Taken as a whole, the results presented here suggest that RA-iTreg cells generated

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with our protocol are highly efficient and functionally stable and therefore fulfill the requirements for a future use in allogeneic cell therapy.

Materials and methods

Mice

CBA.Ca (H2k), CBA-Rag1$^{-/-}$ (H2k), C57BL/6 (H2b), B6SJL-PTPRC (H2b, CD45.1^{+}), BALB/c (H2d), and OT-II (H2b) mice were purchased from Jackson Laboratories and housed under specific pathogen-free conditions. Foxp3-GFP (H2b, CD45.1^{+}) knock-in mice were generously provided by Dr. Alexander Rudensky (Howard Hughes Medical Institute, Memorial Sloan-Kettering Cancer Center, New York). OTII/Foxp3-GFP (CD45.1^{+}/CD45.2^{+}) mice were generated by crossing OT-II mice (CD45.2^{+}) with Foxp3-GFP knock-in mice (CD45.1^{+}). Animal work was carried out under institutional regulations of the Fundacion Ciencia & Vida, Facultad de Ciencias, Universidad de Chile, and the University of Oxford. Animal work was approved locally by ethical review committees.

Antibodies and reagents

The following antibodies from eBiosciences were used: anti-mouse CD4, CD8, B220, Foxp3, CD25, CD103, CD49d, CD101, Lag-3, CD39, CTLA-4, CCR9, α4β7, P-selectin ligand (fusion protein), E-selectin ligand (fusion protein), and anti-human IgG. From Biolegend we used anti-mouse CD45.1 and CD45.2 antibodies. Recombinant mouse IL-2 and recombinant human TGF-β1 (eBioscience) were used at 10 and 2 ng/mL, respectively. All-trans RA (Sigma-Aldrich) was used at 10 nM. $OVA_{323-339}$ peptide (Gene Tel Laboratories) was used at 2 ng/mL. Doses of 1 mg of OVA protein (Sigma-Aldrich) were used for intraperitoneal injections.

Isolation of naive T cells

Splenic CD4^{+} cells were negatively selected using a Miltenyi CD4^{+} T-cell isolation kit following the manufacturer's instructions. CD4^{+} cells were then incubated with PE-anti-CD25 antibody (eBioscience) followed by incubation with anti-PE microbeads (Miltenyi, Biotech, Germany). CD4^{+}CD25^{-} T cells were selected using multi-MACS.

Isolation of splenic APCs

Spleen tissue was fragmented and digested for 45 min at 37°C in the presence of collagenase D (Roche, Germany) and 2 μg/mL of DNAse I (Roche) in complete media. Undigested fibrous material was removed by filtration through a cell strainer. CD11c^{+} cells were obtained by positive selection using anti-CD11c microbeads (Miltenyi). Cells were assayed for the presence of DCs and B cells by flow cytometry, obtaining an average of 60% CD11c^{+} DCs and 40% B220^{+} B cells.

Generation of allogeneic RA-iTreg cells

To generate allogeneic RA-iTreg cells, 0.14 × 10^{6} CD4^{+}CD25^{-} naive T cells from C57BL/6 mice were co-cultured in 96-well plates for 6 days with 2 × 10^{4} allogeneic splenic APCs from BALB/c mice in complete IMDM media supplemented with 10% FCS (Invitrogen), 0.055 mM 2-mercaptoethanol, 0.5 μg/mL fungizone, 2 ng/mL TGF-β, 10 nM RA, and 10 ng/mL IL-2 (TILRA). For skin transplantation experiments, C57BL/6-driven RA-iTreg cells were generated from the co-culture of CBA naive T cells with C57BL/6 APCs in the presence of TILRA.

Generation of OVA-specific RA-iTreg cells

To generate OVA-specific RA-iTreg cells, 0.10 × 10^{6} CD4^{+}CD25^{-} naive T cells from OT-II or OT-II/Foxp3-GFP mice were co-cultured in 96-well plates for 4 days with 2 × 10^{4} APCs from C57BL/6 mice in complete IMDM media supplemented with 10% FCS (Invitrogen), 0.055 mM 2-mercaptoethanol, 0.5 μg/mL fungizone, 2 ng/mL TGF- β, 10 nM RA, and 10 ng/mL IL-2 in the presence of 2 ng/mL $OVA_{323-339}$ peptide and 0.1 mg/mL purified anti-CD3 mAb (145–2C11, eBioscience).

Foxp3 FACS analysis

Cells were blocked with Fc block (Biolegend) and stained with anti-CD4 and CD25 antibodies for 20 min at 4°C. Then, cells were permeabilized for 1 h in Fix/Perm buffer (eBioscience). The cells were washed and stained with anti-Foxp3 clone FJK-16s or isotype control for 30 min at 4°C. The cells were analyzed on a FACSCanto II (BD Bioscience).

CBA immunoassay for Th1, Th2, and Th17 cytokines

Analysis of cytokines from cell-free supernatant samples was conducted using a mouse inflammation CBA kit (BD Biosciences, CA) and assessed on a FACSCanto II flow cytometer. Standard curves were determined for each cytokine within a range of 0–5000 pg/mL.

T-cell suppression assay

The in vitro suppression assay was performed as previously described. Briefly, CD4^{+}CD25^{-} responder T cells (5 × 10^{4}) from C57BL/6 mice were labeled with CFSE or CellTrace Violet and

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stimulated with (5 × 10^4) allogeneic BALB/c or C57BL/6 APCs plus 1 μg/mL anti-CD3 mAb. RA-iTreg cells purified by sorting were added at different ratios. After 3 days, CFSE or CellTrace Violet dilution on responder cells was analyzed by flow cytometry.

Adoptive transfer of RA-iTreg cells for in vivo stability assays

In vitro-generated RA-iTreg cells from OT-II ($CD4^+CD25^{high}CD45.2^+$, 4 × 10^6) or OT-II/Foxp3-GFP mice ($CD4^+CD25^{high}GFP^+CD45.1/2^+$, 3 × 10^6) were sorted (FACS Aria II) and intravenously transferred to B6SJL-PTPRC ($CD45.1^+$) mice. One day after the adoptive transfer, the recipient mice received a dose of 1 mg OVA via intraperitoneal injection. Four days later, splenocytes were recovered and the expression of GFP or Foxp3 on the $CD45.2^+$ population was assessed by flow cytometry. For in vitro suppression assays, OT-II RA-iTreg cells and OT-II/Foxp3-GFP RA-iTreg cells recovered from B6SJL-PTPRC recipient mice were further sorted based on CD45.2, or CD45.2 and GFP expression, respectively. These cells were then assayed at different ratios in in vitro suppression assays.

Adoptive transfer of RA-iTreg cells and skin transplantation

CBA-$Rag1^{-/-}$ mice were reconstituted intravenously with 1 × 10^5 $CD25^-CD4^+$ naive cells from CBA mice with or without 2 × 10^5 C57BL/6-driven RA-iTreg cells. The following day, full-thickness C57BL/6 or third party BALB/c tail skin allografts were transplanted onto graft beds prepared on the left flank. Skin grafts were considered rejected if ≥70% of the surface was necrotic. The end point of the experiment was at 50 days following skin transplantation.

Statistical analysis

Statistical significance was determined by ANOVA using Bonferroni post test and the Kaplan–Meier test for survival curves using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA).

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Abbreviations: **RA**: retinoic acid · **RA-iTreg**: retinoic acid-in vitro induced regulatory T cell · **TILRA**: TGF-β, IL-2, and retinoic acid

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