

# Attenuation of CD8<sup>+</sup> T-Cell Function by CD4<sup>+</sup>CD25<sup>+</sup> Regulatory T Cells in B-Cell Non-Hodgkin's Lymphoma

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## Abstract

**The underlying mechanisms by which tumor cells are resistant to CTL-mediated apoptosis are not clear. Using a human model of B-cell non-Hodgkin's lymphoma (B-cell NHL), we show that intratumoral T<sub>reg</sub> cells inhibit the proliferation and granule production of activated autologous infiltrating CD8<sup>+</sup> T cells. Our results also show that degranulation and subsequent cytotoxic activity of infiltrating CD8<sup>+</sup> T cells exposed to lymphoma B cells is completely attenuated by the presence of intratumoral T<sub>reg</sub> cells. Furthermore, we show that increased numbers of intratumoral T<sub>reg</sub> cells correlates with the number of CD8<sup>+</sup> T cells in biopsy specimens from patients with B-cell NHL, supporting the *in vitro* findings that intratumoral T<sub>reg</sub> cells inhibit proliferation of infiltrating CD8<sup>+</sup> T cells. Taken together, these data indicate that human lymphoma B cells are sensitive to autologous CTL-mediated cell death but are protected by the inhibitory function of intratumoral T<sub>reg</sub> cells.** (Cancer Res 2006; 66(20): 10145-52)

## Introduction

B-cell non-Hodgkin's lymphoma (NHL) is characterized by an accumulation of malignant monoclonal B cells arrested at different stages of differentiation. The microenvironment in which NHL is typically found consists of secondary lymphoid organs that are replete with immune cells. Although numerous studies have focused on the biology of the malignant B cell, there is now a growing literature suggesting that tumor infiltrating immune cells may also play a role in determining the outcome of patients with B-cell NHL (1, 2). The cellular component of NHL consists predominantly of malignant B cells, T cells, and monocytes. The specific role of intratumoral T cells and their significance in the pathogenesis of the NHL is not well understood, but there is increasing evidence indicating their relevance (3). In the nonmalignant scenario, T cells play a critical role in regulation of the immune response; yet, in the tumor microenvironment, these same T cells are unresponsive and fail to function appropriately (4, 5). The presence of an immunosuppressive network within the tumor microenvironment may therefore allow tumor cells to grow unchecked by the immune system. In murine models of NHL, it has been shown that induction of antigen-specific T-cell tolerance is one mechanism by which antitumor immunity is disrupted (6–8). Other underlying mechanisms that foster a poor immune response include reduced quality of tumor-associated antigen (TAA) presentation, aberrant cytokine and inflammatory molecule

expression, and a shift in costimulatory and coinhibitory molecule expression by TAA-presenting, antigen-presenting cells (9).

An additional mean by which tumors escape immunosurveillance is through an imbalance in the number of regulatory T cells (T<sub>reg</sub> cells). An accumulation of T<sub>reg</sub> cells in the tumor microenvironment has been shown to have a significant effect on the functional properties of infiltrating CD4<sup>+</sup> T cells and patient outcome (3, 10, 11). In previous work, we have found that T<sub>reg</sub> cells (CD4<sup>+</sup>CD25<sup>+</sup> T cells with intracellular Foxp3 and CTLA-4 expression) were overrepresented in the tumor sites of NHL. We also found that nearly all CD4<sup>+</sup>CD25<sup>+</sup> T cells isolated from NHL tumors expressed Foxp3, and that intratumoral T<sub>reg</sub> cells could suppress the functional capabilities, proliferation, and production and secretion of IFN-γ and interleukin-4, of autologous infiltrating CD4<sup>+</sup>CD25<sup>+</sup> T cells (3). In accordance with this data, Zhou et al. showed in a murine lymphoma model that tumor induced suppression of CD4<sup>+</sup> T cells was regulated in part by expansion of the T<sub>reg</sub> pool (12). Taken together, these data suggest that the presence of T<sub>reg</sub> cells within the tumor microenvironment results in a dampening of the CD4<sup>+</sup> T-cell response. What remains unanswered is whether or not T<sub>reg</sub> cells directly affect tumor-associated CD8<sup>+</sup> CTL (CD8<sup>+</sup> CTL) function, and more importantly, the ability of CTLs to kill autologous tumor cells.

Previous studies have shown that lymphoma B cells are resistant to CTL-mediated cell death (3–5), and this has been attributed to changes in their genetic or protein expression profile (3–5, 13, 14) as well as to abnormal CTL function (15–17). Additionally, there is accumulating data suggesting that T<sub>reg</sub> cells are capable of inhibiting CD8<sup>+</sup> T cells under different pathologic conditions such as infection (18, 19) or allograft transplantation *in vivo* (20, 21). Murine studies indicate that T<sub>reg</sub> cells can abrogate CD8<sup>+</sup> T cell-mediated tumor rejection by specifically suppressing cytotoxicity of expanded CD8<sup>+</sup> cells (22–24). However, little is known about the effect of intratumoral T<sub>reg</sub> cells on CTL activity in a human NHL model.

