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Efficient Induction and Expansion of Human Alloantigen-Specific CD8 Regulatory T Cells from Naive Precursors by CD40-Activated B Cells

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Although recent studies have focused on CD4+ regulatory T cells (Treg), CD8+ Treg have also been reported to play important roles in the induction and maintenance of immune tolerance. Adoptive transfer of CD8+ Treg in rodents or induction of CD8+ Treg in humans can prevent or treat allograft rejection and autoimmune diseases. However, no approaches have been reported for the generation of human Ag-specific CD8+ Treg at a practical scale for clinical use. Here, we found that two novel CD8+ T cell subsets with different levels of CD8 surface expression, CD8high and CD8low, could be induced from naive CD8+ T cells in vitro by allogeneic CD40-activated B cells, whereas only CD8high T cells were alloantigen-specific Treg with relatively poor alloantigen-specific cytotoxicity. Importantly, alloantigen-specific CD8high Treg could be induced and expanded from naive CD8+CD25− T cells at a large scale after 3 wk of culture without exogenous cytokines. These induced alloantigen-specific Treg were CD45RO+ and CCR7− memory cells, and they expressed Foxp3, CD25, CD27, and CD62L. The induction and expansion of CD8high Treg by CD40-activated B cells were dependent on endogenously expressed IFN-γ, IL-2, IL-4, and CTLA-4. This approach may facilitate the clinical application of CD8+ Treg-based immunotherapy in transplantation and autoimmune diseases. The Journal of Immunology, 2009, 183: 3742–3750.

R egulatory T cells (Treg) are negative regulators of immune responses to self and foreign Ags and are critical for maintaining immune tolerance (1–3). Although extensive studies have focused on the CD4+CD25+Foxp3+ Treg in recent years, CD8+ Treg have also been reported to play important roles in neonatal tolerance (4), tolerance to allograft (5) or xenograft (6), and control of autoimmune diseases, including inflammatory bowel diseases and multiple sclerosis (7–9). Adoptive transfer of CD8+ Treg in rodents or induction of CD8+ Treg in humans have been demonstrated to suppress allograft rejection and to prevent experimental autoimmune encephalomyelitis and autoimmune diabetes (9, 10), suggesting that the endogenous enhancement of CD8+ Treg activity or their adoptive transfer has great potential for the treatment of autoimmunity, allograft rejection, or graft-vs-host disease. Since Ag-specific Treg have higher efficiency and greater specificity for the regulation without the general suppression compared with polyclonal Treg, Ag-specific rather than polyclonal Treg are particularly promising as a means of human immunotherapy (11). However, the clinical application of Treg immunotherapy is hampered by a lack of specific surface markers for Ag-specific Treg and difficulties in isolating and expanding these relatively rare cells.

Several types of adaptive CD8+ Treg have been described in human, such as Qu-1-dependent CD8+, CD8+CD28−Foxp3+, CD8+CD25−CD28Foxp3+, CD8+CD45RO+IL-10−, and CD8+CD25−Foxp3+lymphocyte activation gene-3 (LAG3)+ Treg subsets (12–14). However, the expressions of these surface markers, particularly CD25 and CD45RO, and the lack of CD28 surface expression suggest that these cells are in an “activated” or “memory” state rather than that associated with a regulatory function (15–17). Furthermore, it is still not known whether these different subsets of inducible CD8+ Treg are distinct cell populations, partially overlapping ones, or are essentially derivatives from a common precursor cell type.

Although several approaches for induction of Ag-specific CD8+ Treg have been reported, in which CD8+ T cells were cocultured with allogeneic APC or Ag-pulsed autologous APC (18–20), there is currently no reliable protocol for the ex vivo induction and large-scale expansion of human alloantigen-specific CD8+ Treg. On the other hand, staphylococcal enterotoxin B, anti-CD3 mAb with or without anti-CD28 mAb, autologous monocytes plus GM-CSF and IL-2, or IL-10 and IL-2 have been used to induce polyclonal human CD8+ Treg (21, 22) but not Ag-specific cell populations. Recently, Gilliet et al. reported that human alloantigen-specific CD8+IL-10+ Treg could be induced by allogeneic CD40-activated plasmacytoid dendritic cells (pDC). However, it is difficult to expand these CD8+IL-10+ Treg because of their poor ability to mount secondary proliferation (18).
Compared with pDC, using CD40-activated B cells for Treg induction has obvious advantages in that they can be readily expanded in vitro to relatively large numbers, retain their function after cryopreservation, and are relatively cost-effective to produce (23). Additionally, since B cells stimulated with CD40L-transfected cells or recombinant soluble CD40L (sCD40L) were equally effective at generating alloantigen-specific CD4+ Treg, the use of sCD40L may significantly improve the clinical applicability of the procedure. Taking advantage of the properties of CD40-activated B cells, we previously developed a novel protocol to induce and expand highly efficient human alloantigen-specific CD4+ Treg from naive precursors on a large scale by using allogeneic CD40-activated B cells as stimulators (24).

