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Retargeting of Human Regulatory T Cells by Single-Chain Bispecific Antibodies

Stefanie Koristka,* Marc Cartellieri,* Anke Theil,† Anja Feldmann,* Claudia Arndt,* Slava Stamova,* Irene Michalk,* Katrin Töpfer,‡ Achim Temme,‡ Karsten Kretschmer,† Martin Bornhäuser,§ Gerhard Ehninger,§ Marc Schmitz,§‡† and Michael Bachmann*‡†

Bispecific Abs hold great potential for immunotherapy of malignant diseases. Because the first components of this new drug class are now entering clinical trials, all aspects of their mode of action should be well understood. Several studies proved that CD8⁺ and CD4⁺ effector T cells can be successfully redirected and activated against tumor cells by bispecific Abs both in vitro and in vivo. To our knowledge, this study provides the first evidence that bispecific Abs can also redirect and activate regulatory T cells against a surface Ag, independently of their TCR specificity. After cross-linking, via a bispecific Ab, redirected regulatory T cells upregulate the activation markers CD69 and CD25, as well as regulatory T cell-associated markers, like CTLA-4 and FOXP3. The activated regulatory T cells secrete the immunosuppressive cytokine IL-10, but, in contrast to CD8⁺ and CD4⁺ effector T cells, almost no inflammatory cytokines. In addition, the redirected regulatory T cells are able to suppress effector functions of activated autologous CD4⁺ T cells both in vitro and in vivo. Therefore, the potential risk for activation of regulatory T cells should be taken into consideration when bispecific Abs are applied for the treatment of malignant diseases. In contrast, an Ag/tissue-specific redirection of regulatory T cells with bispecific Abs holds great potential for the treatment of autoimmune diseases and graft rejection. The Journal of Immunology, 2012, 188: 1551–1558.

The development of single-chain bispecific Abs for retargeting of polyclonal T cells has opened the window for a new therapeutic strategy for the treatment of malignant diseases (e.g., Ref. 1). In general, a single-chain bispecific Ab molecule is composed of the variable L and H chains of two distinct mAbs with different Ag specificities linked via flexible peptide chains. Thereby, these molecules can interact simultaneously with two different Ags. Typically, they are directed, on the one hand, to the CD3 complex of T cells and, on the other hand, to a surface Ag on a target cell (e.g., a tumor-associated Ag). By cross-linking T cell and target cell, an activation signal is transmitted to the lymphocyte, which, in turn, triggers its effector functions and eventually leads to the specific elimination of the recognized target cell. Thus, polyclonal T cells are activated independently of their TCR specificity. Bispecific Abs have been successfully applied for retargeting of CD4⁺ and CD8⁺ effector T cells against tumors in vitro and in mice studies (e.g., 2–5). Moreover, the first clinical trials have proven the feasibility and efficacy of this strategy for tumor treatment (6). However, we are not aware of any publication answering the question about whether regulatory T cells (Tregs) can also be redirected and activated with these new therapeutic molecules.

Tregs play a key role in immune homeostasis by establishing and maintaining peripheral tolerance to self-Ags, as well as by controlling the magnitude of immune responses to foreign Ags (7, 8). Nevertheless, in the context of malignant disorders, Tregs may have deleterious effects by suppressing antitumor responses and promoting tumor progression (9, 10). In fact, many studies revealed that T-regulated immunosuppression is one of the main tumor immune-evasion mechanisms. Increased numbers of Tregs have been reported in tumor-bearing patients, and their accumulation correlates with poor survival prognosis (11–13). Consequently, within the scope of tumor therapy with bispecific Abs, the activation of Tregs in the tumor microenvironment is unfavorable and may have to be circumvented.

In contrast, given the fact that Tregs have many beneficial effects (e.g., in preventing autoimmunity and transplant rejection and limiting chronic inflammatory diseases), an Ag/tissue-specific retargeting of these cells via bispecific Abs may open novel therapeutic approaches concerning the aforementioned immunologic disorders. Ongoing efforts for the treatment of autoimmunity and graft-versus-host disease are based on the ex vivo expansion of polyclonal Treg populations and their adoptive transfer back into the patient. This approach has proved successful in preventing or attenuating inflammatory and autoimmune diseases in various animal models (14–16). However, with regard to the transfer of polyclonal Tregs into patients, one has to face the risk for unfavorable pan-immunosuppression. Moreover, experiments in mice showed that Ag-specific Tregs are far more efficient than polyclonal populations (17–19). In light of these arguments, bispecific Abs could provide a promising tool for a target-specific redirection of Tregs in settings like transplantation or autoimmune diseases. The application of polyclonal Tregs redirected in a target-specific manner might lower the risk for unwanted side effects,
likeliness risk for infections and cancers caused by a systemic immunosuppression.

