Pan-Bcl-2 Inhibitor, GX15-070 (Obatoclax), Decreases Human T Regulatory Lymphocytes while Preserving Effector T Lymphocytes: A Rationale for Its Use in Combination Immunotherapy

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Pan-Bcl-2 Inhibitor, GX15-070 (Obatoclax), Decreases Human T Regulatory Lymphocytes while Preserving Effector T Lymphocytes: A Rationale for Its Use in Combination Immunotherapy

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Bcl-2 inhibitors are currently being evaluated in clinical studies for treatment of patients with solid tumors and hematopoietic malignancies. In this study we explored the potential for combining the pan-Bcl-2 inhibitor GX15-070 (GX15; obatoclax) with immunotherapeutic modalities. We evaluated the in vitro effects of GX15 on human T cell subsets obtained from PBMCs in terms of activation, memory, and suppressive function. Our results indicated that in healthy-donor PBMCs, mature-activated T cells were more resistant to GX15 than early-activated T cells, and that GX15 preserved memory but not non-memory T cell populations. Furthermore, GX15 increased the apoptosis of regulatory T cells (Tregs), profoundly downregulated FOXP3 and CTLA-4 in a dose-dependent manner, and decreased their suppressive function. Treating PBMCs obtained from ovarian cancer patients with GX15 also resulted in increased CD8+ Treg and CD4+ Treg ratios. These results support preclinical studies in which mice vaccinated before treatment with GX15 showed the greatest reduction in metastatic lung tumors as a result of increased apoptotic resistance of mature CD8+ T cells and decreased Treg function brought about by GX15. Taken together, these findings suggest that when a Bcl-2 inhibitor is combined with active immunotherapy in humans, such as the use of a vaccine or immune checkpoint inhibitor, immunotherapy should precede administration of the Bcl-2 inhibitor to allow T cells to become mature, and thus resistant to the cytotoxic effects of the Bcl-2 inhibitor. The Journal of Immunology, 2014, 192: 000–000.

GX15-070 (GX15; obatoclax), a pan-Bcl-2 inhibitor, has been widely tested in clinical trials ever since the U.S. Food and Drug Administration granted it orphan drug status for the treatment of chronic lymphocytic leukemia. GX15 has also been tested preclinically and clinically for efficacy in acute myelogenous leukemia (1), mantle cell lymphoma (2), multiple myeloma (3), myelofibrosis (4), and solid tumors such as small-cell lung cancer (5–9).

GX15 is a synthetic derivative of bacterial prodigines belonging to the polypyrrole class of molecules. GX15 mimics the BH3 domain of the antiapoptotic family members of Bcl-2 but differs from other Bcl-2 inhibitors by having consistent binding properties across all antiapoptotic Bcl-2 family members, including Bcl-2, Bcl-xL, Bcl-w, myeloid-cell leukemia differentiation protein-1 (Mcl-1), and Bak, and is thus classified as a pan-Bcl-2 inhibitor. For instance, other Bcl-2 inhibitors such as ABT-737 and ABT-263 have higher binding affinity to Bcl-2 and Bcl-xL than does GX15, but they do not bind to all Bcl-2 family members (most notably, not to Mcl-1) (10, 11). Therefore, tumor cells may become resistant to ABT-737 and ABT-263 by overexpression of Mcl-1, which GX15 has been shown to inhibit (12).

In preclinical studies, a wide range of GX15 concentrations was used depending on the targets to be assayed. For instance, IC50s of GX15 in human lung cancer cell lines ranged from 1.33 to 15.4 μM (8). In clinical studies, Cmax of GX15 was reported to be in the range of 0.03–0.36 μM (11). In a phase I dose-escalation study of GX15 in patients with advanced solid tumors or lymphoma, the maximum tolerated dose using a 3-h i.v. infusion schedule in 27 patients was 20 mg/m2, with Cmax of 0.28 μM and area under the curve of 0.95 μM (5). On the basis of in vitro concentrations of GX15 and pharmacokinetic data derived from a search of the literature, we used GX15 in concentrations ranging from 0.1 to 5 μM. As a single agent, GX15 has thus far shown modest clinical activity (10, 11), leading some investigators to combine GX15 with other anticancer agents such as bortezomib (a proteasome inhibitor) (13, 14) and SNDX-275 (a histone deacetylase inhibitor) (15–17). These combinations have had an enhanced antitumor effect against hematologic malignancies.