In this study, we found that CD40-activated B cells could also induce two T cell subsets with different levels of CD8 surface expression: CD8high or CD8low from naive CD8+ CD25- T cells. Although both CD8high and CD8low T cells had suppressive and cytolytic activities that were mediated via different mechanisms, only CD8high Treg exerted their suppressive and cytotoxic activities in an Ag-specific manner. The potency of CD8high Treg suppression was significantly higher than their cytolytic activity. These CD8high alloantigen-specific Treg exhibited memory CD45RO (for naive CD8+ T cells) and 15% of the viable suspended cells are CD19+ B cells. Ag specificity was examined in the cocultures performed with third-party stimulator PBMC that were fully class I and II HLA-mismatched (target). The purity of sorted cells was routinely >99%. The sorted CD8high/CD8mid/CD8low T cells referred to as “suppressor” were titrated with CD40-activated B cells for Treg induction experiments. In the repeated stimulation experiments, the cell-cell contact dependency of CD8high T cells function was examined by using a Transwell culture system. Briefly, 2 × 105 autologous CD4+ CD25- T cells and allogeneic CD40-activated B cells were cultured in 96-well plates at different effector to target (E:T) ratios and added at the start of MLR coculture consisting of a total of 5 × 104 responder CD4+ CD25+ T cells from the same donor of CD8+ T cells and 5 × 104 γ-irradiated target PBMC from the same donor of allogeneic B cells. Ag specificity was examined in the cocultures performed with third-party stimulator PBMC that were fully class I and II HLA-mismatched with the (target) allogeneic B cells. Proliferation was analyzed by [3H] thymidine incorporation assay as described previously (27, 28). The [3H] thymidine incorporation was expressed as the mean ± SEM counts per 1 min of four to six measurements.

Materials and Methods

Generation of CD40-activated B cells

Human peripheral blood was obtained from healthy donors in accordance with local ethical committee approval. B cells from PBMC were stimulated via CD40 using NIH3T3 cells transfected with the human CD40L (tCD40L) cells as described previously (24). Briefly, PBMC were cocultured with the lethally irradiated (96 Gy) tCD40L cells in the presence of IL-4 (2 ng/ml; R&D Systems) and cyclosporine A (5.5 × 10⁻³ μM) in IMDM (Invitrogen) supplemented with 10% heat-inactivated human AB serum, 50 μg/ml transferrin (Boehringer Mannheim), 5 μg/ml insulin (Sigma-Aldrich), and 15 μg/ml gentamicin (Invitrogen) at 37°C in 5% CO₂. After 14 days of coculture, >95% of the viable suspended cells are CD19+ B cells. The purity of CD19+ B cells was cryopreserved for future use. For coculture with CD4 T cells, CD40-activated B cells were always Ficol-density centrifuged followed by washing with PBS twice to remove nonviable cells, including remaining tCD40L cells.

T cell isolation

Human naive CD8+ and CD4+ CD25- T cells were isolated from healthy donor PBMC by negative selection using a naive CD8+ T cell isolation kit (Miltenyi Biotec) for depletion of CD4, CD14, CD16, CD19, CD36, CD56, CD123, TCRγδ, CD235a, and CD45RO (for naive CD8+ T cells) or depletion of CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCRγδ, and CD235a (for CD4+ T cells). The CD25+ cells were further depleted by positive selection with directly conjugated anti-CD25 magnetic microbeads (Miltenyi Biotec) following double-column depletion procedures. After depletion of CD25+ cells, the purity of CD8+ CD45RA+ CD45RO+ CD25- cells were routinely >99% as determined by flow cytometric analysis.

Allogeneic stimulation assay to induce and expand Treg

Freshly purified CD8+ CD45RA+ CD45RO+ CD25- T cells were cocultured with allogeneic CD40-activated B cells at a T:B cell ratio of 10:1 in RPMI 1640 medium with 10% heat-inactivated human AB serum. For some experiments, T cells were labeled with CFSE before the coculture with CD40-activated B cells. In the repeated stimulation experiments, the allogeneic CD40-activated B cells were added every 7 days of culture. Functional and phenotypic hallmarks of the induced and expanded T cells were examined at indicated times. The expansion of the cells was determined by counting trypan blue negative aliquots.

Flow cytometric analysis

The phenotypes of cells were analyzed by a FACSaria. The following fluorescence-conjugated mAbs were used: anti-CD4-Alexa 405, anti-CD8-PE-Cy7, anti-CD45RA-PE, anti-CD45RO-allophycocyanin (Caltag Laboratories), and anti-CD25-allophycocyanin, anti-CD27PE-allophycocyanin, anti-CD27-PE (BD Biosciences), and their isotype-matched control Abs of irrelevant specificity were purchased from BD Biosciences. Intracellular staining was performed after cell fixation and permeabilization as we described before (25, 26), and the following mAbs were used: anti-glucocorticoid-induced TNF receptor (GITR)-PE, anti-IL-10-PE (R&D Systems), anti-TGF-β-PE (IQ Products), anti-CTLA-4-PE, anti-perforin-PE, anti-granzyme A-PE, anti-granzyme B-FITC, anti-IFN-γ-FITC, and anti-TNF-α-FITC (BD Biosciences). For Foxp3 staining, the human Foxp3 staining kit (eBioscience) was used as we described before (24).

