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

Jian Zheng,* Yinping Liu,* Gang Qin,* Ping-Lung Chan,* Huawei Mao,* Kwok-Tai Lam,* David B. Lewis,† Yu-Lung Lau,2* and Wenwei Tu2*

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+ precursors 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, CD28, and CD62L. The induction and expansion of CD8low 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). 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, CD28, and CD62L. The induction and expansion of CD8low 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.

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3 Abbreviations used in this paper: Treg, regulatory T cell; CMA, concanamycin A; DIO, 3,3’-diiododecylxaro-carboxylicamperphorlate; GITR, glucocoronoid-induced TNFR receptor; pDC, plasmacytoid dendritic cell.

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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 naïve 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 CD8ʰˡ⁻ˡ⁸ᵐ from naïve CD⁸⁻⁺ CD₂⁵⁻ T cells. Although both CD8ʰⁱᵍʰ and CD⁸ʰˡ⁻ˡ⁸ᵐ 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⁺) surface phenotype and also expressed Foxp3, GITR (2⁰), and CD235a (for CD4⁻⁺ T cells) or depletion of CD8, CD14, CD16, CD19, CD23, CD27, and CD28, as well as the lymph node homing receptor CD62L (L-selectin), but they lackedCCR7 expression. Importantly, CD8ʰⁱᵍʰ Treg could be expanded at a large scale, and the induction and expansion of these cells by allogeneic CD40-activated B cells were dependent on endogenously expressed IFN-y, IL-2, IL-4, and CTLA-4.

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 × 10⁻⁷ M) in IMDM (Invitrogen) supplemented with 10% heat-inactivated human AB serum, 5 μg/ml transferrin (Boehringer Mannheim), 5 μg/ml insulin (Sigma-Alrich), 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 were cryopreserved for future use. For coculture with CD⁸ T cells, CD40-activated B cells were always Ficoll-density centrifuged followed by washing with PBS twice to remove nonviable cells, including remaining tCD40L cells.

T cell isolation

Human naïve CD⁸⁺ and CD⁴⁺ CD25⁻ T cells were isolated from healthy donor PBMC by negative selection using a naïve CD⁸⁺ T cell isolation kit or a CD⁴⁺ T cell isolation kit (Miltenyi Biotec) for depletion of CD4, CD14, CD16, CD19, CD36, CD56, CD123, TCRγδ, CD235a, and CD45RO (for naïve CD⁸⁻⁺ T cells) or depletion of CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCRγδ, and CD235a (for CD⁴⁺ T cells). The CD2⁵⁻⁺ T 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⁻⁺ T cells, the purity of CD⁸⁺ CD45RA⁺ CD45RO⁺ CD2⁵⁻⁺ T 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 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-CD28-PE, anti-CCR7-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-CD27-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⁺ CD⁸ʰˡ⁻ˡ⁸ᵐ T cells were sorted by FACSaria after 9, 12, or 21 days of coculture of CD⁴⁺ CD45RA⁺ CD2⁵⁻⁺ T cells and allogeneic CD40-activated B cells (target). The purity of sorted cells was routinely >99%. The sorted CD8ʰⁱᵍʰ⁺ CD⁸ʰˡ⁻ˡ⁸ᵐ T cells referred to as “suppressor” were titrated and added at the start of MLR coculture consisting of a total of 5 × 10⁵ responder CD⁴⁺ CD2⁵⁻⁺ T cells from the same donor of CD⁸ T cells and 5 × 10⁶ γ-irradiated target PBMC from the same donor of allogeneic B cells. In this study, the 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] 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’-dioctadecyloxacarbocyanine 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-CD4) fluorescent target cells, the propidium iodide-negative positive cells were evaluated for the percentage of dead cells.