In a preclinical model, we previously investigated the potential of GX15 to synergize with vaccine-based immunotherapy using a recombinant vaccine (recombinant vaccinia [rV] and recombinant fowlpox [rF]) encoding the tumor-associated carcinoembryonic Ag (CEA) and a triad of costimulatory molecules (rV/F-CEA-TRICOM) in CEA-transgenic mice (18). We determined that the sensitivity of murine lymphocytes to GX15 was dependent on their activation status, as mature-activated CD69+ lymphocytes were more resistant to GX15 than early-activated CD69+ lymphocytes in vitro (18). This finding suggested that GX15 should ideally be administered after lymphocytes have undergone full maturation postvaccination (18). In addition, GX15 impaired the...
suppressing the function of murine regulatory T cells (Tregs) isolated from GX15-treated mice (18). Finally, sequential combination therapy with rVF-CEA-TRICOM vaccine followed by GX15 effectively reduced orthotopic pulmonary tumors (18), providing a rationale for designing similar combination protocols for clinical trials.

In this study, we evaluated the effect of GX15 on specific subsets of human T lymphocytes. Using PBMCs from healthy donors and ovarian cancer patients, GX15 toxicity depended on the activation status of human T lymphocytes, as indicated by CD69 expression. Furthermore, GX15 downregulated expression levels of both FOXP3 and CTLA-4 in human Tregs and decreased their suppressive function. The data obtained from this study provide a further rationale for the clinical translation of the combination of active immunotherapy agents in a temporal regimen with the Bcl-2 inhibitor GX15.

Materials and Methods

Drug preparation
GX15 (obatoclax) was obtained through an agreement between the Cancer Therapeutic Evaluation Program of the National Cancer Institute and Teva Pharmaceuticals (Petah Tikva, Israel). The GX15 was dissolved in DMSO at a concentration of 200 mM. For treatment of human PBMCs or isolated CD8+ T cells, 200 mM GX15 was diluted accordingly and added at 1 µl/10^6 cells/mL at final concentrations ranging from 0.1 to 5 µM.

Isolation of regulatory T cells
Regulatory T cells were isolated from PBMCs from healthy donors using a CD4+CD25+CD127dim/– Regulatory T Cell Isolation Kit II (Miltenyi Biotec, Auburn, CA), according to the manufacturer’s protocol.

Proliferation analysis
CellTrace Violet (CTV) Cell Proliferation Kit (Molecular Probes, Eugene, OR) was used, with some modifications, to label T lymphocytes. First, we prepared a cell suspension of 10^7 cells/mL and a 5 mM stock solution of CTV, then added 0.2 µl of the 5 mM CTV stock solution/1 ml of the cell suspension for a final working concentration of 1 µM. CTV-containing cells were incubated at 37°C in the dark for 10 min. The reaction was stopped by adding 5% volume of cold medium and incubating on ice for 5 min. Cells were spun, resuspended in a prewarmed medium, and incubated at 37°C in the dark for at least 10 min to facilitate acetate hydrolysis.

Early and prolonged activation of T lymphocytes
For early activation of T lymphocytes, 24-well plates were coated with 750 µl anti-CD3 (clone UCHT1; BD Biosciences, San Jose, CA) at 0.5 µg/ml in PBS and then kept at 4°C. Cryopreserved PBMCs from healthy donors were thawed and rested at 37°C with 5% CO2 overnight prior to stimulation. For rest, PBMCs at 1 x 10^6 cells/mL in IMDM containing 10% human serum were added 5 ml/well (5 x 10^6 PBMCs/well) in 6-well plates overnight at 37°C, 5% CO2. After resting overnight, nonadherent cells were collected and then combined with adherent cells, which were gently removed with a cell scraper. PBMCs were then prepared at 1 x 10^6 cells/mL, followed by the addition of soluble anti-CD28 Ab (clone CD28.2; BD Biosciences) at a final concentration of 0.5 µg/mL. Anti-CD3–coated plates were washed once with 1 ml PBS, and 1 ml (1 x 10^6 cells) of the anti-CD28–containing cell suspension in a medium containing IL-7 and IL-15 (PeproTech, Rocky Hill, NJ) at 10 ng/ml for each cytokine was added to each well of the anti-CD3–coated plates. Cells were stimulated for 3 d. For prolonged activation, early-activated cells were transferred to 6-well plates at 1 x 10^6 cells/well (total 3 ml/well) in IL-7/IL-15 containing medium on day 3. Cells were replenished with IL-7 and IL-15 with 3 ml IL-7/IL-15–containing medium/well on day 5. On day 7, cells were harvested, labeled using a CTV Cell Proliferation Kit, and then replated in 24-well plates at 1 x 10^6 cells/well (total 1 ml/well) in fresh IL-7/IL-15 medium. On day 9, cells were replenished with 1 ml of the IL-7/IL-15 medium.

