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Direct Expansion of Human Allospecific FoxP3⁺CD4⁺ Regulatory T Cells with Allogeneic B Cells for Therapeutic Application

Leo C. Chen,* Julio C. Delgado,† Peter E. Jensen,† and Xinjian Chen1†

Compelling evidence from animal studies has demonstrated that allospecific FoxP3⁺CD4⁺ regulatory T (Treg) cells expanded ex vivo can be used as effective therapeutic tools in the treatment of allograft rejection and graft-vs-host disease. Despite the promising results from animal studies, there remain major barriers to developing Treg cell-based immunotherapy in humans. Currently, no effective approach has been established for selective expansion of human allospecific Treg cells ex vivo. Additionally, the very low frequency of Treg cells present in human peripheral blood could pose a formidable challenge to obtaining a sufficient number of Treg cells from a single donor for ex vivo expansion for therapeutic utilization. Extending our recent finding that mouse B cells preferentially induce expansion of alloreactive Treg cells, we report herein that human Treg cells can be expanded ex vivo with allogeneic B cells. The expanded Treg cells express very high levels of FoxP3, maintain anergic phenotype, and are potent suppressors capable of inhibiting the alloproliferation of third-party responder T cells at very low Treg-to-T effector cell ratio in an alloantigen-specific manner. The allostrogenic specificity demonstrated by B cell-expanded Treg cells is not determined by the HLA haplotypes of the Treg cells, but it is induced and determined by the haplotype of the B cells used to expand them. Our findings represent a significant advance in the development of Treg cell-based immunotherapy in humans and raise the possibility of using third-party Treg cells for therapeutic applications. The Journal of Immunology, 2009, 183: 4094–4102.

The naturally arising regulatory FoxP3⁺ CD4 T (Treg)² cells play an essential role in the induction and maintenance of immunological tolerance to self Ags. Loss of this tolerogenic mechanism is associated with fulminate, fatal autoimmunity in both experimental animals and human patients (1–6). In addition to their physiological role, studies have also shown that mouse Treg cells can effectively prevent allograft rejection (7) and graft-vs-host disease (GVHD) when adoptively transferred into recipient hosts (8–11), unveiling the attractive perspective of using Treg cells as therapeutic tools for treatment of transplant rejection and GVHD in humans. As compared with conventional drug-based immunosuppressive therapy, Treg cell-based therapy has the potential to provide long-lasting, nontoxic, and Ag-specific suppression of graft rejection or GVHD without compromising protective immunity in the host against infections and tumors (11). Since the number of Treg cells that can be obtained from donor peripheral blood is limited, freshly isolated Treg cells need to be expanded ex vivo to generate a sufficient number of cells for therapeutic applications. Additionally, expanded Treg cells have been reported to be more therapeutically effective than primary Treg cells (12). Significant success has been made in expanding mouse Treg cells in vitro. Treg cells can be expanded polyclonally with anti-CD3 Ab or in an alloantigen-specific manner with allogeneic APCs. As compared with the anti-CD3-expanded polyclonal Treg cells, allogeneic APC-expanded Treg cells are enriched for allostrogenic specificity, with less potential to induce nonspecific immunosuppression and more therapeutic activity in animal studies (9, 13).

Despite promising results with mouse Treg cells, only limited success has been reported in the direct expansion of human allostrogenic-specific Treg cells with allogeneic APCs. In one study, human CD25⁺ CD4 T cells were successfully expanded indirectly with autologous APCs presenting an allogeneic HLA-A2 peptide, generating T cells with suppressive properties (14). This study provides the first line of evidence that human allostrogenic-specific Treg cells can be expanded. However, the specificity of Treg cells expanded with this approach is limited to those cells with highly restricted indirect allore cognition specificity for allogeneic-derived peptides presented by self HLA molecules. There would be challenges to the general application of this approach given the diversity of clinically relevant donor-recipient HLA mismatches and the relative paucity of information on indirect presentation of specific peptides from allogeneic HLA molecules by recipient HLA class II molecules. Another possible challenge is that the indirectly expanded Treg cells may not be effective in preventing acute organ rejection that is known to be mediated by direct presentation of allostrogenics by the donor APCs (7), or in preventing acute GVHD, which is initiated by direct presentation of host allostrogenics by the host dendritic cells (DCs) (15, 16).