Transwell assay

The cell-cell contact dependency of CD8ʰⁱᵍʰ T cells function was examined by using a Transwell culture system. Briefly, 2 × 10⁶ 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 CD⁸ʰˡ⁻ˡ⁸ᵐ 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 PBMC with 3,3’-dioctadecyloxacarbocyanine 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-CD4) fluorescent target cells, the propidium iodide-negative positive cells were evaluated for the percentage of dead cells.

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), TGF-β (0.5 μg/ml; R&D), IFN-γ (2 μg/ml; R&D Systems), TNF-α (2 μg/ml; R&D Systems), Fast. (10 μg/ml; R&D Systems), IL-2 (1 μg/ml; eBioscience), IL-4 (2 μg/ml; eBioscience), or their isotype-matched controls. The blocking perforin and granzyme assay was performed with recombinant Cama (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 CD8low 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 CD8low 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 alloantigenic 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 allogeic 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 allogeic 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 a CD8high-to-responder T cell ratio of 1:1, priming alloantigen-stimulated proliferation was almost completely inhibited (Fig. 1B). These data demonstrate that CD8high T cells are alloantigen-specific Treg.

*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 CD8<sup>high</sup> and nearly 90% of CD8<sup>low</sup> T cells expressed CD45RO, while only 10% of CD8<sup>mid</sup> cells did. In contrast, all three CD8⁺ T cell subsets were CCR7⁺. CD8<sup>high</sup> and CD8<sup>mid</sup> cells retained CD27, CD28, and CD62L expression at levels similar to those of freshly isolated naive CD8⁺ T cells, whereas most of CD8<sup>low</sup> 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 CD8<sup>high</sup> Treg expressed CTLA-4, GITR, IL-10, and TGF-β compared with only ∼3–8% of CD8<sup>low</sup> T cells. In contrast, CD8<sup>mid</sup> 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 CD8<sup>high</sup> Treg expressed cytolytic granules (perforin, granzymes A and B) compared with only ∼7–13% of CD8<sup>low</sup> T cells. In contrast, the levels of expression of IFN-γ and TNF-α in CD8<sup>high</sup> and CD8<sup>low</sup> T cells were similar. CD8<sup>mid</sup> T cells had minimal to undetectable levels of these molecules (Fig. 2B).

The alloantigen-specific suppression of CD8<sup>high</sup> Treg is cell-cell contact dependent and requires alloantigen-specific stimulation

To understand the mechanisms of CD8<sup>high</sup> Treg suppression, we determined whether CD8<sup>high</sup> 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 CD8<sup>high</sup> Treg in a Transwell culture system (Fig. 3A). Once CD8<sup>high</sup> Treg were activated by stimulator allogeneic PBMC (act CD8<sup>high</sup>) 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 CD8<sup>high</sup> Treg is dependent on cell-cell contact, requires alloantigen-specific stimulation, and soluble molecules released from activated CD8<sup>high</sup> Treg contribute, in part, to their suppression.

The alloantigen-specific suppression of CD8<sup>high</sup> 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 CD8<sup>high</sup> 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 CD8<sup>high</sup> 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 CD8<sup>high</sup> Treg.

To investigate whether cytotoxicity was involved in the suppression mediated by CD8<sup>high</sup> Treg, we first examined their cytotoxic activity in the MLR culture system. As shown in Fig. 3C, CD8<sup>high</sup> Treg had no cytotoxic activity at the E:T ratio of 1:1. Furthermore, the addition of the perforin inactivator CMA, the granzymes 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 CD8<sup>high</sup> Treg suppression in this context (Fig. 3D). These results suggest that the suppression of CD8<sup>high</sup> Treg is not dependent on their cytotoxicity, at least at a 1:1 ratio of Treg to target cells.

CD8<sup>high</sup> 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
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 allogeneic 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 (act CD8<sup>high</sup>), they only showed a minor cytotoxic activity against CD4<sup>+</sup> CD25<sup>−</sup> T cells and allogeneic 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 granzymes A and B inhibitor Bcl-2, or neutralizing mAbs against FasL, IFN-γ, and granzyme (Bcl-2) were added into the system, and results are shown for 3 days of MLR. Data for 4 different experiments are shown (n = 4). Two-tailed unpaired Student’s t tests were used for comparisons (*, p < 0.05; **, p < 0.01).

