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LETTER TO THE EDITOR

Cytotoxic response of human regulatory T cells upon T-cell receptor-mediated activation: a matter of purity

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Regulatory T cells (Tregs) are major players in immune homeostasis, and defects in their number and/or function are associated with a broad range of immunological disorders including autoimmunity, graft-versus-host disease (GvHD), transplant rejection, chronic infections or malignant diseases.[1,2] One way of how Tregs actively damp an immune response might be direct induction of apoptosis in activated antigen-presenting cells as well as in conventional effector T cells (Teffs) by secretion of lytic granzyme- and perforin-containing granula. Despite the substantial progress made in our understanding of Treg biology, the cytotoxic potential of Tregs is still under debate. As Tregs are under intense investigation as cellular therapeutics for treatment of GvHD and graft rejection, and might also interfere with novel antitumor strategies,[3] this question is not a purely academic one but also implies major consequences for the prospective development of clinical treatment strategies involving Tregs. To shed some light on this controversial issue, we decided to analyze this question using peripheral human Tregs, as these cells are one major source for potential therapeutic applications.[1,2] As Tregs are a very rare population in human peripheral blood, it seems currently technically too challenging to isolate sufficient Treg numbers with defined antigen-specificity in a way as it can be done for—for example, virus- or tumor-specific Teffs. Therefore, in this study recombinant bispecific antibodies (bsAb) were applied for antigen-specific redirection of polyclonal human T cells as surrogate for T-cell receptor (TCR)-mediated stimulation. BsAb comprise binding sites for two different target antigens (for example, CD3 and a cell surface antigen) and can directly cross-link T cells and antigen-expressing cells. This results in a major histocompatibility complex (MHC)- and TCR-independent activation and triggering of effector mechanisms of the cross-linked immune cell.[4] T-cell-engaging bsAb have been proven to be highly effective in inducing the lytic capacity of both $CD4^+$ and $CD8^+$ Teffs *in vitro*, *in vivo* and even in clinical trials and closely resemble signal cascades induced upon binding of a TCR to its target peptide/MHC complex.[4–6]

In the current study, peripheral human Tregs were isolated in a two-step procedure to high purity (Figure 1a). First, $CD4^+CD25^+$ Tregs were enriched using magnetic bead isolation (presort population, $80 \pm 10\%$ $FOXP3^+$). In a second purification step, enriched $CD4^+CD25^+$ T cells were further processed to isolate the $CD4^+CD25^+CD127^{low}$ population by flow cytometric cell sorting (postsort population, $95 \pm 3\%$ $FOXP3^+$). The cytotoxic potential of isolated Tregs was evaluated in a standard chromium release assay in comparison to autologous $CD4^+$ or $CD8^+$ Teffs.[5] For antigen-specific redirection of T cells, tumor cells pre-decorated with the human La/SS-B antigen and a cross-linking anti-CD3-anti-La bsAb was used as recently established.[7] The results demonstrate that target cell killing of bsAb-redirected Tregs is highly dependent on the purity of the isolated cell population (Figure 1b). Whereas highly pure, sorted $CD4^+CD25^+CD127^{low}$ Tregs do not elicit any significant cytotoxic effect, Tregs isolated only on the basis of CD25 expression display a considerable killing capacity that can most likely be ascribed to contaminating $CD25^+CD127^+FOXP3^-$ Teffs. The observed killing ability of presort Tregs is as high as the specific lysis provoked by bsAb-engaged autologous $CD4^+$ Teffs. One explanation might be that contaminating $CD25^+$ Teffs within the presort Treg fraction are highly activated and potent memory T cells. Moreover, isolated $CD4^+CD25^+$ T cells were maintained over night in 300 U/ml interleukin (IL)-2 whereas conventional Teffs were cultured in medium supplemented with 50 U/ml IL-2. It is very likely that, the cytotoxic potential of contaminating $CD25^+CD127^+FOXP3^-$ Teffs in the presort Treg population was even potentiated due to pre-conditioning with elevated IL-2 concentrations.