Expansion of Tregs
Isolated Tregs were expanded using a Treg Expansion Kit (Miltenyi Biotec) according to the manufacturer’s protocol. Tregs were activated with CD3/CD28 MACS® Beads (Miltenyi Biotec) for 1 d, then supplemented with IL-2 (PeproTech) at 500 U/ml during the 14-d expansion period.

Treg suppression assay
Tregs were first pretreated with GX15 at 1 µM for 24 h. CD4+/CD25– effector T (Teff) cells (1 x 10^6 cells/ml) were cultured alone or cocultured with Tregs (1 x 10^6, 5 x 10^5, or 1 x 10^5 cells/ml) with 1 µg/ml plate-bound anti-CD3 (clone OKT3; eBioscience, San Diego, CA) and irradiated (3500 rad) T cell–depleted PBMCs (1 x 10^6 cells/ml) in a 96-well flat-bottom plate at 37°C and 5% CO2. On day 4, T cell proliferation was measured by [3H]thymidine (PerkinElmer, Waltham, MA) incorporation at 1 µCi (0.037 MBq)/well and quantified 16 h later using a Wizard 2 gamma counter. Proliferation of CD4+CD25– T cells without Tregs was defined as 100% proliferation. Percent suppression was calculated using the following formula: (cpm of Teff cells alone – cpm of Teff cells treated with Treg)/cpm of Teff cells alone.

In vitro treatment with GX15
GX15 at a concentration of 5 µM was used for early and prolonged activation studies of PBMCs from healthy donors (n = 3). GX15 at a concentration of 1 µM was used for Treg suppression assay. GX15 at concentrations of 0.1, 1.0, and 5.0 µM was used to treat expanded Tregs and PBMCs taken from stage IIIc or IV ovarian cancer patients (n = 4) who had progressed on chemotherapy. All samples were obtained prior to experimental therapy but after enrollment on a National Cancer Institute Institutional Review Board–approved clinical trial (NCT00088413). Equivalent concentrations of DMSO were used as controls in each study.

Flow cytometry analysis; surface and intracellular marker assays
An LSR-II flow cytometer (BD Biosciences) was used for multiparametric flow cytometry analysis. Because GX15 emits a red color, photomultiplier tube settings had to be adjusted, in particular the FITC and PE channels, and appropriate isotype controls or fluorescence minus one controls were used for gating at each GX15 concentration. A Live/Dead Fixable Blue Dead Cell Stain Kit (Molecular Probes) was used to exclude dead cells. The following human mAbs were used to stain PBMCs from healthy donors: FITC-CD45RA, PerCP-Cy5.5 Annexin V, and V500-CD3 (BD Biosciences), PE-Cy7-CD69, APC-CD4, and AF700-CD8 (eBioscience). The following human mAbs were used to stain enriched and expanded Tregs: V500-CD3, Alexa Fluor 647–cleaved poly(ADP-ribose) polymerase (PARP), PE-CD127, PerCP-Cy5.5 Annexin V, APC-Cy7-CD25 (BD Biosciences), Alexa Fluor 700-CD4, and PE-Cy7-FOXP3 (eBioscience). The following mAbs were used to stain PBMCs from ovarian cancer patients: FITC-CD45RA, PerCP-Cy5.5 Annexin V, APC-Cy7-CD25 (BD Biosciences), PE-CD4, APC-CTLA-4, Brilliant Violet 510-CD8, Brilliant Violet 421-CD69, Brilliant Violet 605-CD127 (BioLegend, San Diego, CA); and PE-Cy7-FOXP3 (eBioscience). A FOXP3/Transcription Factor Staining Buffer set (eBioscience) was used for intracellular staining of FOXP3, CTLA-4, and cleaved PARP.