MLR assay

The suppressor capacity of allogeneic CD40-activated B cell-induced and -expanded cells was studied in an MLR coculture suppression assay as we described before with some modifications (24). CD8high/CD8mid/CD8low T cells were sorted by FACSaria after 9, 12, or 21 days of coculture of CD8+ CD45RA+ CD25- T cells and allogeneic CD40-activated B cells (target). The purity of sorted cells was routinely >99%. The sorted CD8high/CD8mid/CD8low T cells referred to as “suppressor” were titrated and added at the start of MLR coculture consisting of a total of 5 × 10⁴ responder CD4+ CD25+ T cells from the same donor of CD8+ T cells and 5 × 10⁴ γ-irradiated target PBMC from the same donor of allogeneic B cells. Ag specificity was examined in the cocultures performed with third-party stimulator PBMC that were fully class I and II HLA-mismatched with the (target) allogeneic B cells. Proliferation was analyzed by [3H] thymidine incorporation assay as described previously (27, 28). The [3H] thymidine incorporation was expressed as the mean ± SEM counts per 1 min of four to six measurements.

Cytotoxicity assay

Cytotoxic capacity of the induced and expanded cells was determined by the Live/Dead cell-mediated cytotoxicity kit (Molecular Probes) (24). Similar coculture system as described in “MLR assay” was set except that PBMC were not irradiated but labeled with 3,3′-diodotyrosine-L-tyrosine perchlorate (DiO), while responder CD4+ CD25- T cells were labeled with anti-CD4-Alexa 405. After 6 h of culture with propidium iodide, cells were analyzed by flow cytometry. Back gating on the green (DiO-PBMC) and blue (Alexa 405-CDS4) fluorescent target cells, the propidium iodide-negative cells were evaluated for the percentage of dead cells.

Transwell assay

The cell-cell contact dependency of CD8high T cell function was examined by using a Transwell culture system. Briefly, 2 × 10⁵ autologous CD4+ CD25- T cells were cocultured with 2 × 10⁵ allogeneic PBMC (for cytotoxicity) or 2 × 10⁵ γ-irradiated allogeneic PBMC (for MLR) in the lower compartment of the cell culture wells while 4 × 10⁵ CD8high T cells (for cytotoxicity) or 2 × 10⁵ CD8high T cells (for MLR) were cultured in the Transwell inserts (0.4 μm pore size; Millicell, Millipore). On hour 6 (for cytotoxicity) or day 3 (for MLR) after coculture, the percentage of dead targets or proliferation of autologous CD4+ CD25- cells was measured as described above. Moreover, to discriminate the cell-cell contact dependency of recognition from that of execution stages, we replaced intact intact CD8high T cells with activated ones, which were sorted from the coculture with autologous CD4+ CD25- cells and allogeneic PBMC 2–4 h after coculture.

Blocking assay

Blocking studies were performed in the presence of the neutralization mAbs against CTLA-4 (1 μg/ml; Ancell), IL-10 (1 μg/ml; eBioscience), GITR (2 μg/ml; R&D Systems), TGFB-β (0.5 μg/ml; R&D), IFN-γ (2 μg/ml; R&D Systems), TNF-α (2 μg/ml; R&D Systems), Fasl. (10 μg/ml; R&D Systems), IL-2 (1 μg/ml; eBioscience), IL-4 (2 μg/ml; eBioscience), or their irrelevant isotype controls. The blocking perforin and granzyme assay was performed with concanamycin A (CMA) (10 μg/ml; Sigma-Aldrich) and Bcl-2 (2 μg/ml; R&D Systems).

Statistical analysis

Graphs and statistical analyses were performed with Prism 5.00 for Windows software (GraphPad Software). Values of p < 0.05 or less were considered significant.
Results

Novel CD8 Treg subsets with different levels of CD8 surface expression are induced by CD40-activated B cells

Purified naive (CD45RA+CD45RO−) CD8+CD25+ T cells (purity >99%) were cocultured with allogeneic CD40-activated B cells without exogenous cytokines for 12 days. Interestingly, a new subset with substantially higher levels of CD8 surface expression (CD8high) compared with those basally was induced after 9 days of allostimulation (Fig. 1A). Another new subset with significantly decreased levels of CD8 surface expression (CD8low) was induced after 12 days of allostimulation (Fig. 1A). Accompanying these changes of CD8 expression, CD25 and Foxp3 expressions were also significantly up-regulated in alloantigen-stimulated CD8high T cells, whereas alloantigen-stimulated CD8low T cells had only modest levels of CD25 and Foxp3 expression (Fig. 1A). Most CD8high and CD8low T cells had undergone seven or eight cell divisions by 12 days of allostimulation (Fig. 1A). In contrast, most T cells that retained basal levels of CD8 expression (hereafter referred to as CD8mid T cells) had not undergone mitosis.