To obtain sufficient Treg material for a clinical application, *in vitro* expansion of this rare cell population is inevitable. However, previous studies pointed out that the only population allowing for an efficient *in vitro* expansion over a long-lasting period without losing phenotypic and functional Treg characteristics is the $CD4^+CD25^+CD127^{low}CD45RA^+$ population.[8] Therefore, we expanded highly pure, sorted $CD4^+CD25^+CD127^{low}CD45RA^+$ Tregs over a 12-day expansion period and subsequently analyzed their cytotoxic potential. The purity of the expanded Treg population was confirmed by flow cytometry analysis to be on average $95 \pm 3\%$ $FOXP3^+$ cells (Figure 1c). Redirecting expanded Tregs with a bsAb does not result in tumor-cell elimination. By contrast, bsAb-redirected expanded $CD4^+$ and $CD8^+$ Teffs efficiently lyse antigen-positive target cells (Figures 1d and f). To confirm these results, we repeated the chromium release assays using another bsAb, which

Figure 1. Highly pure, freshly isolated or expanded human Tregs do not display a cytotoxic potential. (**a**) Representative example of the purity of freshly isolated cells. $CD4^+CD25^+$ Tregs were isolated by magnetic activated cell sorting technology (presort). A fraction of cells was further sorted to obtain $CD4^+CD25^+CD127^{dim}$ Tregs (postsort). Cell fractions were maintained over night in complete RPMI 1640 medium in the presence of 300 U/ml IL-2 and purity was confirmed by flow cytometry analysis. (**b**) Presort or postsort Tregs were incubated with chromium-labeled PC3 cells at a 5:1 or 10:1 ratio in the presence or absence of either the La protein or 6 pmol of the cross-linking bsAb CD3-La for 48 h. As positive control, freshly isolated autologous $CD4^+$ or $CD8^+$ Teffs were used. (**c**) Representative example of the purity of expanded T cells. Sorted $CD4^+CD25^{high}CD127^{low}CD45RA^+$ Tregs were expanded as recently established in the absence of rapamycin.[3] Isolated $CD4^+$ and $CD8^+$ Teffs were stimulated for 4 days with αCD3/CD28-coated beads in the presence of 200 U/ml recombinant IL-2. (**d**, **f**) Expanded T-cell populations were incubated with chromium-labeled PC3 cells at a 5:1 or 10:1 ratio in the presence or absence of La protein or 6 pmol of the bsAb CD3-La. After 24 h, tumor-cell lysis was determined. (**e**, **g**) Cocultivation of expanded Teffs or Tregs with $PSCA^+$ tumor cells and 6 pmol of a cross-linking CD3-PSCA bsAb. As control, cells were stimulated with conventional αCD3/CD28-coated beads at a ratio of 1:5 beads per cell. One representative donor (**d**, **e**) as well as summary of four (**g**) or five (**f**) independent donors (T cell to tumor cell ratio 10:1) is shown. Statistical significance was determined using one-way analysis of variance with Bonferroni multiple comparison test. Error bars represent mean ± s.d. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ (n.s., not significant).

targets the prostate stem cell antigen (PSCA) and mediates efficient cancer cell lysis upon cross-linkage of Teffs to PSCA$^+$ tumor cells.[5] Again, Tregs are not capable of eliminating target cells (Figures 1e and g), proving that the lack of cytotoxicity is independent of the chosen target antigen or bsAb. Next, we wanted to exclude that an additional costimulatory signal is