Over the past years, we successfully developed a series of recombinant bispecific Abs for redirecting immune effector cells against tumor cells (e.g., Refs. 4, 5). Performing binding studies to test the stability of these constructs at 37˚C, we noticed that the bispecific Abs bound homogeneously to all CD4+ T cells in culture, and no second peak could be observed (Supplemental Fig. 1). These results prompted us to investigate whether singe-chain bispecific Abs also bind to and, consequently, activate CD4+ Tregs.

To our knowledge, we provide the first experimental evidence that both freshly isolated and in vitro-expanded human CD4+ CD25+ Tregs can be efficiently activated via bispecific Abs in a target-specific, but TCR-independent, manner. We demonstrate that incubation of freshly isolated Tregs with a bispecific construct in the presence of the respective target Ag leads to the upregulation of CD69, CD25, CTLA-4, and FOXP3, as well as the secretion of the immunosuppressive cytokine IL-10. Furthermore, bispecific Ab-activated Tregs are hyporesponsive and proliferate only in the presence of high doses of IL-2. Importantly, the redirected Tregs are able to suppress the proliferation and cytokine production of autologous CD4+ effector T cells both in vitro and in vivo.

Materials and Methods

Cell lines

The human embryonic kidney (HEK) cell line 293T was transduced with the reading frames of the single-chain bispecific diabodies (scBsDbs) CD3xPSCA and CD3xE7B6 and used for stable expression of both bispecific Abs (4). The prostate cancer cell line PC3 was genetically modified for stable expression of the prostate stem cell Ag (PSCA), as also recently described (4), and used as PSCA+ target cells for the redirection of Tregs.

Transductions were performed, as previously described, using a retroviral system based on Moloney murine leukemia virus (20). The modified cell line PC3xPSCA was maintained in complete RPMI 1640 medium (Biochrom, Berlin, Germany), supplemented with 10% FCS, 100 µg/ml penicillin/streptomycin, 1% nonessential amino acids, 2 mM N-acetyl-l-alanyl-l-glutamine, and 1 mM sodium pyruvate (Biochrom). Bispecific Ab-secreting HEK293T cells were grown in DMEM (Invitrogen, Karlsruhe, Germany), supplemented with 10% FCS and 100 µg/ml penicillin/streptomycin. The original cell lines were purchased from the American Type Culture Collection. All cell lines were cultured at 37˚C in a humidified atmosphere of 5% CO2.

Production and purification of soluble scBsDbs

The scBsDbs used in the current study were constructed, expressed, and purified, as described elsewhere (4, 5). Briefly, culture supernatant of stably transduced HEK293T cells was eluted with ice-cold imidazole containing PBS. Eluted fractions were dialyzed overnight against PBS. The purity of the elution fractions was analyzed after SDS-PAGE and subsequent staining with Coomassie brilliant blue solution. The concentration of the scBsDbs in the fractions was determined by comparison to a BSA standard curve on the same SDS gel (data not shown, see Ref. 4).

Flow cytometric analysis

Flow cytometry was performed on a FACSCalibur (BD Biosciences) and a MACSQuant Analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany). mAbs against human CD4 (RPA-T4), CD25 (2A3), CD45RA (H100), CD69 (FN50), and CD152 (CTLA-4, BNI3) were obtained from BD Biosciences (Heidelberg, Germany). A mAb against human CD127 (BioRDR5) was obtained from eBioscience (Frankfurt, Germany). mAbs against human FOXP3 (259D) and the respective isotype control (IgGl1x) were purchased from BioLegend (Uithoorn, The Netherlands). Staining for cell surface markers was carried out prior to intra-cellular staining for FOXP3 and CTLA-4, according to the manufacturer’s instructions.

The binding of the scBsDbs was assessed by indirect immunofluorescence analysis using unfixed PC3-PSCA cells, LA-decorated PC3 cells, PBMCs, or isolated CD4+ T cells. Binding was visualized by incubation with a FITC-conjugated mAb against the C-terminal myc-tag of the scBsDbs (AbD Serotec, Düsseldorf, Germany), as described elsewhere (4, 5). mAbs against CD3 (BW264/56; Miltenyi Biotec), human La-E7B6, and human PSCA (7F5) (produced at the Institute of Immunology, Medical Faculty of the Technical University Dresden) served as positive controls.

T cell isolation and expansion

Human PBMCs were prepared by Ficoll-gradient centrifugation from buffy coats (supplied by German Red Cross, Dresden, Germany) of healthy volunteers after their informed consent. The study was approved by the local ethics committee of the university hospital of the medical faculty of Carl Gustav Carus Technical University Dresden (EK27/022006). CD4+ T cells were freshly isolated from PBMCs by negative selection and separated into CD25+ and CD25− cells by magnetic bead-activated cell sorting using the CD4+CD25+ Treg isolation kit from Miltenyi Biotec. To increase the purity of isolated CD4+CD25+ cells, two consecutive column runs were performed. The purity of the isolated Th cell and Treg populations was confirmed by FACS analysis and ranged between 85 and 94% (CD4+CD25+) and 86 and 95% (CD4+CD25−), respectively. Before application, freshly isolated cells were maintained overnight in complete RPMI 1640 medium in the presence of 300 U/ml IL-2 (ImmunoTools, Friesoythe, Germany).