**Figure 3.** Mechanisms for suppression of CD8<sup>high</sup> alloantigen-specific Treg. A. The alloantigen-specific suppression of CD8<sup>high</sup> Treg is cell-cell contact dependent and requires Ag-specific stimulation. Autologous CD4<sup>+</sup> CD25<sup>−</sup> cells (2 × 10<sup>5</sup>) (R, responder) and 2 × 10<sup>5</sup> γ-irradiated allogeneic PBMC (S, stimulator) were cocultured in the lower compartment of the cell culture wells, and 2 × 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. On day 3 after coculture, proliferation of CD4<sup>+</sup> and GITR, or their relevant isotype controls, were added into MLR culture system. Proliferation (y-axis) is shown for 3 days of MLR. B. The alloantigen-specific suppression of CD8<sup>high</sup> Treg partially relies on IL-10, TGF-β, CTLA-4, and GITR. Neutralizing mAbs against IL-10, TGF-β, CTLA-4, and GITR, or their relevant isotype controls, were added into MLR culture system. Proliferation (y-axis) is shown for 3 days of MLR. C and D. Suppression of CD8<sup>high</sup> Treg did not depend on cytotoxicity at low ratio of effector (E) to target (T) cells. Target cells (CD4<sup>+</sup> and GITR, or their relevant isotype controls) were added into MLR culture system. Proliferation (y-axis) is shown for 3 days of MLR. Data for 4 different experiments are shown (n = 4). Two-tailed unpaired Student’s t tests were used for comparisons (*, p < 0.05; **, p < 0.01).

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 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 granzymes A and B inhibitor Bcl-2, or neutralizing mAbs against FasL, IFN-γ, and granzyme (Bcl-2) were added into the system, and results are shown for 3 days of MLR. Data for 4 different experiments are shown (n = 4). Two-tailed unpaired Student’s t tests were used for comparisons (*, p < 0.05; **, p < 0.01).
CD8<sup>high</sup> alloantigen-specific Treg can be continuously expanded by CD40-activated B cells on a large scale without loss of function

We examined the ability of 3 wk of coculture of naïve CD8<sup>+</sup> CD25<sup>−</sup> T cells with allogeneic CD40-activated B cells to generate CD8<sup>high</sup> Treg, in which freshly generated CD40-activated B cells were added weekly. As shown in Fig. 6A, ~9.8 × 10<sup>6</sup> (range, 9.2–11.3 × 10<sup>6</sup>) of CD8<sup>high</sup> Treg could be generated from every 1 × 10<sup>6</sup> naïve CD8<sup>+</sup>CD25<sup>−</sup> T cells based on study of 10 healthy randomly selected adult blood donors. Furthermore, expansion of CD8<sup>high</sup> 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<sup>high</sup> Treg on a large scale.

The induction of CD8<sup>high</sup> 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<sup>high</sup> 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 naïve CD8<sup>+</sup>CD25<sup>−</sup> 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<sup>high</sup> Treg by 9 days of culture. However, blockade of IL-10 and TGF-β did not affect the generation of CD8<sup>high</sup> Treg. These data indicate that the induction and expansion of CD8<sup>high</sup> Treg is mediated by IFN-γ, IL-2, IL-4, and CTLA-4.