As an alternative approach, irradiated, human PBMCs were found to be able to induce modest proliferation of allogeneic CD25⁺ CD4 T cells in the presence of exogenous IL-2 plus IL-15. The proliferative CD25⁺ cells, especially the CD27⁺ subset, when isolated from the culture, could potentely suppress alloploproliferation of autologous responder T cells in an alloantigen-specific manner (17). With two cycles of stimulation by alloantigen combined with anti-CD3/CD28 Abs, and in the presence of IL-2 and IL-15, an

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2 Abbreviations used in this paper: Treg cells, naturally arising FoxP3⁺ CD4⁺ T cells; GVHD, graft-vs-host disease; DC, dendritic cells; MFI, mean fluorescence intensity; BMT, bone marrow transplantation.

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average 780-fold expansion could be obtained, generating highly suppressive cells consisting of >90% CD4+ T cells, most of which retained FoxP3 expression (18). Allogeneic PBMCs, especially monocytes, in the presence of IL-2 and IL-15 also induce Treg cells to differentiate into IL-17-producing cells (19), while monocyte-derived DCs have the capacity to induce autologous Treg cells to undergo a limited degree of proliferation (20). In a more recent study, it is reported that CD40L-activated allogeneic B cells can induce a fraction of human CD25+ naïve CD4 T cells to become allospecific FoxP3+ CD4 T cells with suppressive function (21). While the findings of the study are very promising, data from other studies indicate that induced human FoxP3+ Treg cells do not have inhibitory function (22) and they are unstable because the FoxP3 gene in induced Treg cells remains methylated (23).

We have recently reported that mouse B lymphocytes preferentially expand allogeneic Treg cells in MLCs (24). In the present study, we used human B cells to directly expand allogeneic Treg cells. The phenotype and function of the expanded cells were characterized, and the possibility of generating allospecific Treg cells with capacity to suppress HLA-unrelated third-party responder T cells was explored.

Materials and Methods
Isolation of CD25+ and CD25− CD4 T cells, B cells, and monocytes
Leukocytes were back-flushed out of leukopheresis filters with cold Ca2++ and Mg2+-free PBS. Lymphocytes were enriched with Ficol gradients and labeled with 10 μM CFSE. Total CD4 T cells were isolated by magnetic depletion of other lineage-positive cells using a CD4 T cell isolation kit (Miltenyi Biotec). CD25+ cells were isolated from CD4 T cells by positive selection with anti-CD25 microbeads (Miltenyi Biotec). Cells were positively selected with CD14 beads (Miltenyi Biotec) and cultured with 50 ng/ml each of GM-CSF and IL-4 for 6 days with addition 500 ng of LPS on day 5 to generate mature DCs.

Cell cultures and flow cytometry
The culture medium was made of MEM-α plus GlutaMAX MEM (Invitrogen) supplemented with 50 μM 2-ME, HEPES, pyruvate, nonessential amino acids, penicillin/streptavidin, and 10% FCS. For expansion of allospecific Treg cells, CD25+ cells were cultured for various times in 96-well round-bottom plates at 2 × 103 to 1 × 104 per well with allogeneic B cells at indicated B-to-T cell ratio in the presence of IL-2 and stimulatory anti-CD28 Ab CD28.2 (BioLegend) at the indicated concentrations. For expansion of polyclonal Treg cells, CD25+ cells were cultured with anti-CD3 Ab (OKT3) (1 μg/ml) plus CD28.2 (0.5 μg/ml). For restimulation, the cells were cultured for 4 h with PMA (10 ng/ml) plus ionomycin (300 ng/ml). Inhibition assays were performed by culturing CD25+ CD4 responder cells with allogeneic DCs at a 1:5 DC-to-T cell ratio in the presence of various numbers of Treg cells expanded with B cells of the same or different donor origin as the DCs. The Treg cells in the cultures were enumerated by combining cell counts and flow cytometry. B cells constituted only a small fraction (<10%) of the cells in the expanded product at the end of culture, and they were not removed from expanded Treg cells used in suppressor assays. OKT3 plus CD28.2 was also used to stimulate the cultures as indicated. Abs for flow cytometry were purchased from BD Biosciences, except for anti-FoxP3 (256A/E7), which was from eBioscience. Intracellular staining was performed using Perm/Fix kits (eBioscience). The data were collected on FACSCalibur and analyzed with FlowJo software.