For some experiments, highly pure expanded CD45RA− Tregs were used. Prior to expansion, the isolated CD4+CD25− cells were sorted on a FACSaria II cell sorter (BD Biosciences) to obtain the CD45RA− Treg subpopulation. After sorting, the resulting population showed >95% purity (CD4+CD25−/CD45RA−). The expansion protocol was adapted and slightly modified from Putnam et al. (21). In brief, 2.5 × 10⁶ cells were plated in X-Vivo 15 medium (Lonza, Cologne, Germany), supplemented with 10% human AB serum (PAA Laboratories, Co¨ lbe, Germany) and stimulated using αCD3/αCD28-coated DynaBeads (Invitrogen) at a ratio of 1:1 bead/cell at days 1 and 9. Every 2–3 d, 300 U/ml recombinant clinical grade IL-2 (Proleukin S; Novartis Pharmaceuticals, Horsham, U.K.) was added, assuming consumption of the cytokine. During the first week of expansion, 100 ng/ml rapamycin (Axxora Deutschland, Lôrrach, Germany) was added, together with IL-2, to further minimize the risk for CD4+ effector T cell contaminations (22). After a 14-d expansion period, the resulting cell population showed a purity >96% (CD4+CD25+CD127low FOXP3+).

Activation assays with bispecific Abs

A total of 5 × 10⁴ Tregs or autologous Th cells was cocultured with the prostate cancer cell line PC3-PSCA (1 × 10⁶ cells) at a ratio of 5:1 in 96-well round-bottom plates in the presence or absence of 6 µmol the cross-linking scBsDb CD3xPSCA in a total volume of 200 µl RPMI 1640 medium, without additional cytokines. Bispecific Ab CD3xE7B6 was used as a control, which binds to an epitope of the intracellular autoantigen La (23), which is not available on the target cells. Tregs were co-cultured with αCD3/αCD28-coated DynaBeads (Invitrogen) at a ratio of 1:1 bead cell, served as a positive control. All samples were analyzed as triplicates.

CFSE-based suppression assays

Prior to their use in an in vitro suppression assay, CD4+CD25− Th cells were labeled with 1 µM CFSE (Sigma-Aldrich, Steinheim, Germany) for 10 min at 37˚C and washed twice. For a suppression assay, 5 × 10⁴ labeled autologous responder T cells were incubated with 1 × 10⁵ PC3-PSCA tumor cells and the scBsDb CD3xPSCA in 96-well round-bottom plates alone or in the presence of unlabeled autologous responder cells, at indicated ratios, in a total volume of 200 µl RPMI 1640 medium, without adding any additional cytokines. Bispecific Ab CD3xE7B6 was used as a control, which binds to an epitope of the intracellular autoantigen La (23), which is not available on the target cells. Tregs were co-cultured with αCD3/αCD28-coated DynaBeads (Invitrogen) at a ratio of 1:1 bead cell, served as a positive control. All samples were analyzed as triplicates.

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Determination of cytokine concentration

To determine amounts of secreted IFN-γ, IL-10, TNF, and IL-2, supernatants were collected 24 h after the start of an activation or suppression assay and analyzed for cytokine secretion using OptEIA ELISA Sets (BD Biosciences), according to the manufacturer’s protocol. The absorption of the samples was measured after 30 min, and the obtained values were used to calculate the concentration of the cytokines in the samples, according to the values obtained for the standard series provided by the manufacturer.
Quantification of absolute cell numbers

In order to assess expansion rates of different T cell populations in activation or suppression assays, absolute T cell numbers were quantified by flow cytometry. At the indicated time points, cocultures in 96-well plates were carefully resuspended and an aliquot of 10 µl was removed for further analysis. Aliquots were diluted 1:10 with 1 µg/ml propidium iodide (Sigma-Aldrich) immediately before measuring the cell number/ml of the different T cell subpopulations using the MACSQuant Analyzer and MACSQuantify software (Miltenyi Biotec). Living cells were distinguished from dying cells and cellular debris by size exclusion and being propidium negative.