To determine the significance of T<sub>reg</sub> cells on CD8<sup>+</sup> T-cell function, we characterized the role of T<sub>reg</sub> cells in modulating antitumor responses. We show that intratumoral T<sub>reg</sub> cells isolated from NHL tumors completely inhibit proliferation and granule production of infiltrating CD8<sup>+</sup> T cells obtained from the same tumor specimen. Suppression of CD8<sup>+</sup> function by T<sub>reg</sub> cells results in an inability of tumor specific CD8<sup>+</sup> T cells to kill autologous tumor cells. We therefore show for the first time that infiltrating CD8<sup>+</sup> T cells from NHL tumors are functional, and that their activity is suppressed when T<sub>reg</sub> cells are present.

## Materials and Methods

**Patient samples.** Patients providing written informed consent were eligible for this study if they had a tissue biopsy that on pathologic review showed B-cell lymphoma and adequate tissue to perform experiments. The biopsy specimens were reviewed and classified using the WHO Lymphoma classification. This study was approved by the Institutional Review Board of

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the Mayo Clinic/Mayo Foundation. Biopsy specimens from a total of 28 patients were used for the study (see Table 1).

**Cell isolation and purification.** CD8<sup>+</sup> T cells and CD19<sup>+</sup> lymphoma B cells were isolated by using CD8 and CD19 microbeads (positive selection). CD4<sup>+</sup>CD25<sup>-</sup> or CD4<sup>+</sup>CD25<sup>+</sup> T-cell subsets were purified by using CD4<sup>+</sup>CD25<sup>+</sup> Regulatory T-cell Isolation kit (Miltenyi Biotec, Auburn, CA) as previously described (3). Purity was checked by fluorescence-activated cell sorting (FACS) analysis and was typically >95%.

**Immunohistochemistry.** Paraffin-embedded tissue was obtained from Mayo Clinic Tissue Registry and cut serially at 5  $\mu$ m. The tissue sections were deparaffinized in three changes of xylene and cleared through graded ethanol series. Endogenous peroxidase was quenched by incubation in 50% methanol/H<sub>2</sub>O<sub>2</sub>. After rinsing with tap water, all sections were pretreated 30 minutes with 50 mmol/L EDTA (pH 8) using a steamer and cooled for an additional 5 minutes. All immunohistochemical staining was done automatically on DAKO (Carpinteria, CA) Autostainer<sup>plus</sup> using the following antibodies and their corresponding detection systems: Foxp3 (Abcam, Cambridge, MA; 1  $\mu$ g/mL, DAKO Advance<sup>+</sup> DAB<sup>+</sup>), CD25 (Novocastra, Norwell, MA; 1:100, DAKO Advance<sup>+</sup>, DAB<sup>+</sup>), CD8 (DAKO; 1:100, DAKO Advance<sup>+</sup>, DAB<sup>+</sup>), CD3 (DAKO polyclone; 1:200, DAKO Advance<sup>+</sup>, DAB<sup>+</sup>), CD4 (Novocastra; 1:300, DAKO Advance<sup>+</sup>, DAB<sup>+</sup>), CD20cy (DAKO; 1:60, DAKO Advance<sup>+</sup>, DAB<sup>+</sup>), or mouse IgG1 control (DAKO; 1  $\mu$ g/mL, DAKO Envision<sup>+</sup>, DAB<sup>+</sup>). All sections were stained with hematoxylin and rinsed well in tap water. All slides were observed with light microscopy (Olympus AX70,  $\times$ 200/aperture 0.46,  $\times$ 400/aperture 0.75,  $\times$ 600/aperture 0.80; Olympus America, Melville, NY) with images captured with a SPOT RT camera and software (Diagnostic Instruments, Burlingame, CA).

**Flow cytometry and intracellular staining.** Cells ( $1 \times 10^6$ ) were washed in PBS containing 0.5% bovine serum albumin and incubated with CD3-, CD4-, CD8-, CD19-, and CD25-specific fluorochrome-conjugated antibodies and analyzed on a FACSCalibur flow cytometry (Becton

Dickinson, San Diego, CA). For intracellular staining of perforin (PFN) and granzyme B (GzmB), cells were fixed with 2% paraformaldehyde for 15 minutes, washed, permeabilized with 0.5% saponin for 30 minutes, and stained with FITC-CD3, Percp-CD8, and phycoerythrin-conjugated PFN (1:1,000 dilution; BD Pharmingen, San Diego, CA), or APC-conjugated Gzm B (1:200 dilution; GB12; Caltag, Burlingame, CA) for 30 minutes at 4°C. After washing, cells were analyzed by flow cytometry. Isotype controls were done for each sample.

**Carboxyfluorescein succinimidyl ester labeling and T-cell proliferation assay.** Cells were washed, counted, and resuspended at  $1 \times 10^7$ /mL in PBS. A stock solution of carboxyfluorescein succinimidyl ester (CFSE; 5 mmol/L) was diluted 1:100 with PBS and added to the cells for a final concentration of 5  $\mu$ mol/L. After 10 minutes at 37°C, cells were washed thrice with 10 volumes of PBS containing 10% fetal bovine serum. CFSE-labeled responding cells were cocultured with or without stimulating cells in the presence or absence of phytohemagglutinin (PHA; 2.5  $\mu$ g/mL) at 37°C and 5% CO<sub>2</sub>. Cells were harvested at day 3, washed, and stained with fluorochrome-conjugated antibodies for detection of surface markers for 30 minutes at 4°C. Cells were analyzed by flow cytometry. The percentage of CFSE<sup>dim</sup> was measured and calculated as the percentage of proliferated cells.