Molecular HLA typing
Genomic DNA was prepared from PBMCs using a standard phenol-chloroform extraction procedure. HLA-I (HLA-A, HLA-B) and HLA-II (HLA-DR) alleles were identified in PCR-amplified products of exons 2 and 3 of the HLA-A and HLA-B loci and exon 2 of the HLA-DR locus by sequence-specific oligonucleotide probe hybridization using a LumineX platform (Tepnel Life Sciences), as previously reported (25).

Results
Primary human B cells can efficiently expand allogeneic Treg cells
Based on our previous finding that freshly isolated mouse B cells can expand isolated allogeneic CD25+ CD4+ T cells at a B-to-T cell ratio of 4:1 in the absence of exogenous IL-2 (24), we set up similar cultures with human B cells and magnetic bead-sorted allogeneic CD25+ CD4+ T cells at various B-to-T cell ratios in the presence or absence of exogenous IL-2. No significant Treg cell proliferation was obtained when the Treg cells were cultured with allogeneic B cells alone (Fig. 1a), and supplementation of IL-2 resulted in only minimal Treg cell proliferation (Fig. 1b) in multiple attempts. The lack of significant Treg cell expansion under these culture conditions raised the possibility that human Treg cells might require costimulation, which was not required by mouse Treg cells, for activation and proliferation. Freshly isolated human B cells were analyzed for expression of CD80 and CD86, and neither of these costimulatory molecules was detected (Fig. 1e). To provide costimulation, stimulatory anti-CD28 Ab CD28.2 was supplemented in the cultures along with exogenous IL-2, and this resulted in robust proliferation of FoxP3+ cells (Fig. 1c). The concentration of IL-2 and the B cell-to-Treg cell ratio influenced the degree of Treg cell expansion, but the impact was modest (Fig. 1, f and g), since a 25-fold change in IL-2 concentration and 16-fold change in B-to-T cell ratio resulted in <2-fold change in expansion of Treg cells. The kinetics of allogeneic B cell-induced Treg cell proliferation was slow (Fig. 1h). Very little cell division was detected at day 3, with minimal division observed on day 5. Robust cell proliferation was observed at later time points. A 20- to 40-fold expansion of the cells could be obtained within 14 days without further addition of B cells to the cultures. The expanded Treg cells expressed high levels of FoxP3 (with a mean fluorescence intensity (MFI) of 1630) (Fig. 1c), which was 3-fold higher than that of the nondividing input CD25+ CD4 cells (MFI of 550).

Several studies have reported that activated human non-Treg cells also express Foxp3 (26–33) and that TGF-β can convert CD45RA− CD25+ naïve CD4 T cells to FoxP3-expressing cells (22), but neither activated non-Treg cells nor the TGFβ-converted human FoxP3-expressing CD4 T cells are suppressive, complicating the previously proposed defining role of FoxP3 in determining the Treg cell phenotype (34–37). To understand the reason for the dissociation of FoxP3 expression from the Treg cell phenotype, we examined activated non-Treg CD4 cells for FoxP3 expression. While the high level of expression of FoxP3 in the B cell-expanded Treg cells (Fig. 2) was much lower (>20 fold) than that detected in the B cell-expanded Treg cells or in the nondividing input Treg cells. Therefore, the expression of FoxP3 in B cell-expanded Treg cells was quantitatively different from that of activated non-Treg cells.