In vivo-suppression assay of cytotoxic T cell activity in nude mice

Female 9-wk-old NMRInu/nu mice were provided by the animal facility of the Technical University of Dresden. Animals were kept under specific pathogen-free conditions. All experiments were carried out in accordance with the German animal protection law and were approved by the local authorities (Landesdirektion Dresden). A total of 1 × 10⁶ mouse-adapted PC3-PSCA tumor cells was injected s.c. into the right flank of the mice. In some of the animals, 1 × 10⁶ CD4⁺CD25⁻ Th cells and/or equal numbers of autologous, in vitro expanded CD4⁺CD25⁻CD45RA⁺ Tregs and/or 10 µg the scBsDb CD3xPSCA were coinjected with the PSCA⁺ tumor cells. Established tumors were measured weekly in both directions using a digital measuring slide. Mice were killed for ethical reasons when tumors exceeded 18 mm in one direction.

Statistical analysis

Differences in the phenotype of Th cell and Treg populations or in the proliferation of responder T cells were analyzed using the two-tailed Student t test for independent samples. Statistical significance of differences in tumor growth between the bispecific Ab-treated groups was also calculated using the Student t test. In addition, data were analyzed by one-way ANOVA and Bonferroni multiple comparison test using GraphPad Prism software. The p values < 0.05 were considered significant.

Results

Tregs upregulate activation-associated markers after a cross-linkage with target cells via bispecific Abs

To elucidate whether the activation of Tregs can be induced by cross-linking bispecific Abs, CD4⁺CD25⁺ Tregs or autologous CD4⁺CD25⁻ Th cells of eight donors were cocultured with the prostate cancer cell line PC3-PSCA (which was transduced for permanent expression of PSCA; Materials and Methods) in the presence or absence of the scBsDb CD3xPSCA. To exclude that the binding of the bispecific Ab to the CD3 complex itself generates an activation signal, a control scBsDb (CD3xE7B6) was used, which binds to the same CD3 epitope on T cells as does the CD3xPSCA scBsDb but is not able to mediate cross-linking to the tumor cells, because its second arm is directed to an intracellular Ag (4). After 24 h of coculture, the T cell populations were harvested, and the expression pattern of the activation markers CD69 and CD25 was examined. Furthermore, intracellular stainings for the Treg-associated molecules FOXP3 and CTLA-4 were performed (Fig. 1). With regard to the expression of the early activation marker CD69, both CD4⁺CD25⁺ Tregs and CD4⁺CD25⁻ Th cells upregulated CD69 in the presence of the CD3xPSCA scBsDb (63.3 ± 9.1% and 74.8 ± 10.6%, n = 8, respectively). In contrast, without any bispecific Ab or with the unspecific control scBsDb CD3xE7B6, no considerable upregulation of CD69 could be observed. Similar results were obtained for the IL-2Rα–chain (CD25). Although Tregs constitutively express high levels of this molecule on their cell surface, upon incubation with the cross-linking bispecific Ab, the expression level is remarkably enhanced (relative increase in mean fluorescence intensity [MFI]: 42.3 ± 10.1%, n = 8). Th cells also exhibited increased CD25 expression following activation (61.8 ± 17.2% positive cells, n = 8). Similar to the enhanced expression of CD25 on the cell surface, redirected Tregs also exhibited increased intracellular amounts of the transcription factor FOXP3 after cross-linking to target cells via the CD3xPSCA bispecific Ab (relative increase in MFI: 58.6 ± 12.2%, n = 8). In comparison, CD4⁺ Th cells showed only a moderate upregulation of FOXP3, from 5.8 ± 2.5% to 13.2 ± 7.0% FOXP3⁺ cells (n = 8). A characteristic feature of Tregs is their constitutive expression of high amounts of intra- and extracellular CTLA-4. In the presence of the CD3xPSCA scBsDb, Th cells (41.2 ± 17.7%, n = 8), as well as Tregs, exhibited a markedly increased expression level of intracellular CTLA-4 (relative increase in MFI: 31.9 ± 13.0%, n = 8). Altogether, to our knowledge, these results provide the first evidence that Tregs can be activated via cross-linking of bispecific Abs.

Additionally, we performed experiments in which we compared the activation status of Tregs cross-linked to target cells via the bispecific Ab CD3xPSCA with Tregs stimulated with αCD3/ αCD28-coated beads. Analyzing the upregulation of the afore-
mentioned markers CD69, CD25, FOXP3, and CTLA-4, no considerable differences between the two activating agents could be observed (Supplemental Fig. 2).

