Abstract

A major mechanism by which human regulatory T cells (Tregs) have been shown to suppress and kill autologous immune cells is through the granzyme-perforin pathway. However, it is unknown whether Tregs also possess the capacity to kill tumor cells using similar mechanisms. Bispecific antibodies (bscAb) have emerged as a promising class of therapeutics that activate T cells against tumor antigens without the need for classical MHC-restricted T-cell receptor (TCR) recognition. Here, we show that a bscAb targeting the tumor-specific mutation of the EGF receptor, EGFRvIII, redirects human CD4+CD25+FoxP3+ Tregs to kill glioblastoma cells. This activity was significantly abrogated by inhibitors of the granzyme-perforin pathway. Notably, analyses of human primary glioblastoma also displayed diffused infiltration of granzyme-expressing FoxP3+ T cells. Together, these data suggest that despite their known suppressive functions, tumor-infiltrating Tregs possess potent cytotoxic mechanisms that can be co-opted for efficient tumor cell lysis. Cancer Immunol Res; 1(3); 1–5. ©2013 AACR.

Introduction

Despite maximal surgical resection, dose-intensive radiotherapy and multimodal chemotherapy, glioblastoma remains uniformly lethal with a median survival of less than 15 months (1). Novel therapies are desperately needed to improve the prognosis of this disease and immunologic targeting of tumor-specific mutations has emerged as a promising approach (2).

A major barrier that has impeded translation of efficacious immunotherapy is the inability to overcome profound immunosuppression associated with malignant disease (3). Regulatory T cells (Tregs) in particular are thought to play a central role in tumor escape from immune-mediated rejection. One mechanism by which Tregs are known to suppress and even kill autologous immune cells is through the granzyme-perforin pathway (4, 5). Despite this well-characterized cytotoxic capacity, whether Tregs can co-opt cytotoxic mechanisms to kill tumor cells has yet to be evaluated.

T-cell–activating bispecific antibodies (bscAb), particularly those of the bispecific T-cell engager (BiTE) subclass, represent a new therapeutic strategy that has the potential to treat even bulky, invasive disease (6, 7). BiTEs are tandem single-chain molecules that possess dual specificity for tumor-associated surface antigens and the CD3 complex on T cells, which allows them to divantly bind and afford potent, specific target cell lysis (8). Because CD3 is universally expressed among T cells, BiTEs have the theoretical capacity to redirect and activate even Tregs that are elevated and present in tumors of patients with cancer.

Among the few known tumor-specific antigens, perhaps the most widely characterized is the truncated mutant EGF receptor variant type III (EGFRvIII). EGFRvIII is a constitutively activated tyrosine kinase that is frequently expressed on the surface of glioblastoma and other common neoplasms but is completely absent from healthy tissues (9).

In this study, we show that an EGFRvIII–specific BiTE, bscEGFRvIIIxCD3 (7), successfully redirects highly purified Tregs and activates them in the presence of tumors expressing EGFRvIII. Despite their known suppressive properties, purified CD4+CD25+CD127dim/− T cells efficiently lysed EGFRvIII-expressing glioblastoma in vitro upon redirection and activation with bscEGFRvIIIxCD3. This activity was found to be dependent on the granzyme-perforin pathway. Immunohistochemical (IHC) analysis from human primary GBMs also displayed diffuse infiltration of activated, granzyme-producing FoxP3+ cells, showing that Tregs with potent effector functions may already be present in tumors even under natural conditions.

Previous efforts to enhance antitumor immunity via Treg depletion have been limited, in part due to an inability to efficiently eliminate suppressive cells that infiltrate tumor tissue (10). Alternatively, our data suggest that BiTEs can activate even suppressive Tregs to lyse tumor cells by redirecting their natural granzyme-mediated cytotoxic potential. These findings not only highlight a new mechanism by which
BiTEs may circumvent certain aspects of T_{reg}^-mediated suppression, but also have broader implications with regard to the natural functional role of activated, tumor-infiltrating T_{reg}^- that express granzyme and perforin in the tumor microenvironment.