B cell-expanded CD25+ cells remain anergic and are potent suppressors
The high level of expression of FoxP3 in the B cell-expanded Treg cells suggested that these cells maintained the properties of Treg cells. To confirm this possibility, B cell-expanded Treg and activated non-Treg cells were restimulated in IL-2 containing media for 2 days and then restimulated with PMA plus ionomycin. The restimulation induced production of high levels of IL-2 and IFN-γ in the non-Treg but not in the Treg cells (Fig. 2, a–d), indicating that the expanded Treg cells remained anergic, one of the key distinguishing features of Treg cells (22, 38). To determine whether the
B cell-expanded Treg cells were suppressive, CD25⁺ responder CD4 T cells were cultured with allogeneic monocyte-derived, LPS-matured DCs in MLCs in the presence or absence of Treg cells. In the absence of Treg cells, the responder cells underwent vigorous proliferation, generating a large number of dividing T cells in the cultures (Fig. 2e). This intensive proliferation, however, was abrogated by the presence of the B cell-expanded Treg cells at a Treg-to-responder cell ratio of 1:5 (Fig. 2f) or 1:20 (Fig. 2g). Even at a very low Treg-to-responder cell ratio of 1:80, the proliferation of responder cells was partially inhibited (Fig. 2h). The inhibitory potency of B cell-expanded Treg cells was compared with that of the Treg cells expanded with anti-CD3/CD28 Abs (OKT3 and CD28.2) plus IL-2. At a Treg-to-responder cell ratio of 1:5, the OKT3-expanded Treg cells only partially inhibited proliferation of the responder cells (Fig. 2i), and the inhibition was no longer detectable at Treg-to-responder cell ratio of 1:20 (Fig. 2j) or 1:80 (Fig. 2k). Therefore, the B cell-expanded Treg cells were much more potent than OKT3-expanded polyclonal Treg cells in suppressing alloreactivity of responder T cells. Since the low inhibitory capacity of OKT3-expanded Treg cells could be due to low frequency of alloantigen-specific Treg cells in the expanded cells, OKT3 plus CD28.2 instead of allogeneic DCs was used as a global stimulus to provide polyclonal activation to both Treg and responder T cells in the cultures. Stronger inhibition was observed with OKT3-expanded Treg cells (Fig. 2l), as compared with alloanigenic stimulation (Fig. 2, i–k). Nevertheless, the B cell-expanded Treg cells still demonstrated stronger inhibition than OKT3-expanded Treg cells (Fig. 2l). These results taken together suggest

**FIGURE 1.** Direct expansion of Treg cells with allogeneic B cells. CFSE-labeled CD25⁺ CD4 T cells were cultured for 7 days with allogeneic B cells at a B-to-T cell ratio of 4:1 either alone (a), in the presence of IL-2 (100 U/ml) (b), or IL-2 plus stimulatory anti-CD28 Ab CD28.2 (0.5 μg/ml) (c). CFSE-labeled CD25⁺ CD4 non-Treg cells were cultured for 3 days with allogeneic DCs (d). To examine expression of costimulatory molecules (e), primary B cells (solid thick lines) were stained with anti-CD80, CD86m or isotype-control Abs (dotted line); EBV-transformed B cells were used as positive controls. CD25⁺ CD4 T cells were cultured for 7 days with allogeneic B cells at a B-to-T cell ratio of 1:1 in the presence of 0.5 μg/ml CD28.2 plus various concentrations of IL-2 (f), or cultured with allogeneic B cells at different B-to-T cell ratios in fixed concentrations of IL-2 (120 U/ml) and CD28.1 (1 μg/ml) (g), and enumerated by flow cytometry. CD25⁺ CD4 T cells were cultured with allogeneic B cells at B-to-T cell ratio of 1:1 in the presence of 200 U/ml of IL-2 and 1 μg/ml CD28.1, and enumerated at various days of culture (h). The results are representative of more than three experiments.