**Degranulation assay.** Degranulation assays were done by determining CD107a surface mobilization on infiltrating CD8<sup>+</sup> T cells from B-cell NHL during activation or exposure to lymphoma B cells. The assay was set up based upon a similar approach described by Betts et al. with a few modifications (25). Lymphoma B cells were plated at  $2 \times 10^5$  per well in a 48-well plate and incubated at 37°C for overnight. On the next day, the culture supernatant was removed from the wells, and the effector cells were added to the wells at various effector/target (E/T) ratios. Effector cells: (a) CD8<sup>+</sup> T cells without any treatment (CD8-R), (b) CD8<sup>+</sup> T cells activated with PHA (2.5  $\mu$ g/mL) for 5 days, (c) CD8<sup>+</sup> T cells cocultured with CD4<sup>+</sup>CD25<sup>-</sup> T cells in the presence of PHA (2.5  $\mu$ g/mL) for 5 days, (d) CD8<sup>+</sup> T cells

**Table 1. Patient characteristics**

No.	Age	Gender	Histology	Treatment	Site of disease
MF1059	66	male	Mantle cell lymphoma	Untreated	Extranodal
MF1065	52	male	Follicular grade 1	Chemotherapy	Extranodal
MF1076	69	male	Large cell lymphoma	Untreated	Lymph node
MF1077	67	female	Follicular grade 3	Chemotherapy	Lymph node
MF1093	42	male	Follicular grade 1	Untreated	Lymph node
MF1115	72	male	Small lymphocytic lymphoma	Untreated	Extranodal
MF1128	60	male	Follicular grade 2	Chemotherapy	Extranodal
MF1139	58	female	Marginal zone lymphoma	Untreated	Extranodal
MF1143	44	male	Small lymphocytic lymphoma	Rituximab	Lymph node
MF1160	86	male	Small lymphocytic lymphoma	Chemotherapy	Lymph node
MF1171	62	male	Follicular grade 2	Chemotherapy	Extranodal
MF1196	64	male	Small lymphocytic lymphoma	Untreated	Extranodal
MF1200	62	male	Small lymphocytic lymphoma	Untreated	Extranodal
MF1201	54	male	Mantle cell lymphoma	Chemotherapy	Lymph node
MF1240	59	female	Marginal zone lymphoma	Untreated	Extranodal
MF1243	58	female	Follicular grade 3	Chemotherapy	Lymph node
MF1252	67	male	Lymphoplasmacytic	Untreated	Lymph node
MF1260	70	female	Mantle cell lymphoma	Chemotherapy	Extranodal
MF1261	64	male	Follicular grade 1	Untreated	Extranodal
MF1267	69	female	Marginal zone lymphoma	Untreated	Extranodal
MF1270	54	female	Small lymphocytic lymphoma	Untreated	Lymph node
MF1275	61	female	Marginal zone	Chemotherapy	Extranodal
MF1297	70	male	Follicular grade 2	Untreated	Extranodal
MF1314	73	male	Marginal zone lymphoma	Untreated	Extranodal
MF1319	59	male	Large cell lymphoma	Chemotherapy	Lymph node
MF1327	50	male	Small lymphocytic lymphoma	Untreated	Extranodal
MF1332	72	male	Marginal zone lymphoma	Untreated	Extranodal
MF1341	62	male	Mantle cell lymphoma	Chemotherapy	Lymph node

cocultured with intratumoral  $T_{reg}$  cells in the presence of PHA (2.5  $\mu$ g/mL) for 5 days. Control wells containing either only effector cells or tumor targets were also set up with each assay. CD107a-PE (10  $\mu$ L) was added to each well at the same time as addition of effector cells. The plate was then centrifuged for 3 minutes at 1,000 rpm to facilitate immediate contact between the effector cells and the tumor targets at the bottom of the wells and incubated at 37°C for overnight. At the end of the incubation period, the cells were stained with anti-human CD8-Percp antibody and analyzed using a FACSCalibur flow cytometry.