Materials and Methods

**Tumor cell lines and reagents**

The human glioma cell line U87MG and its subline U87MG.ΔEGFR, which expresses EGFRvIII, are described elsewhere and were kind gifts from Dr. Webster Cavenee at University of California, San Diego (11). EGFRvIII expression was verified by flow cytometry using the LS64 antibody (12). These cell lines were regularly confirmed to be Mycoplasma-free by nucleic acid hybridization assay (GEN-PROBE MTC-NI). Characterization, production, and purification of bscEGFRvIIIxCD3 as well as control antibody constructs were conducted as previously described by us (7). Antibodies to CD4 (RPA-T4), CD25 (M-A251), CD69 (L78), CD152 (BN3), Granzyme A (CB9), Granzyme B (GB11), and Perforin (6G9) were purchased from BD Bioscienes. Anti-FoxP3 antibody (PCH101) and the FoxP3 Staining Buffer Set were purchased from eBioscience and intracellular staining was conducted according to manufacturer’s instructions. Antibodies against human FoxP3 (259D, BioLegend) and granzyme B (Cat No. ab4059) were used for IHC staining.

**T_{reg}^- isolation and preparation**

All human samples were obtained at Duke University Medical Center (Durham, NC) from individuals who had given written, informed consent. Human PBMCs were prepared by density gradient centrifugation from buffy coats of healthy donor leukapheresis. Highly purified regulatory T cells were isolated from PBMC by magnetic separation using the CD4^+ CD25^+ CD127dim/- Regulatory T Cell Isolation Kit II and AutoMACS Separator (Miltenyi Biotec) according to the manufacturer’s instructions. Following isolation, purity was confirmed by flow cytometric analysis to be more than 95% (CD4^+ CD25^+ FoxP3^+ ). Where noted, purified T_{reg}^- were prepared with the T_{reg}^- Expansion Kit (Miltenyi Biotec) at a MACSibead Particle-to-T_{reg}^- Ratio of 4:1 according to manufacturer instructions, with recombinant interleukin 2 (riL-2) at 500 U/mL, without rapamycin. Cells were verified for their suppressive ability in vitro before all cytotoxicity assays.

**In vitro activation and functional assays**

Activation, proliferation, cytokine secretion, and measures of specific lysis were conducted as previously described by us (7). In assays assessing activation and proliferation, freshly thawed and sorted lymphocytes were incubated in 96-well round-bottom plates with 1 × 10^5 target tumor cells and EGFRvIII BiTE [E:T ratio, 20:1; incubation time, 48 hours at 37°C; (bscEGFRvIIIxCD3), 10 μg/mL] in a total volume of 200 μL. Supernatant was removed after incubation and analyzed by cytometric bead array analysis according to manufacturer instructions (BD Biosciences). Cells were also harvested and assessed for surface and activation markers as above. In experiments assessing proliferation 1 μCi, ³H-thymidine was added to each well of a 96-well round-bottom plate for an additional 24 hours and cells were collected by a cell harvester. Counts were conducted using a Wallac 1450 Microbeta Trilux Liquid Scintillation/Luminescence Counter (Perkin-Elmer). Similarly, cytotoxicity assays were conducted by a standard chromium release assay as previously described by us (7). In brief, target cells were labeled with ⁵¹Cr and incubated with EGFRvIII BiTE and effector cells [E:T ratio, 20:1; incubation time, 18 hours at 37°C; (bscEGFRvIIIxCD3), 10 μg/mL] in a total volume of 200 μL. Following incubation, supernatants were removed and measured by gamma counter. Where noted, for inhibition of the granzyme-perforin axis, concanamycin A (CMA, 100 nmol/L; Sigma), Granzyme B Inhibitor I (Z-AAD-CMK, 50 μmol/L; Calbiochem), or ethylene glycol tetracetic acid (EGTA, 4 mmol/L; Calbiochem) was added to each well. Inhibitor concentrations were chosen based on previously published studies to establish dependence of cell-mediated cytotoxicity on perforin-granzyme activity (4, 13, 14). Before use, each inhibitor was found to have insignificant effects on the viability of T_{reg}^- following 18 hours incubation at 37°C as assessed by LIVE/DEAD Fixable Violet Dead Cell Stain Kit (Invitrogen). Blockade of FasL- and TRAIL-mediated apoptosis was carried out with antibody clones NOK-1 (25 μg/mL; BD Biosciences) and RIK-2 (25 μg/mL; BD Biosciences), respectively.