B cell-expanded Treg cells were suppressive, CD25⁻ responder CD4 T cells were cultured with allogeneic monocyte-derived, LPS-matured DCs in MLCs in the presence or absence of Treg cells. In the absence of Treg cells, the responder cells underwent vigorous proliferation, generating a large number of dividing T cells in the cultures (Fig. 2e). This intensive proliferation, however, was abrogated by the presence of the B cell-expanded Treg cells at a Treg-to-responder cell ratio of 1:5 (Fig. 2f) or 1:20 (Fig. 2g). Even at a very low Treg-to-responder cell ratio of 1:80, the proliferation of responder cells was partially inhibited (Fig. 2h). The inhibitory potency of B cell-expanded Treg cells was compared with that of the Treg cells expanded with anti-CD3/CD28 Abs (OKT3 and CD28.2) plus IL-2. At a Treg-to-responder cell ratio of 1:5, the OKT3-expanded Treg cells only partially inhibited proliferation of the responder cells (Fig. 2i), and the inhibition was no longer detectable at Treg-to-responder cell ratio of 1:20 (Fig. 2j) or 1:80 (Fig. 2k). Therefore, the B cell-expanded Treg cells were much more potent than OKT3-expanded polyclonal Treg cells in suppressing alloreactivity of responder T cells. Since the low inhibitory capacity of OKT3-expanded Treg cells could be due to low frequency of alloanigen-specific Treg cells in the expanded cells, OKT3 plus CD28.2 instead of allogeneic DCs was used as a global stimulus to provide polyclonal activation to both Treg and responder T cells in the cultures. Stronger inhibition was observed with OKT3-expanded Treg cells (Fig. 2l), as compared with alloanigenic stimulation (Fig. 2, i–k). Nevertheless, the B cell-expanded Treg cells still demonstrated stronger inhibition than OKT3-expanded Treg cells (Fig. 2l). These results taken together suggest
that the high inhibitory potency of B cell-expanded Treg cells is the result of high frequency of alloantigen-specific Treg cells in the expanded products and high inhibitory capacity of individual cells.

**Allogenic B cell-expanded Tregs are enriched for alloantigen specificity**

Ideally, for therapeutic application, the Treg cells administered to a transplant patient should selectively inhibit pathological immunity against allografts or the host tissue in the case of allogeneic bone marrow transplantation (BMT) without compromising protective immunity. This might be accomplished by generating Treg cells that are specific for donor or host alloantigens. To determine whether allogeneic B cell-expanded Tregs have specificity for the stimulating alloantigens, CD25+CD4 T cells from a given donor (no.32; DRB1*0701/*1322) were divided and expanded separately with B cells from two unrelated donors designated “Y and Z”, whose HLA-DR haplotypes were DRB1*0701/*0301 and DRB1*1201/*0101, respectively (Table I). Each expanded Treg population was then used to inhibit alloproliferation of the CD25+CD4 responder T cells from an unrelated donor (no. 40; DRB*04AMAD/*0407) elicited by mature DCs derived from either donor Y or Z. When the Treg cells expanded by B cells of donor Y were added to the culture, they imposed significant inhibition at a Treg-to-responder cell ratio of 1:5 (Fig. 3d), but at the ratio of 1:50, only marginal inhibition was observed (Fig. 3e). The lack of substantial inhibition by the Z-B cell-expanded Treg cells at this low Treg-to-responder cell ratio of 1:50 was not due to an intrinsic low inhibitory capacity of the Treg cells, since, when added to MLCs elicited by Z-DCs at similar Treg-to-responder cell ratios, the Treg cells imposed strong inhibition to the alloproliferation (Fig. 3, g and h). In contrast, the Treg cells expanded by B cells of donor Y imposed only minimal inhibition to the MLCs elicited by DCs of donor Z at a Treg-to-responder cell ratio of 1:50 (Fig. 3j), in contrast to strong inhibition in MLCs elicited by DCs from donor Y (Fig. 3c). Similar results were obtained in multiple cultures involving different allogeneic B Cell/DC and Treg cell combinations.