Discussion

In this study, we describe two novel CD8<sup>+</sup> T cell subsets induced by CD40-activated B cells with different levels of CD8 surface expression (i.e., CD8<sup>high</sup> and CD8<sup>low</sup>), whereas only CD8<sup>high</sup> 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<sup>high</sup> Treg. To the best of our knowledge, this is the first report to generate human Ag-specific CD8<sup>+</sup> Treg on a large scale. By repeated stimulation of naïve CD8<sup>+</sup>CD25<sup>−</sup> T cells with allogeneic CD40-activated B cells for 3 wk, we could generate ~9.8 × 10<sup>6</sup> alloantigen-specific CD8<sup>high</sup> Treg without loss of their suppressive function from every 1 × 10<sup>6</sup> naïve CD8<sup>+</sup>CD25<sup>−</sup> 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<sup>+</sup> Treg, which would 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<sup>high</sup> Treg for a single dose of transplantation.

Different from the previously reported CD8<sup>+</sup> T cells induced by allogeneic CD40L-activated monocyte-derived DC (DC1) or pDC in which DC1-induced CD8<sup>+</sup> T cells had potent cytotoxicity and secondary proliferation, as well as low suppressive ability to allogeneic targets, whereas pDC-induced CD8<sup>+</sup> Treg had poor cytotoxicity and secondary proliferation and highly Ag-specific suppressive ability to allogeneic targets (18), the CD8<sup>high</sup> and CD8<sup>low</sup> 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<sup>high</sup> and CD8<sup>low</sup> T cells induced by allogeneic CD40-activated B cells had suppressive and cytolytic capacities, whereas only CD8<sup>high</sup> Treg exerted their suppressive and cytotoxicity in an Ag-specific manner. In contrast, the CD8<sup>high</sup> Treg expressed high levels of CD25 and Foxp3, whereas the CD8<sup>low</sup> Treg only expressed medium levels of these common Treg molecules (Fig. 1). These data suggest that CD8<sup>high</sup> Treg might be a marker for alloantigen-specific CD8<sup>+</sup> Treg. Importantly, the alloantigen-specific CD8<sup>high</sup> 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<sup>high</sup> and CD8<sup>low</sup> T cells are still unclear. One plausible explanation is that these two subsets represent different stages of CD8<sup>+</sup> T cell differentiation: CD8<sup>high</sup> Treg may represent memory cells, which require specific Ag recognition to exert their function, whereas CD8<sup>low</sup> T cells may represent effector cells, which can directly exert their function without further Ag recognition. Indeed, the fact that CD8<sup>low</sup> T cells come from CD8<sup>high</sup> Treg (supplemental Fig. S1) also supports this speculation.

The alloantigen-specific CD8<sup>high</sup> Treg also showed unique phenotypes. Although both CD8<sup>high</sup> and CD8<sup>low</sup> Treg generated in our system were CD45RO<sup>+</sup> and CCR7<sup>−</sup> memory/effector cells, only...
alloantigen-specific CD8\textsuperscript{high} Treg expressed high levels of lymph node homing receptor CD62L (Fig. 2A), which is different from the induced CD8\textsuperscript{low} Treg reported by others (30–32). The expression of CD62L suggests that CD8\textsuperscript{high} 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\textsuperscript{low} Treg and non-Treg (35), there was no difference about the CD27 expression between CD8\textsuperscript{high} Treg and CD8\textsuperscript{mid} T cells in our system. Another costimulatory receptor, CD28, was reported to be lost in the induced CD8\textsuperscript{low} Treg (36). However, CD8\textsuperscript{high} Treg induced in our system expressed CD28, but CD8\textsuperscript{low} Treg did not. CD28 signaling was reported to be essential for T cell proliferation and survival (37). Therefore, the expression of CD28 on CD8\textsuperscript{high} Treg is important for Treg-based immunotherapy because it could be expanded through CD28 signaling. In fact, we also demonstrate that CD8\textsuperscript{high} Treg has much higher proliferative capacity than do CD8\textsuperscript{low} Treg, and it could be expanded on a large scale.