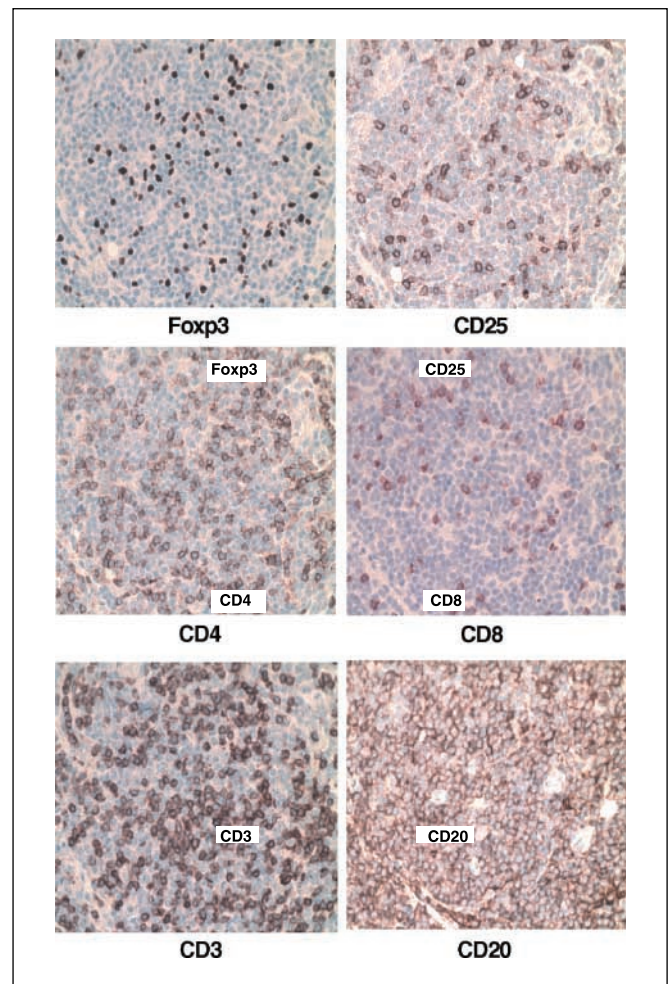
**Cytotoxicity assay.** A flow-based cytotoxicity assay was used to measure *in vitro* cellular cytotoxicity of infiltrating  $CD8^+$  T cells against lymphoma B cells as previously described (26, 27). Target cells (autologous lymphoma B cells or lymphoma B-cell lines) were labeled with 250 nmol/L of CFSE and added to 48-well plate ( $2 \times 10^5$  per well) along with different amount of effector cells in complete RPMI for 24 hours. Effector cells: (a)  $CD8^+$  T cells without any treatment (CD8-R), (b)  $CD8^+$  T cells activated with PHA (2.5  $\mu$ g/mL) for 5 days, (c)  $CD8^+$  T cells cocultured with  $CD4^+CD25^-$  T cells at 2:1 ratio in the presence of PHA (2.5  $\mu$ g/mL) for 5 days, (d)  $CD8^+$  T cells cocultured with intratumoral  $T_{reg}$  cells at 2:1 ratio in the presence of PHA (2.5  $\mu$ g/mL) for 5 days.  $CD8^+$  T cells cocultured with  $CD4^+CD25^{+/-}$  T cells were subjected to CD8-positive selection on the day  $CD8^+$  T cells were exposed to target cells. In parallel, target cells were incubated alone to measure basal apoptosis. Immediately before analysis, 1  $\mu$ g/mL (final concentration) of 7-amino-actinomycin D (7-AAD; Calbiochem, La Jolla, CA) was added to each sample and incubated for 20 minutes at 4°C in the dark. The percentage of apoptotic (7-AAD<sup>lo</sup>+7-AAD<sup>hi</sup>) cells are used to calculate the percentage of specific lysis according to the following formula: % specific lysis =  $100 \times (\% \text{ sample lysis} - \% \text{ basal lysis}) / (100 - \% \text{ basal lysis})$ . Sample lysis is the cell lysis in the presence of effectors at a given E/T ratio, and basal lysis is the cell lysis in the absence of effectors.

**Statistical analysis.** Fisher's exact test was used to compare differences in nominal variables, whereas the rank sum test, paired Student's *t* test, or the Kruskal-Wallis test was used for continuous variables.  $P < 0.05$  was considered significant. Direct correlation between percentage of  $CD4^+CD25^+$  T cells and  $CD8^+$  T cells was done with the use of regression analysis.

## Results

**Foxp3 expression in B-cell NHL.** In previous work, we identified a subset of  $CD4^+CD25^+$  Foxp3<sup>+</sup> T cells in the biopsy specimens of B-cell NHL by flow cytometry (3). In the present study, we did immunohistochemistry to confirm the presence of regulatory T cells in NHL tissue. A series of tissue slides (serial sections) from NHL tumor biopsy specimens ( $n = 11$ ) were stained with antibodies specific for Foxp3, CD25, CD4, CD8, CD3, and CD20. Each specimen also stained with an isotype control and minimal to no staining was detected (data not shown). As shown in a representative case in Fig. 1,  $CD3^+$  T cells, including both  $CD4^+$  and  $CD8^+$  subsets, are present, together with malignant  $CD19^+$  B cells, in lymphoma tissue. As expected, cells expressing Foxp3 or CD25 were also present. Although Foxp3-expressing cells could be detected in all specimens tested, the number and pattern, perifollicular or intrafollicular, varied between specimens (data not shown). The staining pattern of Foxp3<sup>+</sup> cells correlated well with the staining of  $CD25^+$  cells. This data is in accordance with our flow cytometry results showing that the vast majority of  $CD4^+CD25^+$  T cells in NHL tumors express Foxp3 and are therefore  $T_{reg}$  cells (3).