To determine the origin of CD8low T cells, the CD8high and CD8mid T cells after 9 days of allostimulation were sorted by FACS and cocultured with the original CD40-activated B cells for another 3 days. Interestingly, CD8low T cells were induced from CD8high rather than from CD8mid T cells after stimulation with CD40-activated B cells (supplemental Fig. S1). We also determined the secondary proliferative capacities of these CD8 subsets after sorting on day 12 of allostimulation and found that CD8high and CD8low T cells had significantly higher proliferative capacities compared with CD8mid T cells, and CD8high T cells had the highest proliferative capacity (supplemental Fig. S2). CFSE staining showed that a major fraction of CD8low T cells (>60%) experienced seven or eight divisions, whereas only 36% of CD8high T cells underwent seven or eight divisions (Fig. 1A), suggesting that most CD8low T cells might be converted directly from CD8high T cells. Taken together, our results suggest that CD8low T cells may be mainly derived from CD8high T cells and this differentiation is accompanied by mitosis.

Only CD8high T cells are alloantigen-specific Treg

To determine whether the CD8+ T cell subsets induced by CD40-activated B cells are suppressive, the MLR assay was used. As shown in Fig. 1B, CD8high, CD8mid, and CD8low T cells generated by 12 days of allostimulation and sorted by FACS were added to autologous CD4+CD25− T cells (responder cells) and allogeneic irradiated PBMC (stimulator cells) in the MLR system. Both CD8mid and CD8low T cells inhibited the proliferation of responder T cells in response to allogeneic stimulator cells derived from the same donor used for CD40-activated B cells, hereafter referred to as the Treg priming donor. However, both subsets also inhibited the MLR proliferation in which third-party allogeneic PBMC were used, although the suppressive effect of CD8mid T cells in this context was lower than that of CD8low T cells. In contrast, CD8high T cells significantly inhibited the proliferation of responder T cells induced by allogeneic PBMC from the Treg priming donor, but had little effect on third-party-mediated allogenec stimulation of the responder T cells (Fig. 1B). Importantly, these CD8high T cells had potent suppressive potential: even at a ratio as low as 1:512 for CD8high T cells to the autologous responder T cells (CD4+CD25−), there was an obvious suppression (Fig. 1B). At

The online version of this article contains supplemental material.
Characteristics of CD8+ T cell subsets induced by CD40-activated B cells

We further characterized the phenotype of the induced CD8+ T cell subsets. Compared with naive CD8+ T cells, >95% of induced CD8high and nearly 90% of CD8low T cells expressed CD45RO, while only 10% of CD8mid cells did. In contrast, all three CD8+ T cell subsets were CCR7+. CD8high and CD8mid cells retained CD27, CD28, and CD62L expression at levels similar to those of freshly isolated naive CD8+ T cells, whereas most of CD8low generated after 12 days of allostimulation were CD28− and CD62L−, and only ~45% of these cells were CD27+ (Fig. 2A).

We next examined the expression of molecules previously implicated in the suppression of Treg, including CTLA-4, GITR, IL-10, and TGF-β by intracellular staining. Fig. 2B shows that significantly higher frequencies (~10–20%) of CD8high Treg expressed CTLA-4, GITR, IL-10, and TGF-β compared with only ~3–8% of CD8low T cells. In contrast, CD8mid T cells only expressed minimal amounts of these four molecules.

As cytolytic activity has been proposed to mediate regulatory T cell function in some contexts (9, 29), we determined the expression of perforin, granzymes A and B, IFN-γ, and TNF-α by CD8+ T cells following 12 days of allostimulation. A relatively high proportion (17–22%) of CD8high Treg expressed cytolytic granules (perforin, granzymes A and B) compared with only ~7–13% of CD8low T cells. In contrast, the levels of expression of IFN-γ and TNF-α in CD8high and CD8low T cells were similar. CD8mid T cells had minimal to undetectable levels of these molecules (Fig. 2B).

The alloantigen-specific suppression of CD8high Treg is cell-cell contact dependent and requires alloantigen-specific stimulation

To understand the mechanisms of CD8high Treg suppression, we determined whether CD8high Treg-mediated suppression requires cell-cell contact. Suppression was completely lost when the responder T cells and stimulator allogeneic PBMC were physically separated from the CD8high Treg in a Transwell culture system (Fig. 3A). Once CD8high Treg were activated by stimulator allogeneic PBMC (act CD8high) their suppression to the responder T cells was partially reversed, even when they were separated from the responder T cells by Transwell. Taken together, these data suggest that the suppression mediated by CD8high Treg is dependent on cell-cell contact, requires alloantigen-specific stimulation, and soluble molecules released from activated CD8high Treg contribute, in part, to their suppression.

The alloantigen-specific suppression of CD8high Treg partially relies on IL-10, TGF-β, GITR, and CTLA-4 expression, but not on cell-mediated cytotoxicity

To determine which molecules contributed to the alloantigen-specific suppression mediated by CD8high Treg, we added neutralization mAbs against IL-10, TGF-β, CTLA-4, and GITR into the culture of MLR. Blockade of cytokines partially inhibited the ability of CD8high Treg to suppress the MLR, with Abs against IL-10, TGF-β, GITR, and CTLA-4 reversing ~60, 26, 30, and 42% of the suppression, respectively (Fig. 3B). These data indicate that all of the above cytokines contribute to the suppression mediated by CD8high Treg.