**Tregs secrete high amounts of IL-10, but almost no IFN-γ, TNF, or IL-2, upon TCR stimulation with a bispecific Ab**

To analyze the cytokine-production profile of the different T cell populations, culture supernatants were collected 24 h after the beginning of a coculture assay, and levels of secreted IL-10, IFN-γ, TNF, and IL-2 were measured by ELISA. Fig. 2A shows the cytokine-production profile of Tregs and Th cells of one representative donor. As expected, incubation of the cells without any bispecific Ab or with the CD3xE7B6 control construct did not result in cell activation; hence, no cytokines could be detected in the supernatants. In contrast, culturing the cells in the presence of the bispecific CD3xPSCA Ab led to the induction of cytokine production. Although Th cells secreted substantial amounts of the inflammatory cytokines IFN-γ and TNF, as well as growth-promoting IL-2 and immunomodulatory IL-10, only the latter cytokine is produced in considerable amounts by CD4+CD25+ Tregs under the same culturing conditions. To compare the results of eight donors, the respective amount of cytokines secreted by redirected Th cells was set to 100% and the corresponding amount of cytokines secreted by Tregs was calculated and given as a percentage (Fig. 2B). The redirected Tregs of all donors produced more IL-10 than did redirected Th cells (116 ± 36%) but, compared with the Th cells, very low amounts of inflammatory cytokines (IFN-γ: 13.2 ± 7.8%, TNF: 9.5 ± 5.2%, IL-2: 15.5 ± 6.1%).

**Redirected Tregs proliferate only in the presence of high doses of exogenous IL-2**

For several years, it has been well known that Tregs are hypoproliferative in response to TCR stimulation. A prerequisite for their expansion is strong TCR signaling combined with high doses of IL-2. To evaluate whether cross-linking of Tregs via a bispecific Ab could induce an activation signal strong enough to initiate proliferation of the Tregs, the doubling of Treg or Th cell populations was assessed by coculturing them in the presence or absence of the CD3xPSCA scBsDb, with or without the addition of exogenous IL-2 (Fig. 3). Cell numbers were determined every 24 h using a MACSQuant Analyzer (Miltenyi Biotec), and the expansion rate of the respective cell population was calculated. Incubation of Th cells or Tregs with target cells alone or with the control bispecific Ab CD3x7B6E did not induce proliferation of either cell population (Fig. 3A). However, culturing Th cells with target PC3-PSCA cells and the bispecific CD3xPSCA Ab resulted in a significant expansion (4 ± 1-fold) of the initial cell population (Fig. 3A, 3B). In contrast, Tregs incubated under the same conditions did not proliferate. Next, we investigated whether this lack of proliferation was IL-2 dependent, although Tregs are activated under these conditions, as already demonstrated. For this purpose, two concentrations of IL-2 (50 and 500 U/ml) were added to the

![FIGURE 2. Cytokine production profile of redirected CD4^+CD25^+ Tregs or CD4^+CD25^- Th cells. A. Freshly isolated Th cells (black bars) or Tregs (white bars) were cocultured with PC3-PSCA cells and incubated without any scBsDb (-), with an unspecific CD3x7B6E (control), or with the cross-linking CD3xPSCA scBsDb (+). Culture supernatants were collected 24 h later and analyzed by ELISA to determine the levels of secreted IL-10, IFN-γ, TNF, and IL-2. Data shown represent means ± SD of triplicate wells. The results of one representative donor are shown. B, Summary of the results of eight donors. The respective amount of cytokines secreted by Th cells was set to 100% and the corresponding relative amounts of cytokines secreted by Tregs were calculated. ***p < 0.001, Student t test.]
cocultures (Fig. 3C). Only in the presence of the CD3xPSCA bispecific Ab and high doses of IL-2 (500 U/ml) did Tregs expand >4-fold within 1 wk.

**Bispecific Ab-redirected Tregs are capable of suppressing proliferation and cytokine production of autologous Th cells**

So far, these results supported the notion that activation of freshly isolated Tregs can be achieved in an Ag-specific manner by using bispecific Ab constructs. A prerequisite for the use of Tregs in clinical trials is their efficient in vitro expansion to sufficient numbers without losing phenotypic and functional characteristics. Previous studies showed that only highly pure human CD45RA⁺ Tregs maintain their suppressive phenotype over a long-lasting cultivation period (24, 25). Therefore, we next tested whether expanded Tregs of three donors could also be activated via bispecific Abs and whether they exerted a suppressive capacity. CD45RA⁺ Tregs were isolated by FACS sorting and expanded over 14 d using CD3/CD28-coated beads and high-dose IL-2. In an αCD3/αCD28 bead-stimulated (1:75) suppression assay, these cells suppressed proliferation of autologous (data not shown), as well as allogeneic, CD4⁺CD25⁻ effector T cells at suppressor/effector ratios of 1:1 to 1:32 (Supplemental Fig. 3). Using the bispecific CD3xPSCA Ab for the Ag-specific, but TCR-independent, activation of Tregs also resulted in a significant reduction in autologous T cell proliferation capacity (Fig. 4A, 4B).