**Immunohistochemistry**

Tissues for IHC analysis were derived from human brain tumor biopsy material. Paraffin-embedded tissues were fixed, cut into 5 μm sections, mounted on glass slides and subjected to primary and secondary staining using the MACH 2 Double Stain I polymer Detection Kit (Biocare Medical) according to the manufacturer’s instructions. Diaminobenzidine (DAB) and Aminoethyl Carbazole (AEC) were used to detect granzyme B and FoxP3, respectively.

**Statistical analysis**

Groups were compared using a two-sample two-tailed t test and statistical significance was determined at a value of P<0.05.

**Results and Discussion**

The EGFRvIII BiTE, bscEGFRvIIIxCD3, has previously been shown to redirect polyclonal T-cell populations against EGFRvIII-expressing gliomas (7); however, whether this BiTE also signals activation of FoxP3^+ T_{reg}^-, which are not only elevated among tumor-infiltrating lymphocytes (15) but also known to possess potent suppressive properties in the setting of glioblastoma (16), is currently unknown. To evaluate the impact of the EGFRvIII BiTE on human T_{reg}^-, we first isolated naturally occurring human T_{reg}^- according to phenotypic CD4^+ CD25^+ CD127dim/- expression (17) and cultured these cells in the presence of EGFRvIII BiTE and glioma cells expressing EGFRvIII (U87MG.ΔEGFR). A control BiTE directed against a nonspecific antigen was also tested to exclude the possibility that the CD3-binding portion of bscEGFRvIIIxCD3 alone was sufficient for activation. After 24 hours, T cells were harvested and examined for surface and intracellular activation markers.

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In line with previous literature, isolation of CD4+ CD25+ CD127dim− cells yielded a highly pure population of T cells expressing elevated levels of transcription factor FoxP3, consistent with the phenotypic definition of the Treg subset (18) (Fig. 1A). Following coculture with target cells expressing EGFRvIII, flow cytometric analysis of sorted Tregs showed that, in the presence of control BiTE, surface activation markers CD69 and CD25 remained unchanged. However, in wells cultured with EGFRvIII BiTE, Treg exhibited significant upregulation of both early activation associated glycoprotein, CD69, as well as IL-2 receptor alpha chain, CD25 (Fig. 1B). These results were also consistent on repeat using sorted Tregs isolated from three separate healthy donors (P < 0.05; Fig. 1C). On the basis of this evidence of activation, we also sought to determine functional characteristics of Tregs stimulated by the EGFRvIII BiTE in the presence of tumor. Importantly, we found that following BiTE-mediated activation, Treg remained refractory both in terms of their ability to produce inflammatory cytokines (Fig. 1D) and proliferate when compared with CD4+ CD25+ helper T cells (Fig. 1E). Together, these results indicate that although the EGFRvIII BiTE clearly has the capacity to activate Tregs, the functional outcome of this activation may be similar to what would otherwise be expected through standard TCR-restricted engagement of Tregs in vitro.

Despite their lack of classical proinflammatory mechanisms, it is well-known that even naturally activated Tregs possess significant cytotoxic capacity, largely through the production of high levels of granzyme B expression (19). In fact, the perforin-granzyme axis is a major mechanism by which Tregs are known to suppress immune responses, through the direct killing of effector T cells (4, 13). Given this well-characterized cytotoxic function, we hypothesized that the EGFRvIII BiTE might actually have the ability to co-opt the perforin-granzyme axis in Tregs to kill cells other than lymphocytes, resulting in the redirected lysis of even tumor cells expressing a target antigen of interest, in this case EGFRvIII.