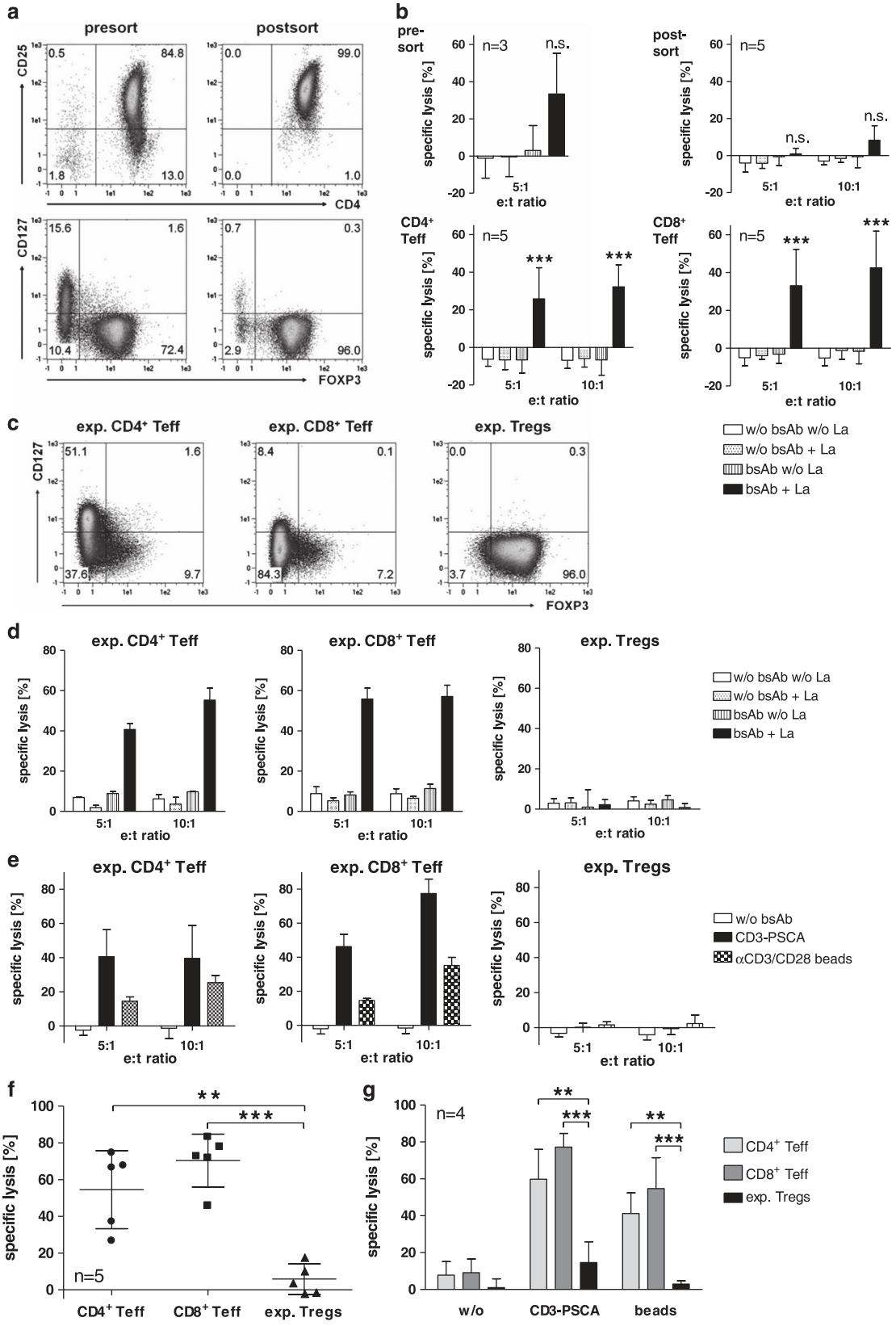

Figure 1. For caption see page 1.