**Table I. HLA allele typing of HLA-DR, A and B loci of the blood donors**

<table>
<thead>
<tr>
<th></th>
<th>DRB1</th>
<th>A</th>
<th>B</th>
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<tbody>
<tr>
<td>Donor Y</td>
<td>0701/0301</td>
<td>0101</td>
<td>0201/0201</td>
</tr>
<tr>
<td>Donor Z</td>
<td>1201/0101</td>
<td>0101</td>
<td>2301/5101</td>
</tr>
<tr>
<td>Donor 31</td>
<td>0404/1301</td>
<td>0101</td>
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<td>0701/1322</td>
<td>0301</td>
<td>2402/2402L</td>
</tr>
<tr>
<td>Donor 38</td>
<td>0409/0101</td>
<td>0201</td>
<td>2402/0702</td>
</tr>
<tr>
<td>Donor 39</td>
<td>0404/0301</td>
<td>0101</td>
<td>3101/0801</td>
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<tr>
<td>Donor 40</td>
<td>0401/0407</td>
<td>0101</td>
<td>6801/0702</td>
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*DRB*0701 is a shared allele between donor Y and no. 32; DRB1*0404 is a shared allele between donor nos. 31 and 39.
The results demonstrate that allogeneic B cell-expanded Treg cells are highly suppressive and, at a relatively high Treg-to-responder cell ratio (1:5), they are capable of imposing potent inhibition on alloreactive, third-party responder T cells in a haplotype nonspecific manner. At a very low Treg-to-responder cell ratios (1:50), however, the Treg cells lose their capacity to potently inhibit alloreactivity elicited by nonspecific alloantigens while remaining highly potent in inhibiting alloproliferation of responder cells elicited by specific alloantigens. The alloantigen specificity demonstrated by B cell-expanded Treg cells is not determined by the HLA haplotypes of the Treg cells but is induced and determined by the haplotype of the B cells used to expand them.

B cell-expanded Treg cells can inhibit HLA-unrelated “third-party” responder T cells

We used third-party responder T cells in the above inhibition experiments based on previous reports that mouse Treg cells, when activated, could suppress responder T cells of different Ag specificities or MHC allotypes (39–41). If B cell-expanded human Treg cells can inhibit HLA-unrelated responder T cells in an alloantigen-specific manner, then it opens the possibility of using alloantigen-specific third-party Treg cells for therapeutic applications. The frequency of FoxP3+ CD4 T cells in human peripheral blood is very low (~1% of CD4 T cells) (42), and this could pose a formidable challenge if a large number of Treg cells need to be harvested from a single donor for ex vivo expansion for therapeutic utilization. While the information regarding the availability of Treg cells from a single donor is scant in the literature, in our experience, with magnetic beads sorting, only a very small number (1 to 3 × 10⁵) of CD25+ cells of purity >90% FoxP3+ cells could be obtained from a leuko-filter of 1 U (500 ml) of blood. Although the number of Treg cells could be expanded ex vivo substantially, excessive expansion has been shown to increase the frequency of FoxP3− T cells in the expanded product (18, 43). Since transfer of activated effector T cells is potentially harmful to the recipients, the requirement for highest quality of expanded Treg cells might pose a limit to the extent that Treg cells could be expanded ex vivo, and thus make it necessary to have a sufficient number of freshly isolated Treg cells as starting input cells for expansion. Another potential barrier to the use of Treg cells in transplantation could be related to the time needed to expand Treg cells. According to all current protocols, at least 2 wk are needed to expand Treg cells on a large scale, which makes expansion of Treg cells specific for alloantigens of unexpected donors, such as cadaver donors, impossible. The use of third-party Treg cells expanded for specificity for various major target HLA haplotypes opens the potential for “off the shelf” cell products that could be rapidly employed in therapy.
To include a variety of combinations of HLA-unrelated Treg and responder cells in inhibition experiments, CD25<sup>+</sup> CD4<sup>T</sup> cells from three HLA-unrelated blood donors, nos. 38, 39, and 40 (Table I), were used in cultures with CD25<sup>+</sup> CD4<sup>T</sup> cells from each of two HLA-unrelated donors, nos. 31 and 32, which had been expanded separately with B cells from donors Y and Z (Table I). Although the HLA haplotypes of responder T cells were disparate from that of Treg cells at both class I and II alleles (Table I), the B cell-expanded Treg cells potently inhibited alloproliferation of the responder T cells in all Treg and responder cell combinations at a Treg-to-responder T cell ratio of 1:5, or 1:50 when the Treg cells were stimulated with the same alloantigens against which the Treg cells were expanded (Fig. 4, a and b). When the B cell-expanded Treg cells were stimulated with DCs expressing different alloantigens from which they were expanded (Fig. 4, c and d), the Treg cells still substantially inhibited alloproliferation of the responder cells at Treg-to-responder cell ratio of 1:5. At a Treg-to-responder cell ratio of 1:50, however, only partial or marginal inhibition was observed. These results demonstrate that when used as third-party suppressor cells, the B cell-expanded Treg cells can potently inhibit alloproliferation of HLA-unrelated responder T cells. Effective inhibition was obtained with either Ag-specific or nonspecific Treg cells at a relatively high Treg-to-responder cell ratio; at a low Treg-to-responder cell ratio, however, potent inhibition was only associated with Ag-specific Treg cells.