To test this hypothesis, we first sought to evaluate the impact of bscEGFRvIIIxCD3-mediated activation on the expression of perforin and granzymes among Tregs in the presence of target tumor cells expressing EGFRvIII. Using flow cytometric analysis of CD4+ CD25+ FoxP3+ cells, we found that Tregs cultered with control BiTE and target tumor cells expressed only low levels of perforin, granzyme A (GrA), and granzyme B (GrB). However, Tregs activated in the presence of bscEGFRvIIIxCD3 exhibited significant upregulation of these cytotoxic molecules (Fig. 2A). These results were repeated with consistent and significant upregulation of GrA, GrB, and perforin in samples isolated from three separate healthy donors (P < 0.05; Fig. 2B). Moreover, when analyzed for surface markers, back-gating analysis of GrB-positive cells (dots) displayed a distinct population with dual positivity for Treg phenotypic markers, FoxP3 and CD25 (Fig. 2C). The level of FoxP3 expression did not appear to change significantly when gating for Treg with either high or low levels of GrB (data not shown). Although the impact of BiTEs on Treg expression of GrB has not been previously described, these results are consistent with previous studies which have shown substantial GrB upregulation in Treg on stimulation with anti-CD3 antibody (5), which in theory provides the same signal delivered through the T-cell engaging arm of bscEGFRvIIIxCD3.

Because of the significant upregulation of the perforin-granzyme pathway in BiTE-activated Tregs, we next decided to test whether the EGFRvIII BiTE could redirect the cytotoxic payload expressed in Treg to lyse target tumor cells expressing EGFRvIII. CD4+ CD25+ CD127dim− T cells were expanded between 4 and 7 days to provide sufficient numbers for in vitro cytotoxicity, a period of time known to preserve both suppressive capacity and FoxP3 expression (20). Under these conditions, sorted Treg efficiently lysed target cells expressing EGFRvIII when redirected by the EGFRvIII-specific BiTE. Importantly, this effect was not detected in the presence of a nonspecific control bsAb or Treg alone (Fig. 3A). Moreover, redirected Treg-mediated lysis was shown to be actually dependent on the perforin-granzyme pathway, as cytotoxicity was not impacted by TRAIL or FasL blockade, but significantly abrogated in the presence of previously characterized

Figure 1. BscEGFRvIIIxCD3 activates highly purified Tregs but does not reverse defects in cytokine secretion and proliferation in response to stimulation. A, representative flow cytometric analysis of purified CD4+ CD25+ CD127dim− Treg shows more than 97% purity as determined by FoxP3 and CD25 phenotypic markers. B, purified Tregs express elevated levels of activation markers CD69 and CD25 in response to bscEGFRvIIIxCD3 specifically in the presence of EGFRvIII-expressing tumors when compared with cells incubated with nonspecific, control BiTE. These activities were consistent and statistically significant among lymphocyte donors from three separate individuals (C). D, supernatants from wells containing U87MG.EGFR, bscEGFRvIIIxCD3, and Treg contained significantly lower levels inflammatory cytokines compared with wells in which responder cells consisted of purified CD4+ CD25− helper T cells (T). E, proliferation of Treg and TH in response to bscEGFRvIIIxCD3 and solid phase EGFRvIII as measured by [3H]-thymidine incorporation shows that proliferative defects in the Treg compartment persist following activation with bscEGFRvIIIxCD3. Statistical analysis in (D) and (E) were conducted in triplicate wells with lymphocytes from a single donor and all experiments were repeated twice. Horizontal bars represent a statistical significance of P < 0.05.

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Tregs might be redirected to kill tumor cells through engagement with a bscAb. One previous study has explored the impact of Tregs suppresses effector cell proliferation and abrogates antitumor efficacy (22), innovative methods to reappropriate the Treg compartment without ablating these cells completely could provide an attractive alternative to currently available depletive strategies. As such, we have provided evidence and a mechanism by which attractive alternative to currently available depletive strategies. Moreover, because their work employed the use of rapamycin to expand Tregs, ex vivo, an additive which is known to completely suppress GrB expression in Tregs (19), it may ultimately not be feasible to directly compare the results of our studies. One limitation of our work is the use of a glioblastoma cell line that may elicit low-level allogeneic responses and thus a permissive effect on the mechanisms of Treg activation explored here. Further studies will be necessary to explore the degree to which this is the case, likely through the use of syngeneic mouse models or autologous human tumors.