In functional analysis, we demonstrated that the alloantigen-specific CD8\textsuperscript{high} 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\textsuperscript{+}CD25\textsuperscript{-} T cells and 50,000 allologeneic PBMC stimulators. These effects are more potent than those previously reported in CD8\textsuperscript{+} Treg and even most CD4\textsuperscript{+} 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\textsuperscript{high} Treg in adoptive immunotherapy. The mechanisms of suppressive function are different depending on the type of induced CD8\textsuperscript{+} Treg. We demonstrate the Ag-specific suppression of CD8\textsuperscript{high} 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\textsuperscript{high} Treg in adoptive immunotherapy. The mechanisms of suppressive function are different depending on the type of induced CD8\textsuperscript{+} Treg. We demonstrate the Ag-specific suppression of CD8\textsuperscript{high} 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\textsuperscript{high} Treg in adoptive immunotherapy. Some previous studies have also suggested that the suppression of CD8\textsuperscript{+} Treg may be mediated by their cytotoxicity (22). However, we found that the suppression of CD8\textsuperscript{high} 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\textsuperscript{high} T cells in the system. These functions would help reducing the dose of CD8\textsuperscript{high} Treg needed and avoiding general destruction in recipient organs caused by them.
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 (5:1). 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.

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 (5:1). 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.

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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}CD25\textsuperscript{−} alloantigen-specific 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
Supplementary figure legend

**Supplementary Figure S1:** CD8\textsuperscript{low} T cells came from CD8\textsuperscript{high} T cells but not from CD8\textsuperscript{mid} T cells. CD8\textsuperscript{low} cells came from CD8\textsuperscript{high} alloantigen-specific Treg. Freshly purified naïve CD8\textsuperscript{+}CD25\textsuperscript{−} T cells were co-cultured with CD40-activated B cells for 9 days, then CD8\textsuperscript{high} and CD8\textsuperscript{med} subsets were sorted and co-cultured with CD40-activated B cells from either the same donor (A) or a third party (B) respectively for another 3 days. Results showed that CD8\textsuperscript{low} cells could only be induced from CD8\textsuperscript{high} but not CD8\textsuperscript{med} cells.

**Supplementary Figure S2:** Proliferation of CD8\textsuperscript{+} populations co-culturing with B cell for 3 days after sorting out on day 12. CD8\textsuperscript{high} Treg had the highest secondary proliferation. Freshly purified naïve CD8\textsuperscript{+}CD25\textsuperscript{−} T cells were co-cultured with CD40-activated allogeneic B cells for 12 days and CD8\textsuperscript{high}/CD8\textsuperscript{med}/CD8\textsuperscript{low} were sorted and co-cultured with CD40-activated allogeneic B cells respectively for another 3 days. Results showed that CD8\textsuperscript{high} and CD8\textsuperscript{low} T cells had significantly higher proliferation than CD8\textsuperscript{med} T cells, and CD8\textsuperscript{high} T cells had the highest proliferation.

**Supplementary Figure S3:** Cytotoxicity of supernatant from coculture of CD8\textsuperscript{high} and targets. The cytotoxicity of CD8\textsuperscript{high} alloantigen-specific Treg requires the soluble factors. CD8\textsuperscript{high} T cells were sorted after 12 days of allo-stimulation and co-cultured with targets CD4\textsuperscript{+}CD25\textsuperscript{−} and PBMC at the E: T ratio of 20: 1 for 4 hours. Then the supernatants of cultures were collected and added to intact target cells for another 2 hours.
Killing efficiency was determined by PI staining and analyzed by FACS as described in “Cytotoxicity assay.” Data for 4 different experiments are shown (n=4). The 2-tailed unpaired Student $t$ tests were used for comparison (*$P<0.05$. **$P<0.01$).
$\text{CD8}^{\text{low}}$ T cells came from $\text{CD8}^{\text{high}}$ T cells but not from $\text{CD8}^{\text{mid}}$ T cells.
Proliferation of CD8 populations co-culturing with B cell for 3 days after sorting out on day 12
Cytotoxicity of supernatant from coculture of CD8^{high} and targets