Human Regulatory T Cells Kill Tumor Cells through Granzyme-Dependent Cytotoxicity upon Retargeting with a Bispecific Antibody

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Abstract
A major mechanism by which human regulatory T cells (Treg) have been shown to suppress and kill autologous immune cells is through the granzyme-perforin pathway. However, it is unknown whether Treg 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 diffuse 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); 163–7. ©2013 AACR.
BiTEs may circumvent certain aspects of Treg-mediated suppression, but also have broader implications with regard to the natural functional role of activated, tumor-infiltrating Tregs, 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 LA4 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 (BN13), Granzyme A (CB9), Granzyme B (GB11), and Perforin (G9) were purchased from BD Biosciences. Anti-FoxP3 antibody (PCH101) and the FoxP3 Staining Buffer Set were purchased from eBioscience and BD Biosciences. Anti-FoxP3 antibody (PCH101) and the FoxP3 Staining Buffer Set were purchased from eBioscience and BD Biosciences. Anti-FoxP3 antibody (PCH101) and the FoxP3 Staining Buffer Set were purchased from eBioscience and BD Biosciences. Anti-FoxP3 antibody (PCH101) and the FoxP3 Staining Buffer Set were purchased from eBioscience and BD Biosciences. Anti-FoxP3 antibody (PCH101) and the FoxP3 Staining Buffer Set were purchased from eBioscience and BD Biosciences. Anti-FoxP3 antibody (PCH101) and the FoxP3 Staining Buffer Set were purchased from eBioscience and BD Biosciences. Anti-FoxP3 antibody (PCH101) and the FoxP3 Staining Buffer Set were purchased from eBioscience and BD Biosciences. Anti-FoxP3 antibody (PCH101) and the FoxP3 Staining Buffer Set were purchased from eBioscience and BD Biosciences. Anti-FoxP3 antibody (PCH101) and the FoxP3 Staining Buffer Set were purchased from eBioscience and BD Biosciences. Anti-FoxP3 antibody (PCH101) and the FoxP3 Staining Buffer Set were purchased from eBioscience and BD Biosciences.

Treg 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 PBMCs 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% (CD25+ FoxP3). Where noted, purified Treg were prepared with the Treg Expansion Kit (Miltenyi Biotec) at a MACS™ bead Particle-to-Treg 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 × 10^6 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 ^51^Cr 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 Tregs 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 1 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+ Tregs, 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 Tregs, we first isolated naturally occurring human Tregs 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.
Redirected Regulatory T Cells Kill Tumors

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, Tregs 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, Tregs remained refractory both in terms of their ability to produce inflammatory cytokines (Fig. 1D) and proliferate when compared with CD4⁺ CD25⁺ helper T cells (Teff; 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 cultured 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 Tregs 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 Tregs 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 Tregs 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 Tregs 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 bscAb or Tregs 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...
Tregs might be redirected to kill tumor cells through engagement of bscAbs on Tregs and shown that bscAb-mediated activation of 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 of P < 0.05 between groups of three donors each defined by the presence of either EGFRvIII-specific or control BITE.

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 Treg suppression 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 Treg might be redirected to kill tumor cells through engagement with a bscAb. One previous study has explored the impact of bscAbs on Treg and shown that bscAb-mediated activation of Treg suppresses effector cell proliferation and abrogates antitumor efficacy (23). However, the direct effects of bscAb-redirected Treg on target tumor cells were not discussed.

Furthermore, because their work employed the use of rapamycin to expand Treg in vivo, an additive which is known to completely suppress GrB expression in Treg (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 alleloimmunogenic 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.

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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.

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