In the experiment, 5 × 10⁵ CFSE-labeled responder Th cells were cocultured in the presence of unlabeled responder Th cells and the bispecific Ab, leading to a considerable proliferation of 34% (1:1 ratio) and 40% (4:1 ratio) of the CFSE-labeled Th population after 120 h (Fig. 4A). When the unlabeled responder Th cells were replaced by Tregs at the same effector/suppressor ratios, proliferation decreased significantly (Fig. 4A, 4B). Furthermore, bispecific Ab-redirected Tregs influenced proliferation, as well as cytokine production, of autologous Th cells (Fig. 4C). Analysis of the supernatants revealed that, in the presence of equal numbers of Tregs, Th cells secreted only 27% (IFN-γ) and 2% (TNF) of the amount of inflammatory cytokines that they produced in their absence. Moreover, under the same culturing conditions, almost no IL-2 could be detected in the culture supernatants, either because of IL-2 consumption by the Tregs or suppression of IL-2 production of Th cells.

**Bispecific Ab-redirected Tregs cause an extensive downregulation of CD25 and CD127 on autologous Th cells**

To analyze the suppressive capacity of redirected Tregs in more detail, surface stainings for the α-chains of the receptors for the common γ-chain (γc) cytokines IL-2 and IL-7 on suppressed Th cells were performed (Fig. 4D). With regard to the IL-2–α-chain CD25, upregulation on the surface of activated Th cells was markedly reduced in the presence of Tregs. A similar effect was observed for the α-chain of IL-7R. In response to CD3 stimulation caused by the bispecific CD3xPSCA Ab, Th cells downregulated CD127 expression, which is thought to be a homeostatic mechanism to prevent excessive T cell expansion (26). Adding Tregs to the culture strikingly enhanced the downregulation of the CD127 molecule. In summary, bispecific Ab-redirected Tregs were capable of suppressing proliferation and cytokine production of autologous Th cells and negatively influenced the expression of the high-affinity receptor chains of the γc-cytokines IL-2 and IL-7.

**Bispecific Ab-redirected Tregs suppress Th cell-induced antitumor response in nude mice**

The experiments conducted thus far showed that single-chain bispecific Abs activated Tregs and triggered their suppressor function in vitro. To confirm the data in vivo, we tested whether redirected Tregs suppressed a Th cell-mediated antitumor response in athymic nude mice. All animals were injected with PC3-PSCA tumor cells into their right flank, and developing s.c. tumors were measured weekly. Coinjection of PSCA⁺ prostate tumor cells with equal amounts of CD4⁺CD25⁻ Th cells and the scBsDb CD3xPSCA resulted in reduced tumor outgrowth in this group of mice compared with those groups in which tumor cells were injected alone or with Th cells in the absence of the bispecific Ab. When autologous in vitro-expanded Tregs were added, tumor development was comparable to that of both aforementioned control groups. However, adding Tregs at an effector/suppressor ratio of 1:1 in the presence of the activating bispecific Ab strongly enhanced tumor growth (Fig. 5). Taken together, these results indicated that bispecific Ab-redirected Tregs can execute their suppressive function in vitro, as well as in vivo posttransfer.

**Discussion**

Bispecific Abs were originally designed for the recruitment of cytotoxic immune cells against target cells in an Ag-specific
manner. Usually, T cells are redirected to target cells by cross-linking the activating CD3 receptor of T cells and any chosen Ag on the cell surface of the target cells. It was demonstrated, both in vitro and in mouse models, that, in this way, cytotoxic T cells can be very efficiently redirected and activated against tumor cells (e.g., Refs. 1–5), leading finally to the lysis of the target cells and production of inflammatory cytokines by the redirected T cells. Recently, the first clinical trials proved that this concept can be successfully applied for the treatment of malignant diseases.

However, among various mechanisms of immune suppression by tumors, the recruitment of Tregs to tumor sites plays an important role. Accumulation of Tregs in tumors suppresses the activation and proliferation of immune effector cells, and increased numbers of Tregs correlate with poor survival prognosis for tumor patients. At this translational point toward a clinical application of bispecific Abs for tumor treatment, there is an urgent need to answer the question whether Tregs will be activated by bispecific Abs in a similar way as their effector counterparts.

Using a recently described CD3xPSCA scBsDb, we demonstrated that effector T cells, as well as CD4+CD25+ naturally occurring Tregs, can be activated via bispecific Ab constructs. Experimental evidence was provided by monitoring the expression of different activation markers upon the incubation of Tregs with the scBsDb CD3xPSCA in the presence of PSCA+ target cells. Only in the presence of the cross-linking bispecific Ab and target cells was an upregulation of the early activation marker CD69 observed on both Tregs and Th cells. Moreover, the expression levels of CD25 and CTLA-4 were markedly enhanced in both

![FIGURE 4](http://www.jimmunol.org/)