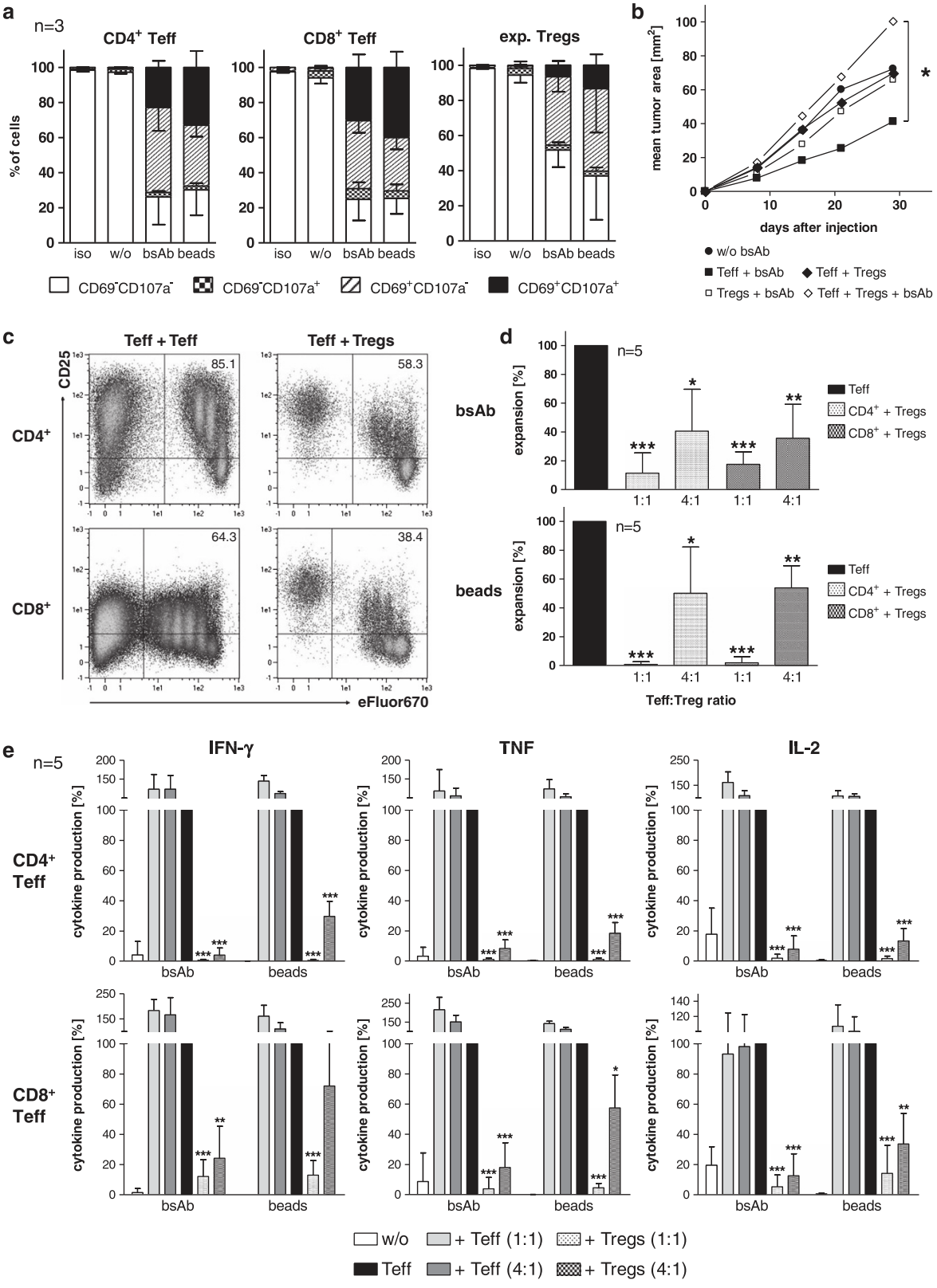

Figure 2. For caption see page 4.

necessary to trigger the cytotoxic potential of Tregs. Therefore, we used conventional αCD3/CD28-coated beads for T-cell activation. However, as opposed to autologous CD4$^+$ and CD8$^+$ Teffs, Tregs do not kill cocultured tumor cells even upon polyclonal bead stimulation (Figures 1e and g).