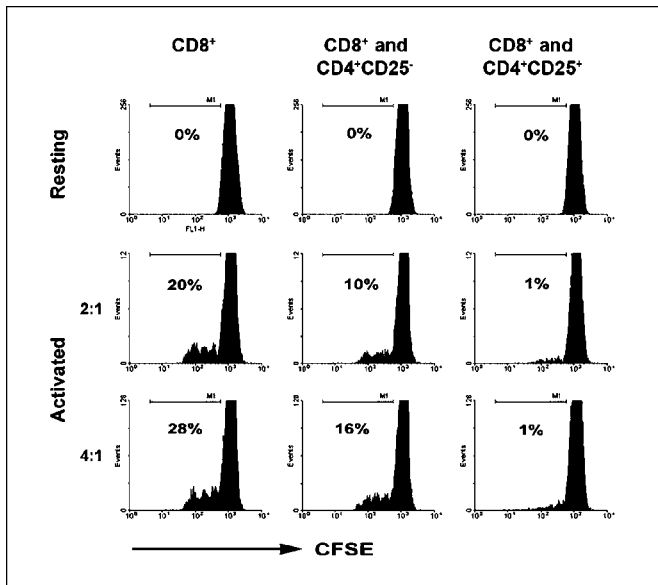
**Intratumoral  $T_{reg}$  cells suppress proliferation of autologous infiltrating  $CD8^+$  T cells in B-cell NHL.** We next wanted to determine the influence of  $T_{reg}$  cells on proliferation of  $CD8^+$  T cells. Infiltrating  $CD8^+$  T cells ( $n = 3$ ; responding cells) were labeled with CFSE and cultured either alone or with intratumoral  $T_{reg}$  cells (stimulating cells) in the presence (activated) or absence (resting) of PHA at a ratio of 4:1 and 2:1 (responding cells/stimulating cells). As a control,  $CD8^+$  T cells were also cultured in



**Figure 1.** Identification of Foxp3-expressing cells in biopsy specimens of patients with B-cell NHL. Immunohistochemical analysis of Foxp3 expression in NHL tumor tissue was done as described in Materials and Methods. Expression of CD25, CD4, CD8, CD3, and CD20 was also performed. From the same region of a serial section of the tumor specimen. Original magnification,  $\times 40$ . Representative specimen ( $n = 11$ ).

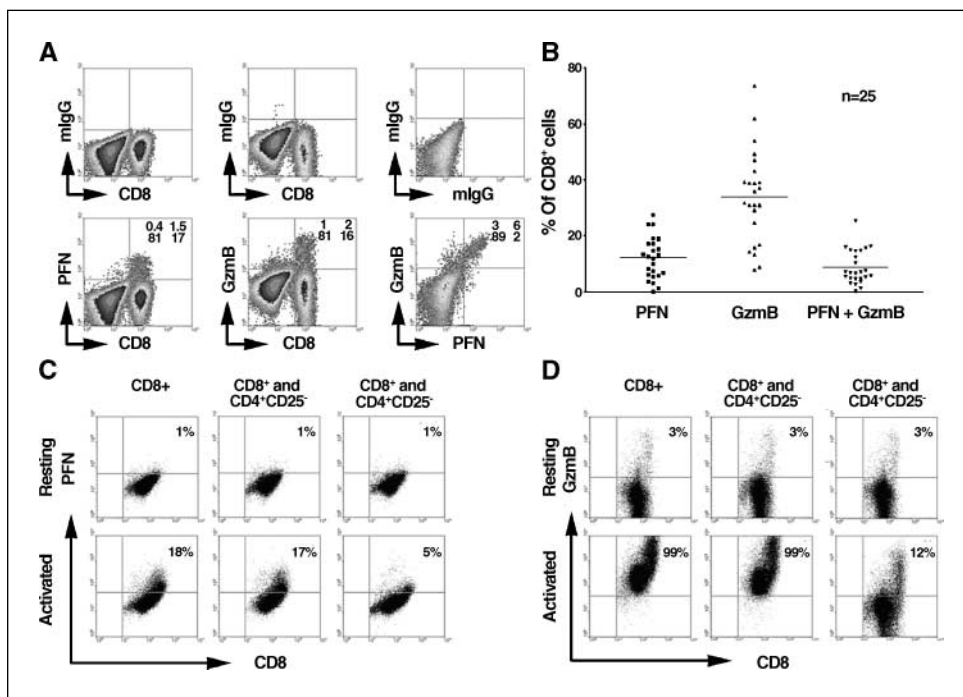
the same conditions with infiltrating  $CD4^+CD25^-$  T cells. Our results show that intratumoral  $T_{reg}$  cells completely suppress proliferation of PHA-activated infiltrating  $CD8^+$  T cells at both a 4:1 and 2:1 ratio (Fig. 2). Of note, infiltrating  $CD4^+CD25^-$  T cells partially inhibited proliferation of PHA-activated infiltrating  $CD8^+$  T cells, reducing it from 28% to 16% at a 4:1 ratio and 20% to 10% at a 2:1 ratio when compared with  $CD8^+$  T cells cultured alone, suggesting that some infiltrating  $CD4^+CD25^-$  T cells in biopsy specimens of B-cell NHL also exhibit inhibitory function.