**FIGURE 4.** Suppressive capacity of bispecific Ab-redirected Tregs in vitro. A total of $5 \times 10^6$ CFSE-labeled CD4+ responder T cells were cultured with PC3-PSCA target cells and either unlabeled autologous CD4+ Th cells or unlabeled autologous expanded Tregs at effector/suppressor ratios of 1:1 or 4:1 in the presence or absence of the CD3xPSCA scBsDb. A, Proliferation of the labeled Th cells was monitored by levels of CFSE dilution. The graphs show the CFSE staining 120 h after starting the coculture in the presence (+) or absence (-) of the CD3xPSCA scBsDb. The percentage of proliferating cells is indicated in each graph. B, Relative proliferation of CFSE-labeled Th cells in the presence of unlabeled Tregs or T cells after 96 h. The amount of proliferating cells in the presence of redirected Th cells was equalized to 100%, and the relative proliferation of labeled Th cells in the presence of redirected Tregs was calculated. Mean and SD from experiments with three donors are shown. *p < 0.05, **p < 0.001, Student t test. C, Culture supernatants from the above-described experiments were collected after 24 h and analyzed by ELISA to determine the levels of secreted IFN-γ, TNF, and IL-2. Results for one representative donor are shown; error bars represent SD from the triplicate wells. D, CD25 and CD127 surface expression of responder T cells 120 h after beginning of the coculturing period in the presence (solid line) or absence (dashed line) of the cross-linking Ab. Numbers represent the MFI of total cells and percentage of positive cells under the marker for each condition (filled gray area: isotype control). Data shown are representative for one of three donors.

![FIGURE 5](http://www.jimmunol.org/)

**FIGURE 5.** Suppressive capacity of bispecific Ab-redirected Tregs in vivo. Nude mice were inoculated s.c. with mouse-adapted PC3-PSCA tumor cells ($1 \times 10^6$). Animals received $10 \mu g$ of bispecific CD3xPSCA Ab and either $1 \times 10^6$ CD4+ Th cells ($\bullet$, n = 5) or $1 \times 10^6$ CD4+ Th cells and in addition $1 \times 10^6$ in vitro-expanded Tregs ($\circ$, n = 5). As controls, tumor cells were injected alone ($\blacklozenge$, n = 5) or were coinjected with Th cells or Th cells and Tregs without the cross-linking scBsDb ($\blacklozenge$ and $\bigodot$, respectively, n = 5). Tumor size was measured weekly. Statistical significance was determined for the bispecific Ab-treated groups with or without Tregs at the final time point of the experiment using the Student t test and ANOVA with the Bonferroni multiple comparison test. *p < 0.05.
populations under these conditions. In addition, an increased intracellular level of the transcription factor FOXP3 was observed in the activated Tregs. In previous studies, it was shown that FOXP3 enhances the expression of CD25 and CTLA-4 (27, 28), which might explain the increasing levels of both markers after activation in Tregs. Our observations of increased levels of these markers in Tregs after activation are in accordance with experiments in which αCD3/CD28-coated beads were used for Treg stimulation, resulting in increased FOXP3, CD25, CTLA-4, and CD69 expression levels in activated Tregs (29). It was demonstrated by a number of studies that the cross-linkage of conventional T cells to target cells via bispecific Abs triggers the secretion of inflammatory cytokines (4, 5). In accordance with these observations, our study showed that only in the presence of the specific bispecific Ab did the cross-linkage of conventional CD4+ T cells and Ag+ target cells result in the secretion of significant amounts of IFN-γ, IL-2, and TNF. In contrast, redirected Tregs secreted almost no proinflammatory cytokines or IL-2 upon cross-linkage, although the flow cytometric analysis demonstrated the upregulation of activation markers under these conditions. This is in line with findings that constituently expressed FOXP3 in Tregs acts as a transcriptional repressor that blocks the production of a wide range of effector cytokines (30). Nevertheless, the activation of freshly isolated Tregs by bispecific Abs triggered the release of the immunosuppressive cytokine IL-10. It should be noted that expanded CD45RA+ Tregs did not produce any IL-10 upon cross-linkage with the bispecific Ab (data not shown), which correlates with studies by Hoffmann et al. (25), who did not detect this cytokine in culture supernatants after in vitro expansion and TCR stimulation of expanded CD45RA+ Tregs.

Activation of (memory) T cells via bispecific Abs results in profound proliferation as does normal TCR triggering, despite the assertion that bispecific Abs can be used as activating molecules of exogenous IL-2 induced their expansion, further validating the positive capacity of Tregs upon Ag-specific activation with bispecific Abs. In contrast, redirected Tregs secreted almost no proinflammatory cytokines or IL-2 upon cross-linkage, although the flow cytometric analysis demonstrated the upregulation of activation markers under these conditions. This is in line with findings that constitutively expressed FOXP3 in Tregs acts as a transcriptional repressor that blocks the production of a wide range of effector cytokines (30). Nevertheless, the activation of freshly isolated Tregs by bispecific Abs triggered the release of the immunosuppressive cytokine IL-10. It should be noted that expanded CD45RA+ Tregs did not produce any IL-10 upon cross-linkage with the bispecific Ab (data not shown), which correlates with studies by Hoffmann et al. (25), who did not detect this cytokine in culture supernatants after in vitro expansion and TCR stimulation of expanded CD45RA+ Tregs.