To investigate whether cytotoxicity was involved in the suppression mediated by CD8high Treg, we first examined their cytotoxic activity in the MLR culture system. As shown in Fig. 3C, CD8high Treg had no cytotoxic activity at the E:T ratio of 1:1. Furthermore, the addition of the perforin inactivator CMA, the granymes A and B inhibitor Bcl-2, or neutralizing mAbs against FasL (CD95L), IFN-γ, and TNF-α into the culture of MLR did not reverse CD8high Treg suppression in this context (Fig. 3D). These results suggest that the suppression of CD8high Treg is not dependent on their cytotoxicity, at least at a 1:1 ratio of Treg to target cells.

CD8high Treg have alloantigen-specific cytotoxicity at a high ratio of Treg to target cells

We next determined the capacity of alloantigen-induced CD8+ T cell populations to mediate cytotoxicity at relatively high ratios to
their targets. As shown in Fig. 4, CD8<sup>low</sup> and CD8<sup>mid</sup> T cells showed a similar level of dose-dependent cytotoxicity, and they killed both autologous CD4<sup>+</sup>CD25<sup>+</sup> T cells and PBMC from the priming allogeneic donor or a third-party donor. In contrast, CD8<sup>high</sup> Treg only killed autologous CD4<sup>+</sup>CD25<sup>+</sup> T cells and PBMC from the priming allogeneic donor at a relatively high (>5:1) ratio with respect to these target cells, but they did not exhibit cytotoxicity when PBMC came from a third party. These results indicate that CD8<sup>high</sup> Treg have alloantigen-specific cytotoxicity only at a high ratio of Treg to target cells.

**Alloantigen-specific cytotoxicity of CD8<sup>high</sup> Treg requires alloantigen stimulation and is cell-cell contact dependent**

To determine the mechanisms of cytotoxicity of CD8<sup>high</sup> Treg, sorted CD8<sup>high</sup> Treg were cocultured with autologous CD4<sup>+</sup>CD25<sup>+</sup> T cells and/or PBMC from the priming allogeneic donor at a ratio of 20:1 for CD8<sup>high</sup> Treg to target cells. Similar to the results shown in Fig. 4, CD8<sup>high</sup> Treg killed both autologous CD4<sup>+</sup>CD25<sup>+</sup> T cells and alloantigen PBMC in the coculture of MLR. However, in the absence of alloantigen PBMC in the coculture, CD8<sup>high</sup> Treg had no cytotoxic activity against autologous CD4<sup>+</sup>CD25<sup>+</sup> T cells (Fig. 5A). Additionally, the cytotoxicity of CD8<sup>high</sup> Treg against alloantigen PBMC was significantly reduced when there were no CD4<sup>+</sup>CD25<sup>+</sup> T cells in the coculture (Fig. 5A). Therefore, these data indicate that optimal alloantigen-specific cytotoxicity mediated by CD8<sup>high</sup> Treg requires alloantigen stimulation, and CD4<sup>+</sup> T cells help.

We then applied a Transwell culture system to determine whether the cytotoxicity of CD8<sup>high</sup> Treg need the direct cell-cell contract. Similar to their suppression, the cytotoxicity of CD8<sup>high</sup> Treg was almost completely lost when the autologous CD4<sup>+</sup>CD25<sup>+</sup> T cells and alloantigen PBMC were physically separated from the CD8<sup>high</sup> Treg (Fig. 5B). Once CD8<sup>high</sup> Treg were activated by alloantigen (actCD8<sup>high</sup>), they only showed a minor cytotoxic activity against CD4<sup>+</sup>CD25<sup>+</sup> T cells and alloantigen PBMC when they were separated from the target cells by Transwell (Fig. 5B). Additionally, the supernatants from the culture of the alloantigen-stimulated CD8<sup>high</sup> Treg also exerted cytotoxic activities against CD4<sup>+</sup>CD25<sup>+</sup> T cells and PBMC (supplemental Fig. S3). These data indicate that the cytotoxicity of CD8<sup>high</sup> Treg is dependent on the cell-cell contact, and the soluble molecules released from activating CD8<sup>high</sup> Treg also contribute, at least in part, to their cytotoxicity.