To determine whether Tregs release cytotoxic molecules including granzymes and perforin upon activation, we stimulated T cells either with conventional αCD3/CD28-coated beads or with bsAb in the presence of target cells and assessed expression of the activation marker CD69 and degranulation marker CD107a.

Figure 2. Highly pure, expanded Tregs are not cytotoxic *in vivo* and display a potent suppressive capacity. (**a**) Expanded T cells were cocultured together with PC3 cells either without or with 6 pmol of the CD3-La bsAb. Polyclonal stimulation with αCD3/CD28-coated beads was included as positive control. After 20 h, cells were harvested and stained for CD69 and CD107a. Relative percentage of CD69/CD107a double-positive, CD69 or CD107a single-positive and double-negative cells were summarized for three different donors. (**b**) NMRI$^{nu/nu}$ mice were injected subcutaneously with PC3-PSCA cells. Animals received either CD4$^+$ Teffs or expanded Tregs alone with a CD3xPSCA bsAb or a mixture of both T-cell populations with or without the cross-linking bsAb. Tumor size was measured weekly. The *in vivo* experiment was performed in parallel with previously presented work.[3] Statistical significance was determined using one-way analysis of variance with Bonferroni multiple comparison test. (**c**–**e**) eFluor670 proliferation dye-labeled CD4$^+$ or CD8$^+$ Teffs (5×10^4) were cultured in the presence of La-decorated target cells and 6 pmol bsAb CD3-La together with either 5×10^4 (1:1) or 12.5×10^4 (4:1) unlabeled autologous Teffs or expanded Tregs. (**c**) CD25 surface expression and dilution of proliferation dye was assessed after 96 h. Numbers in upper right quadrant refer to percentage of CD25$^+$ Teffs related to total eFluor670-labeled cells. One representative donor out of five independently performed experiments is depicted. (**d**) Absolute cell number of bsAb- or bead-activated, eFluor670-labeled CD4$^+$ or CD8$^+$ Teffs was measured using a MACSQuant Analyzer (Miltenyi Biotec, Bergisch-Gladbach, Germany) at days 0 and 4. Overall expansion in the presence of unlabeled Teffs was determined, equalized to 100% and relative expansion in the presence of Tregs was calculated. (**e**) Culture supernatants were collected after 48 h and analyzed by enzyme-linked immunosorbent assay. Secreted interferon (IFN)-γ, tumor necrosis factor (TNF) and IL-2 by 5×10^4 bsAb- or bead-activated CD4$^+$ or CD8$^+$ Teffs were set to 100% (black bars) and relative cytokine levels detected in the supernatant in cocultures with autologous Teffs (gray bars) or Tregs (patterned bars) at indicated ratios were calculated. (**d**, **e**) Five independent donors were summarized and statistical significance was determined using one-sample *t*-test. Error bars represent mean ± s.d. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Analysis of three different donors (Figure 2a) revealed that although Tregs become activated (45.5 ± 9.7% and 60.3 ± 26.6% CD69$^+$ cells with bsAb and bead activation, respectively), they only marginally upregulate the degranulation marker CD107a (9.4 ± 1.4% and 16.1 ± 7.0% upon stimulation with bsAb or beads, respectively) in accordance with published results.[9] This suggests that Tregs are not capable of directly inducing apoptosis via release of cytotoxic granula. However, we cannot rule out the possibility that apoptosis in Teffs is induced indirectly for example, via cytokine deprivation. In order to verify our results *in vivo*, athymic nude mice were inoculated with PSCA$^+$ tumor cells and a cross-linking CD3xPSCA bsAb as well as either one of the two T-cell populations or a mixture of both CD4$^+$ Teffs and Tregs. As shown in Figure 2b, injection of expanded Tregs in combination with tumor cells and bsAb did not reduce tumor outgrowth in mice, substantiating that Tregs are not capable of eliminating co-injected cancer cells. Nevertheless, despite the lack of cytotoxic activity bsAb-redirected Tregs hold a great suppressive potential. They are efficiently diminishing CD25 upregulation, proliferation and overall expansion of cocultured autologous CD4$^+$ and CD8$^+$ Teffs (Figures 2c and d). Moreover, bsAb-engaged Tregs substantially inhibit interferon-γ, tumor necrosis factor and IL-2 cytokine release of autologous Teffs (Figure 2e). The suppressive potency of bsAb-redirected Tregs was further confirmed *in vivo*, as Treg administration rather suppressed the antitumor effect of co-injected CD4$^+$ Teffs and significantly enhanced tumor growth (Figure 2b). The same observation holds true for Tregs antigen-specifically engineered with chimeric immune receptors, which instead of contributing to tumor-cell killing efficiently suppress the antitumor reaction of Teffs *in vivo*.[10]