Statistical analysis

Unless specified, results of tests of significance are indicated as p values, derived from a two-tailed Mann–Whitney U test. All p values were derived at 95% using GraphPad Prism 6 statistical software for PCs.

Results

Prolonged activation made enriched CD8+ T cells from healthy-donor PBMCs more resistant to GX15 than early-activated CD8+ T cells
We first evaluated the extent to which GX15 affects human CD4+ and CD8+ T cells from healthy-donor PBMCs (n = 3) after early or prolonged activation (Fig. 1). To test early activation, PBMCs were stimulated with anti-CD3 and anti-CD28 in the presence of IL-7 and IL-15 for 3 d, then treated with GX15 for 24 h at 5 µM (Fig. 1A). To test prolonged activation, PBMCs that had been stimulated for 3 d were maintained in culture with IL-7 and IL-15 for an additional 7 d and then treated with GX15 for 24 h at 5 µM (Fig. 1A). Results showed that GX15 significantly decreased the number and viability of PBMCs after early activation, but had no significant effect after prolonged activation (Fig. 1A). To deter-
FIGURE 1. CD4+ and CD8+ T cells from healthy-donor PBMCs were more sensitive to GX15 after early activation than after prolonged activation. (A) For early activation, healthy-donor PBMCs were stimulated with anti-CD3 and anti-CD28 Abs for 3 d and then treated with GX15 for 24 h at 5 μM on day 3. For prolonged activation, early-activated PBMCs were supplemented on day 3 with IL-7 (10 ng/ml) and IL-15 (10 ng/ml) for an additional 7 d and then treated with GX15 for 24 h at 5 μM on day 10. Bar graphs show the effect of GX15 on cell number and viability of PBMCs from three healthy donors after early and prolonged activation, as measured by trypan blue exclusion. (B) The gating strategy to analyze percentages of early- and prolonged-activated CD4+ and CD8+ T cells undergoing apoptosis, as measured by cleaved PARP expression after GX15 treatment, is shown. Bar graphs show the apoptotic effect of GX15 on early- and prolonged-activated CD4+ and CD8+ T cells from three healthy donors. Error bars represent SE of mean of triplicates. The numbers in parentheses were calculated by dividing the GX15 group by the control group. *p < 0.05, statistical significance.
mine the effect of GX15 on early- and prolonged-activated CD4+ and CD8+ T cells, we performed a flow analysis to measure the level of cleaved PARP in live GX15-treated T cells (Fig. 1B). Results showed that there was a greater increase in apoptosis, as measured by the expression level of cleaved PARP, in both early-activated CD4+ and CD8+ T cells compared with their prolonged-activated counterparts after GX15 treatment (Fig. 1B). Thus, CD4+ and CD8+ T cells that had been activated and then maintained in culture were more resistant to GX15 compared with early-activated CD4+ and CD8+ T cells.

Early-activated (CD69+) T cells from healthy-donor PBMCs were more sensitive to GX15 compared with prolonged-activated (CD69−) T cells

We next examined whether expression of the early-activation marker CD69 on human CD4+ and CD8+ T cells made the cells more sensitive to GX15 after early and prolonged activation (Fig. 2). We analyzed nonapoptotic (cleaved PARP− and annexin V−) cells taken from PBMCs of 3 additional healthy donors who had undergone either early or prolonged activation in vitro (Fig. 2). Results showed that GX15 at 5 μM decreased CD69 expression to a greater extent in early-activated CD4+ (two of three donors) and CD8+ T cells (all donors) than in prolonged-activated CD4+ and CD8+ T cells (Fig. 2). This suggested that CD69+ T cells are more sensitive to GX15 than CD69− T cells, especially after early activation. We then examined the effect of GX15 on T cell proliferation based on CD69 expression. GX15 had a greater inhibitory effect on the highly proliferating (generation 3) CD4+CD69+ (all donors) and CD8+CD69+ (two of three donors) populations after early activation than after prolonged activation (Fig. 2). In addition, GX15 had no effect on the proliferation of the CD69− population after prolonged activation, most notably in CD8+ T cells (Fig. 2).