**Alloantigen-specific cytotoxicity of CD8<sup>high</sup> Treg is mediated by soluble molecules and the Fas/FasL pathway**

To further understand which soluble molecules and pathways are involved in the cytotoxicity of CD8<sup>high</sup> Treg, blocking assays were performed using the perforin inactivator CMA, the granymes A and B inhibitor Bcl-2, or neutralizing mAbs against FasL, IFN-γ, and TNF-α (Fig. 5C). CMA or neutralizing mAbs against FasL, TNF-α, and IFN-γ significantly inhibited the cytotoxicity of CD8<sup>+</sup> Treg, and ~70% of their cytotoxicity was blocked by a combination of CMA, Bcl-2, and neutralizing mAbs for FasL (Fig. 5C). These results suggest that soluble molecules and Fas/FasL pathway are involved in the cytotoxicity of CD8<sup>high</sup> Treg.
described in "Cytotoxicity assay". The percentage of dead cells (determined by propidium iodide staining and analyzed by FACS) from every 106 naive CD8+ T cells were added weekly. As shown in Fig. 6A, ~9.8 × 10^6 (range, 9.2–11.3 × 10^6) of CD8^high Treg could be generated from every 1 × 10^6 naive CD8+CD25^- T cells based on study of 10 healthy randomly selected adult blood donors. Furthermore, expansion of CD8^high Treg for 21 days in culture did not alter their suppressive ability and Ag specificity (Fig. 6B). Additionally, these Treg still maintained Foxp3 and CD25 expressions (data not shown). These data demonstrate that CD40-activated B cells can induce and expand alloantigen-specific CD8^high Treg on a large scale.

The induction of CD8^high alloantigen-specific Treg by CD40-activated B cells is dependent on IFN-γ, IL-4, IL-2, and CTLA-4 expressions

To determine the mechanisms underlying the induction of alloantigen-specific CD8^high Treg by CD40-activated B cells, we added the neutralizing mAbs against IFN-γ, IL-2, IL-4, IL-10, TGF-β, and CTLA-4 in the coculture of naive CD8+CD25^- T cells and allogeneic CD40-activated B cells. As shown in Fig. 7, neutralization of IFN-γ, IL-2, IL-4, or CTLA-4 significantly blocked the generation of CD8^high Treg by 9 days of culture. However, blockade of IL-10 and TGF-β did not affect the generation of CD8^high Treg. These data indicate that the induction and expansion of CD8^high Treg is mediated by IFN-γ, IL-2, IL-4, and CTLA-4.

Discussion

In this study, we describe two novel CD8+ T cell subsets induced by CD40-activated B cells with different levels of CD8 surface expression (i.e., CD8^high and CD8^low), whereas only CD8^high T cells are Ag-specific Treg. Using allogeneic CD40-activated B cells, we are able to induce and expand large numbers of highly efficient alloantigen-specific CD8^high Treg. To the best of our knowledge, this is the first report to generate human Ag-specific CD8+ Treg on a large scale. By repeated stimulation of naive CD8+CD25^- T cells with allogeneic CD40-activated B cells for 3 wk, we could generate ~9.8 × 10^6 alloantigen-specific CD8^high Treg without loss of their suppressive function from every 1 × 10^6 naive CD8+CD25^- T cells, which can typically be isolated from 10–20 ml of peripheral blood. Therefore, this is a practical protocol for the generation of relatively large numbers of human Ag-specific CD8+ Treg, which should facilitate the development of clinical immunotherapy based on the adoptive transfer of Treg. Based on their high efficiency in inhibition, even 10 ml of peripheral blood from donors could be enough for induction of enough CD8^high Treg for a single dose of transplantation.

Different from the previously reported CD8+ T cells induced by allogeneic CD40L-activated monocyte-derived DC (DC1) or pDC in which DC1-induced CD8+ T cells had potent cytotoxicity and secondary proliferation, as well as low suppressive ability to allogeneic targets, whereas pDC-induced CD8+ Treg had poor cytotoxicity and secondary proliferation and highly Ag-specific suppressive ability to allogeneic targets (18). The CD8^high and CD8^low T cells induced by allogeneic CD40-activated B cells had unique characteristics in terms of their capacities of secondary proliferation, suppression, and cytotoxicity. Both CD8^high and CD8^low T cells induced by allogeneic CD40-activated B cells had suppressive and cytolytic capacities, whereas only CD8^high Treg exerted their suppressive and cytotoxicity in an Ag-specific manner. In contrast, the CD8^high Treg expressed high levels of CD25 and Foxp3, whereas the CD8^low Treg only expressed medium levels of these common Treg molecules (Fig. 1). These data suggest that CD8^high might be a marker for alloantigen-specific CD8+ Treg. Importantly, the alloantigen-specific CD8^high Treg had highly secondary proliferative capacity, and thereby they are easy to be expanded on a large scale (supplemental Fig. S2 and Fig. 6).

The reasons underlying the marked difference in Ag specificity between CD8^high and CD8^low T cells are still unclear. One plausible explanation is that these two subsets represent different stages of CD8+ T cell differentiation: CD8^high Treg may represent memory cells, which require specific Ag recognition to exert their function, whereas CD8^low T cells may represent effector cells, which can directly exert their function without further Ag recognition. Indeed, the fact that CD8^low T cells come from CD8^high Treg (supplemental Fig. S1) also supports this speculation.