**Intratumoral  $T_{reg}$  cells inhibit the production of PFN and GzmB by infiltrating  $CD8^+$  T cells in B-cell NHL.** Synthesis of cytolytic granules (PFN and GzmB) by  $CD8^+$  T cells is critical to their ability function as cytotoxic T cells. Therefore, our next goal was to determine the effect of intratumoral  $T_{reg}$  cells on cytolytic granule production by infiltrating  $CD8^+$  T cells. First, we determined the intracellular expression profile of PFN and GzmB in infiltrating  $CD8^+$  T cells from fresh biopsy specimens using multicolor intracellular staining (Fig. 3A). Mononuclear cells isolated from NHL biopsy specimens were costained with antibodies specific to CD3, CD8, and PFN or GzmB. Matched isotype controls were used to distinguish PFN<sup>+</sup>, GzmB<sup>+</sup>, or PFN<sup>+</sup>GzmB<sup>+</sup>



**Figure 2.** Intratumoral T<sub>reg</sub> cells inhibit the proliferation of infiltrating CD8<sup>+</sup> T cells in B-cell NHL. Proliferation of infiltrating CD8<sup>+</sup> T cells was determined by coculturing CFSE labeled CD8<sup>+</sup> T cells with autologous CD4<sup>+</sup>CD25<sup>-</sup> or CD4<sup>+</sup>CD25<sup>+</sup> T cells in the presence (activated) or absence of PHA (resting) for 3 days. The percentage of cells that lost CFSE expression were considered to have undergone proliferation. Representative of three independent experiments.

cells. As shown in Fig. 3A, PFN or GzmB were exclusively expressed by infiltrating CD8<sup>+</sup> T cells. PFN and GzmB expression could be detected individually and in some cases was found to be coexpressed. Data acquired from 25 fresh NHL samples indicate that the mean percentage of CD8-positive T cells that were PFN<sup>+</sup> was 10% (10 ± 6%), GzmB<sup>+</sup> 38% (38 ± 15%), and PFN<sup>+</sup>GzmB<sup>+</sup> 8% (8 ± 6%; Fig. 3B). These data suggest that a majority (>50%) of resting infiltrating CD8<sup>+</sup> T cells do not express PFN or GzmB.

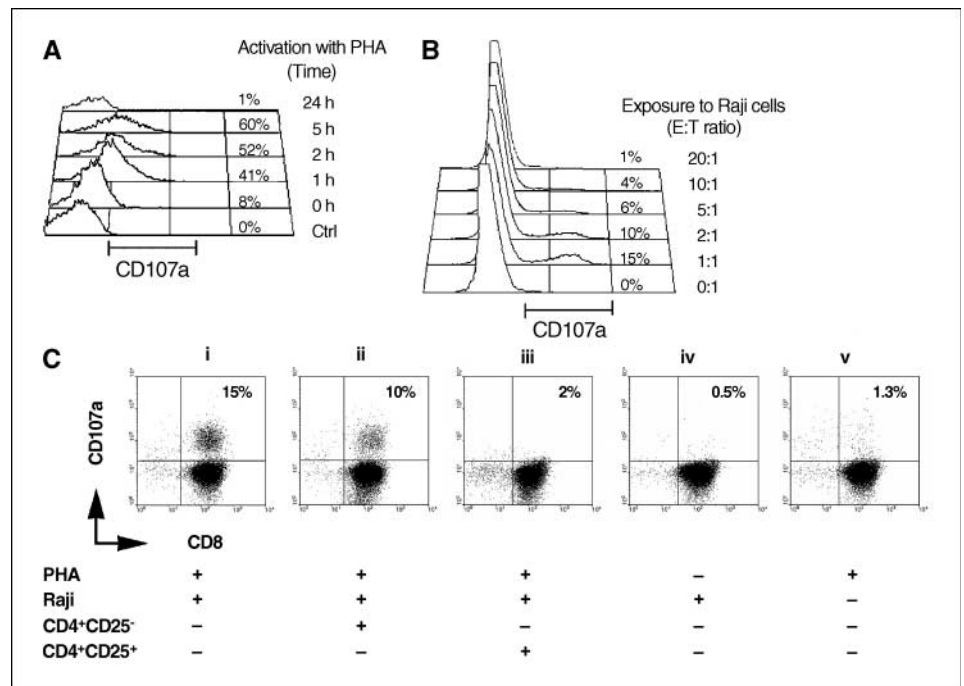


**Figure 3.** Intratumoral T<sub>reg</sub> cells dose-dependently inhibit the production of PFN and GzmB by infiltrating CD8<sup>+</sup> T cells in B-cell NHL. *A*, intracellular staining of PFN or GzmB expression as well as coexpression of PFN and GzmB in CD8<sup>+</sup> T cells from fresh biopsy specimens of B-cell NHL. Isotype controls are shown for each sample (top). *B*, summary of the percentage of CD8<sup>+</sup> T cells expressing PFN, GzmB, or both from 25 patient samples. *C* to *D*, intracellular expression of PFN (*C*) and GzmB (*D*) in infiltrating CD8<sup>+</sup> T cells cocultured either alone or with infiltrating CD4<sup>+</sup>CD25<sup>-</sup> T cells or intratumoral T<sub>reg</sub> cells in the presence or absence of PHA for 3 days. Representative of three independent experiments.