Nonmemory (CD45RA+) T cells were more sensitive to GX15 than memory (CD45RA−) T cells

Our results showed that the activation status of T cells, based on CD69 expression, can determine T cell sensitivity to GX15. Because Bcl-2 has been shown to play a dynamic role in T cell differentiation, memory formation, and survival (19–22), we explored the extent to which the memory status of T cells is affected by treatment with GX15 after early and prolonged activation (Fig. 4). Treatment with 5 μM GX15 resulted in a significant decrease in the proportion of nonmemory (CD45RA−) CD4+ and CD8+ T cells after early activation in all donors, whereas the proportion of memory (CD45RA−) T cells was preserved (Fig. 4). In prolonged activation, 5 μM GX15 resulted in a decrease in CD4+CD45RA− cells in one of three donors, whereas CD8+CD45RA− cells decreased in all donors (Fig. 4). As with CD69 expression, we also examined the effect of GX15 on T cell proliferation based on CD45RA expression. Results showed that GX15 greatly inhibited the highly proliferating (generation 3) CD45RA− and CD45RA+ cells in both CD4+ and CD8+ T cell populations after early activation (Fig. 5). With prolonged activation, CD45RA− proliferation was notably maintained in the CD8+ T cell population (Fig. 5).

Treatment with GX15 resulted in apoptosis and downregulation of FOXP3 in Tregs

To study the effect of GX15 on Tregs from human PBMCs, we first isolated and then expanded human Tregs from healthy-donor PBMCs (Fig. 6A). We determined the purity of isolated and expanded human Tregs by flow cytometry analysis and gating of live CD4+, CD25+, FOXP3+, and CD127− cells (Fig. 6B). Activation of T cells has been shown to expose phosphatidylserine on the cell surface (23, 24), which may confound the results derived from

FIGURE 2. Early-activated (CD69+) T cells were more sensitive to GX15 than CD69− T cells after early and prolonged activation. The gating strategy and representative flow cytometry analysis of the effect of GX15 on CD69+ T cells from PBMCs of three healthy donors. Error bars represent SE of mean of triplicates. *p < 0.05, statistical significance.
FIGURE 3. Proliferation of early-activated (CD69+) T cells was more sensitive to GX15 than that of CD69– T cells after early and prolonged activation. The gating strategy and representative proliferation analysis of CTV-labeled CD69+ and CD69– T cells treated with GX15 (5 μM) after early and prolonged activation are shown. Bar graphs indicate the percentage of proliferating CD69+ and CD69– T cells in generation 3 after GX15 treatment. Error bars represent SE of mean of triplicates. The numbers in parentheses were calculated by dividing the GX15 group by the control group. *p < 0.05, statistical significance.
FIGURE 4. Memory (CD45RA\(^+\)) T cells were resistant to GX15 after early and prolonged activation. The gating strategy and representative flow cytometry analysis of the effect of GX15 on nonmemory (CD45RA\(^+\)) and memory (CD45RA\(^-\)) T cells after early and prolonged activation are shown. Bar graphs indicate GX15-induced sensitivity of CD45RA\(^+\) and CD45RA\(^-\) T cells from PBMCs of three healthy donors. Error bars represent SE of mean of triplicates. The numbers in parentheses were calculated by dividing the GX15 group by the control group. *p < 0.05, statistical significance.
FIGURE 5. Proliferation of nonmemory (CD45RA⁺) T cells was more sensitive to GX15 than that of memory (CD45RA⁻) T cells after early and prolonged activation. The gating strategy and representative proliferation analysis of CTV-labeled CD45RA⁺ and CD45RA⁻ T cells treated with GX15 (5 μM) after early and prolonged activation are shown. Bar graphs indicate the percentage of proliferating CD45RA⁺ and CD45RA⁻ T cells in generation 3 after GX15 treatment. Error bars represent SE of mean of triplicates. The numbers in parentheses were calculated by dividing the GX15 group by the control group. *p < 0.05, statistical significance.
annexin V–based measurements of apoptotic cells. Therefore, we measured the level of apoptosis induced by GX15 in Tregs by cleaved PARP expression on live annexin V– cells (Fig. 6C). We also examined the level of FOXP3 expression in Tregs treated with GX15, because other studies have shown that the mean fluorescence intensity (MFI) of FOXP3 positively correlates with Treg function (25–27). Our results showed that GX15 at 0.1, 1, and 5 μM for 24 h increased cleaved PARP expression in Tregs (Fig. 6D). More interestingly, GX15 treatment noticeably downregulated expression of FOXP3, suggesting that GX15 may potentially impair Treg function (Fig. 6D).