**Discussion**

In the present study, we demonstrate that human Treg cells can be directly expanded with allogeneic B cells. The expanded Treg cells express high levels of FoxP3, maintain an anergic phenotype, and are potent suppressors capable of inhibiting alloproliferation of CD25<sup>+</sup> CD4<sup>T</sup> responder T cells at a very low Treg-to-responder cell ratio in an alloantigen-specific manner. These findings represent an important advance in the development of Treg cell-based immunotherapy, since currently no other successful approach has been reported for direct expansion of human alloantigen-specific Treg cells.

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**FIGURE 4.** B cell-expanded Treg cells potently inhibit alloproliferation of third-party responder T cells in various Treg and responder cell combinations in an alloantigen-specific manner. Treg cells from donor nos. 31 or 32, each of which had been expanded separately with B cells from either donor Z (a and d) or Y (b and c), were used to inhibit alloproliferation of responder T cells derived from three HLA-unrelated donors, nos. 38, 39, and 40, elicited by DCs from either donor Z (a and c) or Y (b and d).
cells. Studies of animal models have clearly demonstrated that Ag-specific Treg cells are significantly more efficient than polyclonal Treg cells in suppressing autoimmunity (44). Similarly, mouse alloantigen-specific Treg cells are proven to be more effective in preventing GVHD than are polyclonal Treg cells (9, 13). Additionally, Ag-specific Treg cells are less likely to impose nonspecific immunosuppression. Therefore, Ag-specific, as compared with polyclonal, Treg cells should represent an attractive population for therapeutic application in humans. However, because of previous barriers in expanding human Ag-specific Treg cells, there has been interest that anti-CD3/CD28-expanded polyclonal Treg cells might be the only approach to generate large numbers of Treg cells for therapeutic applications (43, 45, 46). Over the last few years, great effort has been made to improve anti-CD3/CD28-based expansion of human polyclonal Treg cells with the goal of improving the yield and purity of the expanded cells (45, 47–51). Our findings provide an approach to selectively expand alloantigen-specific Treg cells that can serve as a springboard to future development of alloantigen-specific Treg cell-based therapy in humans.

Our finding that human Treg cells require exogenous anti-CD28-derived costimulation for proliferation indicates that human Treg cells have distinct requirements for proliferation compared with their mouse counterparts. Mouse Treg cells can be induced to proliferate vigorously by nonirradiated allogenic B cells alone (24) or irradiated splenic APCs in the presence of exogenous IL-2 without anti-CD28-derived costimulation (52), suggesting that mouse APCs including B cells are capable of providing all the costimulation required for mouse Treg cell activation and proliferation (53, 54), B7.1 (CD80) and B7.2 (CD86) seemed not to play a major role in CD28 costimulation of mouse Treg cells since B7.1/B7.2−/− APCs were as effective as B7.1/B7.2-sufficient APCs to induce mouse Treg cell activation (55). The inability of human B cells to induce significant allogenic proliferation of Treg cells in the presence of IL-2 suggests that costimulation provided by human B cells is insufficient to activate the full transcriptional program necessary for proliferation of human Treg cells. It is known that TCR stimulation in the absence of costimulation can induce isolated activation and nuclear translocation of NFAT without activation of other major transcription factors required for T cell activation, such as NF-κB and AP1 (56, 57). Activation of all necessary nuclear factors may require costimulation through CD28, which amplifies TCR activation-induced intracellular signaling (58, 59). Apparently, mouse Treg cells can bypass the requirement for CD28 costimulation for activation in the presence of IL-2, but human Treg cells cannot. The nature of the difference in the intracellular signaling between mouse and human Treg cells is yet to be determined, and much remains to be learned about signal transduction during Treg cell activation.