Next, we determined the influence of T<sub>reg</sub> cells on PFN and GzmB expression (Fig. 3C and D). Therefore, we cultured infiltrating CD8<sup>+</sup> T cells alone, with intratumoral T<sub>reg</sub> cells, or with infiltrating CD4<sup>+</sup>CD25<sup>-</sup> T cells at a ratio of 4:1 in the presence or absence of PHA (*n* = 3). The cells were then subjected to intracellular staining for PFN and GzmB and analyzed by flow cytometry. As shown in Fig. 3C and D, 1% of resting purified infiltrating CD8<sup>+</sup> T cells expressed intracellular PFN and 3% expressed GzmB. As expected, activation with PHA dramatically increased intracellular expression of PFN from 1% to 18% and GzmB from 3% to 99%. When CD8<sup>+</sup> T cells were cocultured with intratumoral T<sub>reg</sub> cells, we saw attenuation of activation-induced expression of PFN from 18% to 5%. Similarly, GzmB expression was decreased from 99% to 12%. In the presence of infiltrating CD4<sup>+</sup>CD25<sup>-</sup> T cells, activation-induced expression of PFN and GzmB by infiltrating CD8<sup>+</sup> T cells remained unchanged. These findings indicate that intratumoral T<sub>reg</sub> cells strongly inhibit the production of PFN and GzmB granules by infiltrating CD8<sup>+</sup> T cells in B-cell NHL.

**Intratumoral T<sub>reg</sub> cells inhibit the degranulation of infiltrating CD8<sup>+</sup> T cells in B-cell NHL.** We next sought to determine the effect of intratumoral T<sub>reg</sub> cells on granule release by infiltrating CD8<sup>+</sup> T cells. Degranulation is an essential mechanism by which CD8<sup>+</sup> T cells lyse tumor cells, and it has been shown that granule release is accompanied by the surface mobilization of CD107a (lysosome associated-membrane protein-1) on cytolytic cells. Therefore, CD107a surface expression has been used as a surrogate marker of degranulation by cytolytic cells such as natural killer cells (28, 29) and CTLs (25, 30). When we examined CD107a expression on resting or PHA activated infiltrating CD8<sup>+</sup> T cells, we saw that a negligible amount of surface expression was detectable on resting cells. However, activation of infiltrating CD8<sup>+</sup> T cells using PHA increased the surface expression of CD107a (Fig. 4A). No effect on CD107b expression was seen (data not shown). CD107a surface expression on infiltrating CD8<sup>+</sup> T cells reached its highest level after 5 hours of stimulation and declined to undetectable

**Figure 4.** Intratumoral T<sub>reg</sub> cells inhibit the degranulation of infiltrating CD8<sup>+</sup> T cells in B-cell NHL. **A**, expression of CD107a on PHA-activated infiltrating CD8<sup>+</sup> T cells in freshly isolated biopsy specimens of B-cell NHL at the indicated time points. **B**, CD107a expression on infiltrating CD8<sup>+</sup> T cells exposed to Raji cells at the indicated E/T ratios. **C**, CD107a surface expression on activated CD8<sup>+</sup> T cells exposed to Raji cells alone (*i*) or in the presence of CD4<sup>+</sup>CD25<sup>-</sup> T cells (*ii*) or T<sub>reg</sub> cells (*iii*). Resting CD8<sup>+</sup> T cells (*iv*) or activated CD8<sup>+</sup> T cells in the absence of Raji (*v*) serve as negative controls.



levels after 24 hours. This is in agreement with the observation that CD107a surface expression is transient (31). Furthermore, when infiltrating CD8<sup>+</sup> T cells were exposed to lymphoma B cells (Raji cells), CD107a surface expression was significantly up-regulated, particularly with an E/T ratio of 1:1 (Fig. 4B).

Next, we determined the effect of intratumoral T<sub>reg</sub> cells on CD107a expression on infiltrating CD8<sup>+</sup> T cells exposed to tumor cells (Fig. 4C). Before exposure to Raji cells, PHA-activated CD8<sup>+</sup> T cells were cocultured either alone (*i*) or with infiltrating CD4<sup>+</sup>CD25<sup>-</sup> T cells (*ii*) or intratumoral T<sub>reg</sub> cells (*iii*). Raji cells were added to the cultures, and CD107a expression on CD8<sup>+</sup> T cells was assessed. CD107a expression on resting CD8<sup>+</sup> T cells exposed to Raji cells (*iv*), and activated CD8<sup>+</sup> T cells without exposure to Raji cells (*v*) served as controls. When activated CD8<sup>+</sup> T cells were exposed to Raji cells (*i*), CD107a expression increased (15 ± 2%, *n* = 5) compared with that found on activated cells alone (*v*; 1.3 ± 0.2%, *n* = 5) or on resting cells exposure to Raji cells (*iv*; 0.5 ± 0.15%, *n* = 5). When intratumoral T<sub>reg</sub> cells were added to the culture, CD107a surface expression was significantly reduced (2 ± 0.6%, *n* = 5). We saw a moderate inhibition of CD107a surface expression on activated CD8<sup>+</sup> T cells when the cells were exposed to infiltrating CD4<sup>+</sup>CD25<sup>-</sup> T cells (*ii*; 10 ± 1.6%, *n* = 5). These results indicate that intratumoral T<sub>reg</sub> cells attenuate CD107a surface expression on activated infiltrating CD8<sup>+</sup> T cells during exposure to tumor cells.