The alloantigen-specific CD8^high Treg also showed unique phenotypes. Although both CD8^high and CD8^low Treg generated in our system were CD45RO+ and CCR7^- memory/effector cells, only
alloantigen-specific CD8<sup>high</sup> Treg expressed high levels of lymph node homing receptor CD62L (Fig. 2A), which is different from the induced CD8<sup>high</sup> Treg reported by others (30–32). The expression of CD62L suggests that CD8<sup>high</sup> Treg might potentially be useful for migrating to peripheral lymphoid tissues draining graft sites to suppress T cell-mediated allograft rejection and graft-vs-host disease (24, 33, 34). Differing from previous reports that co-stimulatory receptor CD27 could be used to discriminate the functional CD4<sup>+</sup> Treg and non-Treg (35), there was no difference about the CD27 expression between CD8<sup>high</sup> Treg and CD8<sup>mid</sup> cells in our system. Another costimulatory receptor, CD28, was reported to be lost in the induced CD8<sup>high</sup> Treg (36). However, CD8<sup>high</sup> Treg induced in our system expressed CD28, but CD8<sup>low</sup> Treg did not. CD28 signaling was reported to be essential for T cell proliferation and survival (37). Therefore, the expression of CD28 on CD8<sup>high</sup> Treg is important for Treg-based immunotherapy because it could be expanded through CD28 signaling. In fact, we also demonstrate that CD8<sup>high</sup> Treg has much higher proliferative capacity than do CD8<sup>low</sup> Treg, and it could be expanded on a large scale.

In functional analysis, we demonstrated that the alloantigen-specific CD8<sup>high</sup> Treg generated from our system could completely block the alloantigen stimulated MLR. With these Treg, the marked suppressive effects could occur with as little as ~98 suppressors (1:512 ratios) in a culture of 50,000 responding CD4<sup>+</sup>CD25<sup>+</sup> T cells and 50,000 allogeneic PBMC stimulators. These effects are more potent than those previously reported in CD8<sup>+</sup> Treg and even most CD4<sup>+</sup> Treg, in which the significant suppressions were only found at the ratio of 1:32 to 1:1 for Treg to responding cells (22, 38–42), and again they suggest the potential clinical utility of the CD8<sup>high</sup> Treg in adoptive immunotherapy. The mechanisms of suppressive function are different depending on the type of induced CD8<sup>+</sup> Treg. We demonstrate the Ag-specific suppression of CD8<sup>high</sup> Treg is cell-cell contact dependent, requires Ag-specific stimulation, and partially relies on IL-10, TGF-β, GITR, and CTLA-4 expressions, consistent with previous observations of in vitro induced human CD8<sup>high</sup> Treg in adoptive immunotherapy. The mechanisms of suppressive function are different depending on the type of induced CD8<sup>+</sup> Treg. We demonstrate the Ag-specific suppression of CD8<sup>high</sup> Treg is cell-cell contact dependent, requires Ag-specific stimulation, and partially relies on IL-10, TGF-β, GITR, and CTLA-4 expressions, consistent with previous observations of in vitro induced human CD8<sup>high</sup> Treg (43–45). Some previous studies have also suggested that the suppression of CD8<sup>+</sup> Treg may be mediated by their cytotoxicity (22). However, we found that the suppression of CD8<sup>high</sup> Treg in MLR is not dependent on their cytotoxicity at the ratio of 1:1 for Treg to target cells, which might result from the decrease in releasing of cytolytic substance and efficiency of cell-cell contact caused by the reduction of CD8<sup>high</sup> T cells in the system. These functions would help reducing the dose of CD8<sup>high</sup> Treg needed and avoiding general destruction in recipient organs caused by them.

**FIGURE 5.** Mechanisms for the cytotoxicity of CD8<sup>high</sup> alloantigen-specific Treg. A. The cytotoxicity of CD8<sup>high</sup> Treg requires alloantigen stimulation, and CD4<sup>+</sup> T cells can help their cytotoxicity. Target CD4<sup>+</sup>CD25<sup>+</sup> and PBMC were cultured separately or together, and then effector CD8<sup>high</sup> Treg were added into system as an effector-to-T cell ratio of 20:1. The percentage of dead cells is shown for 6 h of cultures. B. Cytotoxicity of CD8<sup>high</sup> Treg is cell-cell contact dependent and requires soluble molecules released from activating CD8<sup>high</sup> Treg. Autologous CD4<sup>+</sup>CD25<sup>+</sup> cells (2 × 10<sup>5</sup>) (R, responder) and 2 × 10<sup>5</sup> allogeneic PBMC (S, stimulator) were cocultured in the lower compartment of the cell culture wells, and 4 × 10<sup>5</sup> of resting or activated CD8<sup>high</sup> T cells act CD8<sup>high</sup>) were placed in the Transwell inserts. Results are shown for 6 h of cultures. C. Cytotoxicity of CD8<sup>high</sup> Treg is mediated by soluble molecules and the Fas/FasL pathway. Blocking reagents against perforin (CMA), FasL, TNF-α, IFN-γ, and granzyme (Bcl-2) were added into the cytotoxicity system, and results are shown for 6 h of cultures. The percentage of dead cells is shown. Dead cells within the CD4<sup>+</sup>CD25<sup>+</sup> cells and PBMC were determined by propidium iodide staining after gating by Alexa 405 (CD4<sup>+</sup>) and DiO (PBMC)-positive cells and analyzed by FACS as described in “Cytotoxicity assay”. Data for four different experiments are shown (n = 4). Two-tailed unpaired Student’s t tests were used for comparisons (*, p < 0.05; **, p < 0.01).
The same as for their suppressive activities, CD8\textsuperscript{high} Treg also have alloantigen-specific cytotoxicity, whereas the cytotoxicity of CD8\textsuperscript{low} T cells is Ag nonspecific. However, compared with their suppressive capacity in which even at a ratio of 1:512 for Treg to responding cells, CD8\textsuperscript{high} Treg significantly inhibited alloantigen-stimulated proliferation, the potency of CD8\textsuperscript{high} Treg cytotoxicity was relatively poor because CD8\textsuperscript{high} Treg only exert their cytotoxicity at very high ratio of Treg to target cells (1:1). Consistent with previous reports (9, 29), we found that alloantigen-specific cytotoxicity of CD8\textsuperscript{high} Treg is cell-cell contact dependent and requires alloantigen stimulation. We further demonstrated that perforin, granzymes A and B, IFN-\gamma, TNF-\alpha, and the Fas/FasL pathway mediated their cytotoxicity.