Tregs were more sensitive to GX15 than CD4+ Teff cells

To determine whether Tregs and Teff cells have different sensitivity to GX15, we analyzed the effect of GX15 on PBMCs from several cancer patients. PBMCs from ovarian cancer patients (n = 4) were thawed and then rested for 24 h before treatment with GX15 at 0.1, 1, and 5 μM (Fig. 7A). Results indicated that Tregs were significantly more sensitive than CD4+ T cells to GX15 at 1 and 5 μM (Fig. 7B). Furthermore, Tregs expressed a significantly higher level of the early-activation marker CD69+ compared with CD4+ T cells, which could explain the higher sensitivity of Tregs to GX15 compared with CD4+ T cells (Fig. 7C). In addition, at 1 μM GX15 downregulated FOXP3 in Tregs from all four patient samples and further decreased FOXP3 expression at 5 μM (Fig. 7D). CTLA-4 expression in Tregs also decreased after treatment with 0.1 μM GX15, and decreased further in a dose-dependent manner at 1 and 5 μM (Fig. 7D). Taken together, these findings show not only that Tregs are more sensitive than CD4+ T cells to GX15 but also that GX15 treatment likely impairs the functional capacity of Tregs.

Treatment with GX15 resulted in functional impairment of Tregs

Isolated Tregs from healthy-donor PBMCs were tested for their suppressive function after treatment with GX15. We used a 1 μM concentration of GX15 rather than a 5 μM concentration because it more effectively maintained the viability of Tregs (Fig. 8B). In addition, downregulation of FOXP3 with 1 μM GX15 (Fig. 6D) was comparable to that of 5 μM GX15 (Fig. 6D) and significantly decreased CTLA-4 expression in Tregs undergoing apoptosis (Fig. 8C). Results also showed that 1 μM GX15 reduced the suppressive function of Tregs in all three donors (Fig. 8D).

Ratios of both CD4+ and CD8+ T cells to Tregs increased after GX15 treatment

Because Tregs are more sensitive than CD4+ T cells to GX15 (Fig. 7B), we analyzed the extent to which GX15 treatment altered the ratio of CD4+ or CD8+ T cells to Tregs in PBMCs from cancer patients (Fig. 9A). Our results showed that GX15 at 5 μM significantly increased the ratio of CD4+ and CD8+ T cells to Tregs in three of four PBMC samples (Fig. 9B).
Discussion

Because improved tumor control would likely require combining GX15 with another anticancer modality, we decided to examine the potential of GX15 in combination with immunotherapy by analyzing the effects of GX15 on human T lymphocytes. Specifically, we focused on measuring the in vitro sensitivity of differently activated T lymphocytes to the pan-Bcl-2 inhibitor. We also studied the effects of GX15 in vitro treatment on Tregs and on the balance of effector versus suppressor immune elements in the PBMCs of cancer patients. This study follows a preclinical investigation in mice that tested the use of the tumor Ag-specific vaccine rV/F-CEA-TRICOM in an orthotopic pulmonary tumor model (18).

FIGURE 7. Tregs were more sensitive than CD4+ T cells to GX15. (A) Schematic outline of GX15 treatment of PBMCs from ovarian cancer patients (n = 4). PBMCs were rested 1 d prior to treatment and harvested 24 h posttreatment with GX15. (B) Bar graphs show the effect of GX15 at 0.1, 1, and 5 μM on Tregs and CD4+ T cells. Change in percentage of Tregs or CD4+ T cells was calculated as follows: for Tregs (%TregGX15 - %Treg DMSO)/%Treg DMSO × 100. For CD4+ T cells, (%CD4GX15 - %CD4DMSO)/%CD4 DMSO × 100. Average and SE of mean are also indicated. (C) Flow plots show percentages of Tregs and CD4+ T cells expressing CD69. Bar graphs indicate average and SE of mean. (D) Histograms show effect of GX15 on expression of FOXP3 and CTLA-4 in Tregs (red line, GX15 treatment; black line, DMSO control). Bar graphs indicate average and SE of mean.
From this mouse study, we determined that GX15 did not affect mature-activated CD8+/CD69– T cells but did decrease the suppressive function of Tregs, thereby enhancing the vaccine-mediated antitumor efficacy of rV/F-CEA-TRICOM in a Lewis lung tumor model (18). On the basis of these results, the timing of GX15 administration was determined to be critical, and that an immunotherapeutic regimen should precede GX15 treatment to achieve optimal antitumor effects.