Taken together, our data provide strong evidence that human CD4$^+$CD25$^+$CD127^{low} T cells harbor no considerable cytotoxic potential, neither freshly isolated nor after *in vitro* expansion and prolonged culture, but have substantial suppressive capacity and therefore fulfill the criteria to be indeed Tregs. Likewise, human CD4$^+$CD25$^+$CD127^{low} Tregs isolated from synovial fluid of juvenile idiopathic arthritis patients were able to efficiently inhibit Teff activation, but no elevated apoptosis rate of Teffs was observed in cocultures.[11] As shown by others, Tregs do not have to rely on active apoptosis induction in Teffs by release of cytotoxic granula as other suppressive mechanisms known to be utilized by Tregs such as cytokine deprivation, IL-10 secretion or presentation of cell surface-bound TGF-β are sufficient to suppress Teff activation.[11–13] On the other hand, we demonstrate that human T cells isolated on the basis of surface markers CD4$^+$CD25$^+$ exhibit considerable cellular cytotoxicity, most likely due to contamination with CD25$^+$-activated CD4$^+$ Teffs. Our observation might explain previous reports on cellular cytotoxicity of human Tregs, which were based either on isolated CD4$^+$CD25$^+$ T-cell populations or *ex vivo*-induced Treg-like populations.[14,15] Interestingly, recent observations from a transgenic mouse model allowing discrimination between peripherally derived 'induced' Tregs (pTregs) and thymus-derived Tregs (tTregs) indicate that, pTregs, but not tTregs, express granzyme B and therefore are capable of mediating cytotoxic effector mechanisms (Kretschmer, personal communication). One can speculate that CD4$^+$CD25$^+$CD127^{low} Tregs in human peripheral blood represent the human analog to tTregs in mice. Human pTregs might be hidden in the remaining CD4$^+$CD25$^+$ T-cell population, but so far they have not been distinguished from activated CD25-upregulating CD4$^+$ Teffs.

Collectively, at present the CD4$^+$CD25$^+$CD127^{low}CD45RA$^+$ population is the Treg subset of choice for clinical use as these cells have the highest capacity to maintain FOXP3 expression and preserve their suppressive capacity even after prolonged *in vitro* cultivation.[8] In this article, we clearly demonstrate that this population does not have any cytotoxic capacity upon CD3 triggering via conventional activator beads or recombinant antibody derivatives. Our findings indicate that the risk of harming recognized target cells by bsAb-redirected CD4$^+$CD25$^+$CD127^{low} human Tregs is negligible. This should encourage attempts to use bsAb-mediated Treg activation within inflamed tissues for the treatment of autoimmunity, transplant rejection or GvHD.

CONFLICT OF INTEREST

MB and GE hold patents related to the antibodies directed to La and PSCA. They both have founded the start-up company GEMoaB. The remaining authors declare no conflict of interest.

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