Activation of (memory) T cells via bispecific Abs results in profound proliferation as does normal TCR triggering, despite the fact that additional costimulatory signals are missing. As demonstrated in this study, the cross-linkage of conventional CD4+ Th cells to tumor cells via the CD3xPSCA bispecific Ab induced significant expansion of the Th cells, although the target cells used in this study do not express the CD28 ligands CD80/CD86 (data not shown) and, therefore, cross-linked T cells could not get any additional CD28 costimulatory signals from the target cells. In contrast, Ab-redirected Tregs were hyporesponsive and did not expand when cultured without the addition of exogenous cytokines. Most likely, this hyporesponsiveness is due to their inability to produce growth-promoting IL-2. As shown by other investigators, Tregs rely on high amounts of IL-2 and strong TCR ligation to proliferate (31, 32). Incubating Tregs with both CD3xPSCA and high doses of exogenous IL-2 induced their expansion, further validating the assertion that bispecific Abs can be used as activating molecules for Tregs.

One prerequisite for the treatment of graft rejection or autoimmune diseases with bispecific Ab-redirected Tregs is the suppressive capacity of Tregs upon Ag-specific activation with bispecific Abs. Therefore, we investigated the ability of bispecific Ab-redirected Tregs to inhibit the proliferation and cytokine production of autologous Th cells, a well-described characteristic feature of Tregs (33, 34). Indeed, the incubation of Tregs in the presence of a cross-linking bispecific Ab resulted in a significant suppression of both proliferation and cytokine secretion of autologous Th cells. The lack of IL-2, together with our observation that the Ab-activated Tregs strongly reduced the expression of the high-affinity receptor α-chain for IL-2 (CD25), might account for the reduced proliferation capacity of autologous Th cells in the presence of bispecific Ab-activated Tregs. In addition, we observed an enhanced CD127 downregulation on the surface of suppressed effector T cells, as also shown by other investigators (34–36). Finally, bispecific Ab-activated Tregs were able to abrogate the antitumor effector function of redirected Th cells and to promote tumor growth in a mouse model.

Although we clearly demonstrated in this study that bispecific Abs are able to activate Tregs both in vitro and in vivo, the first clinical trials showed their efficacy for cancer treatment (6). CD8+ T cells respond much faster by cross-linking via bispecific Abs than do conventional CD4+ T cells (M. Bachmann, unpublished observations). Similar conclusions can also be drawn from data published by other groups (37, 38). The time gap between the activation of cytotoxic CD8+ T cells and the activation of conventional or regulatory CD4+ T cells might explain why, in the clinical trials performed thus far, the treatment with bispecific Abs was rather successful, despite the most likely presence of Tregs. Nevertheless, strategies helping to circumvent or overcome the potential activation of Tregs should improve the treatment of malignant diseases with CD3-engaging bispecific Abs. One possibility to avoid the unwanted stimulation of Tregs is the adoptive transfer of ex vivo bispecific Ab-loaded effector T cells instead of a direct application of the bispecific Ab into a patient.

In view of their important role in establishing and maintaining peripheral tolerance, an Ag-directed retargeting of Tregs using bispecific Abs may represent a promising therapeutic opportunity for the treatment of autoimmunity and graft rejection. Based on several animal experiments, it is increasingly evident that Ag-specific Tregs are more efficient in autoimmunoprevention and treatment than are polyclonal Treg populations (39, 40). Additionally, adoptively transferred polyclonal Tregs might result in an unfavorable systemic immunosuppression, with an increasing risk for opportunistic infections and/or reactivation of persistent virus infections. In contrast, Tregs with a certain Ag/tissue specificity are able to suppress ongoing self-destructive immune responses at the appropriate sites of inflammation. One of the main obstacles to the clinical application of Ag-specific Tregs is the isolation of sufficient numbers. One possible approach is the generation of genetically modified Tregs, which express an artificial TCR against a desired Ag (e.g., Refs. 19, 41, 42). Still, the most efficient methods for the genetic manipulation of T cells make use of virus-mediated gene transfer, which bears the risk of effecting the expression of oncogenes or tumor-suppressor genes (43). In light of this, bispecific Abs may provide powerful tools to redirect adoptively transferred polyclonal Tregs in an Ag-specific and/or site-specific manner, without the need to genetically manipulate the cells. A potential strategy could be the preloading of polyclonal Tregs ex vivo with a bispecfic Ab directed against a desired Ag (e.g., an autoantigen in the case of an autoimmune disease) and retransfer of the cells into a patient. The bispecific Ab would endow the decorated Tregs with a predefined Ag specificity and trigger their suppressive function locally at the site of the ongoing immune reaction, thereby potentially enhancing the therapeutic effect and minimizing the risk for unspecific immunosuppression.

Disclosures
The authors have no financial conflicts of interest.

References