Initially, we determined that human T lymphocytes were more sensitive to GX15 after early activation than prolonged activation (Fig. 1B). This result was consistent with our preclinical study (18), which found that GX15 should be administered long enough after vaccination to allow activated CD8+ T lymphocytes to undergo sufficient maturation and become resistant to the Bcl-2 inhibitor. We thus determined that sequential treatment with vaccine followed by GX15 would be more immune-favorable than coadministration of both agents or GX15 treatment followed by vaccine.

We further investigated the effect of GX15 on human T lymphocyte subsets after early and prolonged activation, based on their activation, memory, and suppressive function. Ideally, testing a large number of PBMC samples from cancer patients would have been most appropriate. However, due to the scarcity of patient samples available for this study, we felt the best alternative was to use PBMCs from healthy donors. We found that after early and prolonged activation, CD4+ and CD8+ T cells (obtained from PBMCs of healthy donors) that expressed the early-activation marker CD69 were sensitive to GX15 (Fig. 2), which corresponded with data from the mouse study (18). An underlying mechanism for the greater sensitivity of human CD69+ T cells to GX15 could be the increased expression of Mcl-1 induced during early activation of T cells. It has been shown that human T cells upregulate the Mcl-1

**FIGURE 8.** The suppressive activity of Tregs is reduced after GX15 treatment. (A) Schematic outline of GX15 treatment of Tregs isolated from normal donor PBMCs (n = 3). Purified Tregs were treated with 1 μM GX15 for 24 h, after which GX15-treated Tregs were washed and then cocultured with conventional CD4+ T cells for 4 d, after which the addition of 3H was added for 24 h, and then its incorporation was measured. (B) Bar graphs indicate the % change in viability and cell number and in cleaved PARP (cPARP) after GX15 treatment at 1 μM. Average and SE of mean of the percent change are also shown. (C) Bar graphs show the percent change in MFI of FOXP3 and CTLA4 in cPARP+ and cPARP− Tregs. Average and SE of mean of the percent change are also shown. (D) Bar graphs indicate the suppressive activity of GX15-treated Tregs at various effector:Treg ratios.

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FIGURE 9. In PBMCs from ovarian cancer patients, CD4⁺:Treg and CD8⁺:Treg ratios increased after in vitro treatment with GX15. (A) Schematic outline of GX15 treatment of PBMCs from ovarian cancer patients (as described in Fig. 7A). (B) Graphs show changes in the CD4⁺:Treg and CD8⁺:Treg ratios in PBMCs from ovarian cancer patients after treatment with GX15. The CD4⁺:Treg and CD8⁺:Treg ratios were calculated using the percentage of non-Treg CD4⁺ T cells or CD8⁺ T cells and the percentage of Tregs after treatment with GX15 or control. Means and SE of means are indicated. Tables show the number and percentage of patients whose CD4⁺:Treg and CD8⁺:Treg ratios increased (> +20%), decreased (< −20%) or remained unchanged (< +20% and > −20%) after GX15 treatment.

gene within 10 h after TCR ligation, suggesting a role for Mcl-1 in early activation (28). In addition, GX15 noticeably decreased highly proliferating cells in the CD69⁺ T cell population but had less of an effect on low-proliferating CD69⁺ T cells (Fig. 3), suggesting that GX15 has a cytostatic effect (1, 29). In contrast, GX15 altered the proliferation of CD69⁺ T cells to a lesser degree than CD69⁻ T cells, most notably in prolonged activation (Fig. 3).

It has been shown that Bcl-2 sends signals necessary for the survival and maintenance of memory cells by way of the γ-chain cytokines IL-7 and IL-15 (30, 31). For this reason, we examined the effect of GX15 on human memory T cells after prolonged activation upon IL-7/IL-15 in vitro maintenance (Figs. 4, 5). We found that GX15 treatment consistently decreased the nonmemory compartment (CD45RA⁺) of the CD8⁺ T cell population in healthy-
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Disclosures

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