FoxP3 has proven to be a reliable specific marker for mouse Treg cells, for its expression in mouse CD25+CD4+ naive T cells is sufficient to confer Treg cell phenotype and function (34). Ectopic expression of FoxP3 in mouse FoxP3−/− CD4+ T cell lines leads to expression of downstream target genes similar to those observed in FoxP3-expressing primary Treg cells (60, 61). Despite the unequivocal role of FoxP3 in defining the Treg cell phenotype in murine CD4+ T cells, FoxP3 has been regarded as a nonspecific marker for human Treg cells, since it is detected in activated human non-Treg cells and in TGF-β–converted FoxP3+ T cells, where expression of FoxP3 does not give rise to the suppressive properties of Treg cells. Why the expression of FoxP3 in human non-Treg CD4+ T cells is insufficient to confer Treg cell properties is not understood (22). By directly comparing the level of FoxP3 expression, our results show that the level of expression of FoxP3 in activated non-Treg cells was actually much lower than that identified in primary or B cell-expanded Treg cells. It has been reported that Foxp3 regulates Treg cell function in a dose-dependent, non-binary manner, and decreased Foxp3 expression can abolish the immune-suppressive properties of Treg cells (62). Since the level of Foxp3 expressed in activated human non-Treg cells is low, it is likely that the lack of Treg cell properties of these T cells is due to a relatively low subthreshold level of expression. These findings, taken together, suggest that the stable anergic phenotype and high suppressive potency of B cell-expanded human Treg cells is related to their high level expression of Foxp3, and Foxp3 can remain a valid marker for identification of human Treg cells if the level of expression is taken into consideration.

Our results showing that B cell-expanded Treg cells are more potent than anti-CD3/CD28-expanded polyclonal Treg cells in suppressing alloregenic CD4+ cell responses (Fig. 2) is consistent with the previous observation that mouse Ag-specific Treg cells are more effective in suppressing GVHD than polyclonal Treg cells (9, 13). One possible reason for the high inhibitory potency of B cell-expanded Treg cells is that the expanded cells are enriched for T cell receptors with specificity for the alloantigens presented by the B cells used in expansion cultures. This possibility is supported by the finding that Treg cells from a given donor can be rendered specific for different alloantigens after expansion with different alloregenic B cells (Fig. 3). Such plasticity in Ag specificity may have at least two important clinical implications. First, Treg cells from any unrelated donor can be directed (or enriched) toward a desired specificity, and second, unwanted reactivity can be attenuated through B cell-mediated expansion of alloregenic-specific T cell clones. Notably, at a relatively high Treg-to-responder cell ratio, alloregenic-nonspecific Treg cells can also substantially inhibit responder T cells. This is probably due to the presence in the expanded population of a small number of Treg cells that have cross-reactivity toward the nonspecific alloantigens, since at low Treg-to-responder cell ratios, nonspecific Treg cells substantially lose their inhibitory function. The plasticity in Ag specificity of B cell-expanded Treg cells also suggests the possibility that the B cells used to expand Treg cells may not need to be from the organ donors or the recipients, but instead they can be derived from unrelated donors that express the target HLA haplotypes toward which Treg cell activity was needed. This, however, needs to be tested experimentally. The use of HLA haplotype-shared third-party B cells for expansion of alloregenic-specific Treg cells might constitute the only option in the setting of BMT where the recipients cannot be a source of B cells because of ongoing hematological malignancies, such as leukemia or multiple myeloma.

We studied the capacity of B cell-expanded Treg cells to inhibit the alloreactivity of responder T cells of unrelated (third-party) donor origins to explore the possibility of using third-party Treg cells for therapeutic applications. The limited availability of Treg cells from a single donor or cord blood source could constitute a major barrier to the development of Treg cell-based therapy in humans. This barrier, however, might be readily circumvented if third-party Treg cells could be used in place of donor or recipient Treg cells. At blood donation/transfusion centers nation-wide, hundreds of units of blood are routinely processed daily to remove leukocytes from whole blood through leukopheresis for preparation of packed RBC. The leukopheresis-derived leukocytes could potentially provide an unlimited source of cells for Treg cell isolation and expansion. In fact, the Treg cells used in the present study are all derived from leukopheresis performed at the blood donation center of our institution. Using these Treg cells, our data clearly demonstrate that third-party Treg cells, after expansion with allogeneic B cell donors, can suppress the alloreactivity of the
Disclosures

The authors have no financial conflicts of interest.

References