B cells as the weaker APC have been reported to expand CD4\textsuperscript{+} Treg in mice (46). Recently, we also reported that human CD40-activated B cells can induce and expand CD4\textsuperscript{+} Treg from naive CD4 T cells (24). In this study, we first described that CD40-activated B cells can induce and expand alloantigen-specific CD8\textsuperscript{high} Treg on a large scale without any exogenous cytokines. Different from other reports that TGF-\beta and IL-10 are critical for the induction of Treg (18, 47, 48), we found that the induction and expansion of CD8\textsuperscript{high} Treg by CD40-activated B cells was not mediated by TGF-\beta and IL-10. Consistent with previous reports that IL-2 is critical to maintain Treg survival, we also found that IL-2 is essential for the expansion of CD8\textsuperscript{high} Treg. Moreover, we demonstrated that the induction and expression of CD8\textsuperscript{high} Treg were also dependent on IL-4, IFN-\gamma, and CTLA-4 expressions (Fig. 7). This is the first report about the involvement of IL-4, IFN-\gamma, and CTLA-4 in the induction and expansion of Treg, although these cytokines have been reported to play important roles in Treg suppressions (49). Importantly, it was unnecessary to add any exogenous cytokines for inducing and expanding alloantigen-specific CD8\textsuperscript{high} Treg, because CD40-activated B cells can secrete substantial amounts of IL-2 (24), and CD8\textsuperscript{+} T cells can express some IFN-\gamma, CTLA-4, and IL-4 (data not shown). This lack of requirement for exogenous cytokines could significantly reduce the cost for the generation of alloantigen-specific CD8\textsuperscript{+} Treg.

In conclusion, we have developed a simple and low-cost method by using allogeneic CD40-activated B cells to induce and expand highly efficient human alloantigen-specific CD8\textsuperscript{high} Treg with both suppressive and cytolytic capacities from naive CD8\textsuperscript{+}CD25\textsuperscript{-} T cells on a large scale. This may facilitate the clinical applications of Treg-based immunotherapy for the treatment of allograft rejection, autoimmune diseases, and leukemia in the future.

**FIGURE 6.** CD8\textsuperscript{high} alloantigen-specific Treg could be continuously expanded by CD40-activated B cells on a large scale without loss of function. A, The absolute number of CD8\textsuperscript{low}, CD8\textsuperscript{mid}, and CD8\textsuperscript{high} subsets generated from 1 × 10\textsuperscript{6} naive CD8\textsuperscript{+}CD25\textsuperscript{-} T cells during 21 days of culture with replacement of CD40-activated allogeneic B cells every 7 days (n = 10). B, CD8\textsuperscript{high} alloantigen-specific Treg induced and expanded by CD40-activated B cells for 21 days remain functional. The sorted CD8\textsuperscript{high} cells were added into the MLR culture system as described in “MLR assay.” Data shown are representative of four independent experiments.

**FIGURE 7.** Induction and expansion of CD8\textsuperscript{high} Treg depended on IL-2, CTLA-4, and IFN-\gamma, but not IL-4, IL-10, and TGF-\beta. Freshly purified naive CD8\textsuperscript{+}CD25\textsuperscript{-} T cells were cocultured with CD40-activated allogeneic B cells for the indicated time. Neutralizing Abs against IL-2, IL-4, IL-10, IFN-\gamma, CTLA-4, TGF-\beta, and their relevant isotype controls were added in the coculture system with replacement of medium every 3 days. Data for four different experiments are shown (n = 4). Two-tailed unpaired Student’s t tests were used for comparing the absolute numbers of CD8\textsuperscript{high} between groups with neutralizing Abs and those with isotype control at indicated times (*, p < 0.05; **, p < 0.01).
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Disclosures

The authors have no financial conflicts of interest.

References


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