In the current study, redirected cytotoxicity was dependent on the presence of bscEGFRvIIIxCD3; although, it is reasonable to suspect that similar results might be observed upon even endogenous TCR-specific engagement of granzyme-producing Tregs present in the tumor microenvironment. Supporting this, inhibitors of perforin- and granzyme-mediated cytotoxicity (Fig. 3B). Adding clinical relevance to these findings, infiltrating Foxp3+ Tregs in human glioma samples were actually found to coexpress detectable levels of GrB by IHC analysis (Fig. 3C). Consistent with what has been reported elsewhere, GrB expression appeared to be largely cytoplasmic while subcellular localization of Foxp3 was exhibited prominently in the nucleus (21). Overall, these data suggest that activated Tregs with potent effector functions may be present within glioblastomas, and could potentially serve as effector cells upon infusion with T-cell–engaging bscAb therapies in vivo.

In light of recent findings showing that Tregs may actually be required for the priming of high-avidity CD8+ T-cell responses (22), innovative methods to reappropriate the Treg compartment without ablating these cells completely could provide an attractive alternative to currently available depletive strategies. As such, we have provided evidence and a mechanism by which Tregs might be redirected to kill tumor cells through engagement with a bscAb. One previous study has explored the impact of bscAbs on Tregs and shown that bscAb-mediated activation of Tregs suppresses effector cell proliferation and abrogates antitumor efficacy (23). However, the direct effects of bscAb-directed Tregs on target tumor cells were not discussed.

Figure 2. BscEGFRvIIIxCD3 activates Tregs to elevate expression of granzymes and perforin specifically in the presence of tumor cells expressing EGFRvIII. A, human PBMCs were incubated in the presence of U87MG.ΔEGFR and control BiTE (left) or EGFRvIII BiTE (right), harvested, and stained for flow cytometric analysis. CD4+ cells were isolated and gated for high CD25 and FoxP3 expression and then analyzed for Perf, GrA, and GrB expression with positivity determined by isotype control. Plots are representative of at least three repeated experiments. B, percent positive cells expressing Perf, GrA, or GrB were determined to be significantly elevated in the presence of EGFRvIII BiTE over nonspecific control BiTE across lymphocytes from three separate healthy donors. C, flow cytometric plot of backgated GrB-positive cells from (A) corresponds to a discrete population (dots) with dual positivity for CD25 and FoxP3. All experiments were repeated twice. Horizontal bars represent a statistical significance of P < 0.05 between groups of three donors each defined by the presence of either EGFRvIII-specific or control BiTE.

Figure 3. Tregs expressing GrB are present in human glioma and possess cytotoxic activity against EGFRvIII-expressing tumor in the presence of EGFRvIII BiTE. A, upon redirection with bscEGFRvIIIxCD3, but not a nonspecific control bscAb (control), activated Tregs show enhanced lysis against EGFRvIII-expressing tumor (Treg:target, 20:1; incubation time 18 hours; (BiTE) 10 μg/mL). B, specific lysis against target tumor cells expressing EGFRvIII is not significantly inhibited by blockade of Fas ligand- and TRAIL-mediated apoptosis but is significantly abrogated by partial inhibitors of the granzyme-perforin pathway, Z-AAD-CMK, EGTA, and CMA. Pairwise comparisons with respect to Treg, BiTE, and inhibitors of the granzyme-perforin pathway were made. All tests were conducted in triplicate wells and independently repeated. Horizontal bars represent a statistical significance between compared groups of P < 0.05. C, IHC analysis of human GBM shows diffuse infiltration of Foxp3+ Tregs (AEC) expressing detectable levels of GrB (DAE).
previous studies have shown that, in general, BiTE-mediated antitumor activity is indeed contact-mediated, and has the ability to induce cytolytic synapses that are indistinguishable from those naturally occurring between effector TCR and MHC (24). Thus, while our findings here were shown using a single test molecule against EGFRvIII, we expect that BiTEs targeting other tumor antigens may likewise mediate similar effects. Overall, this work advances our understanding of potential functions for suppressive immune cells in the tumor microenvironment; however, the true functional relevance of $T_{reg}$-mediated tumor killing remains unknown. The implications of these findings will need to be explored in future studies across broader areas of autoimmunity and immunotherapy where $T_{reg}$ biology is relevant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

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References

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