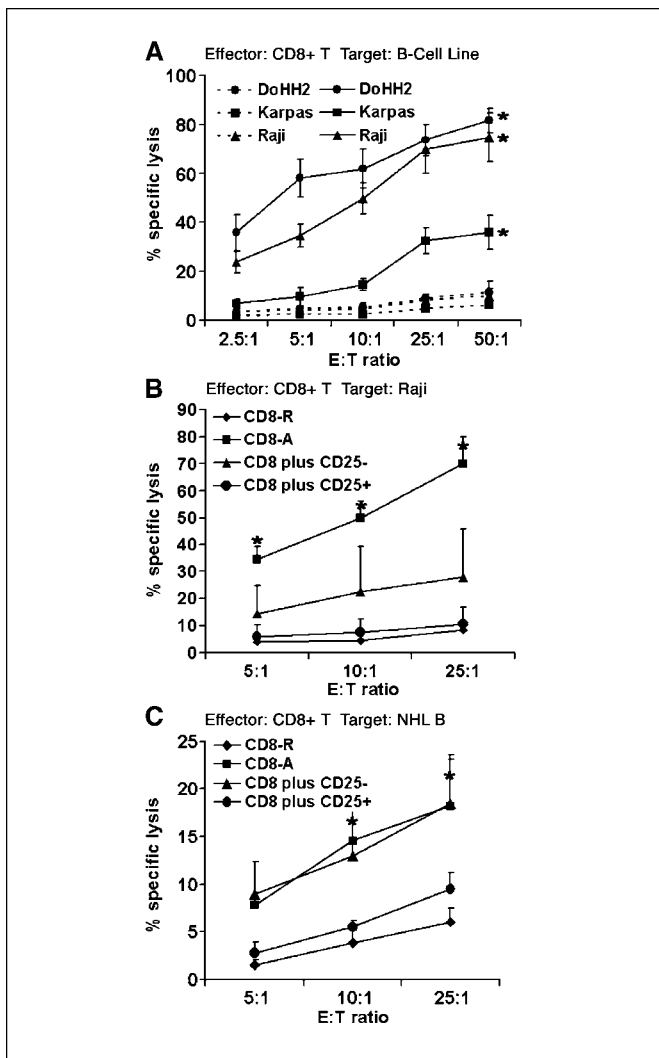
**Intratumoral T<sub>reg</sub> cells inhibit the ability of infiltrating CD8<sup>+</sup> T cells to lyse lymphoma B cells.** Because intratumoral T<sub>reg</sub> cells inhibit the proliferation, granule production, and degranulation of infiltrating CD8<sup>+</sup> T cells, we next wanted to determine the effect of intratumoral T<sub>reg</sub> cells on cytotoxicity of infiltrating CD8<sup>+</sup> T cells when exposed to lymphoma B cells. We first examined the ability of resting and activated CD8<sup>+</sup> T cells to lyse lymphoma B cell lines Raji, DoHH2, and Karpas. As shown in Fig. 5A, resting CD8<sup>+</sup> T cells were not capable of killing target cells. However, activation of infiltrating CD8<sup>+</sup> T cells with PHA induced a significant cytotoxic response to the lymphoma B cell lines compared with resting CD8<sup>+</sup> T cells (*n* = 3, *P* = 0.0002), although the cytotoxic activity was

different among three lines. Activated CD8<sup>+</sup> T cells killed DoHH2 and Raji cells in a dose-dependent fashion with nearly complete destruction of target cells at an E/T ratio 50:1.

We then wanted to determine the effect of intratumoral T<sub>reg</sub> cells on the cytotoxic activity of activated infiltrating CD8<sup>+</sup> T cells (Fig. 5B). Using Raji cells as a target, we cocultured activated infiltrating CD8<sup>+</sup> T cells treated with intratumoral T<sub>reg</sub> cells or infiltrating CD4<sup>+</sup>CD25<sup>-</sup> T cells at E/T ratios of 5:1, 10:1, and 25:1. We found that activated CD8<sup>+</sup> T cells showed significant dose-dependent cytolytic activity toward Raji cells. Coculture of activated infiltrating CD8<sup>+</sup> T cells with intratumoral T<sub>reg</sub> cells (*n* = 3) attenuated the cytotoxic activity against Raji cells (11 ± 6.06% at E/T ratio 25:1) compared with activation-induced CD8<sup>+</sup> T cells alone (69.8 ± 10.01% at E/T ratio 25:1, *P* = 0.00183) or with CD4<sup>+</sup>CD25<sup>-</sup> cells (27.7 ± 7.9% at E/T ratio 25:1, *P* = 0.056). In the presence of intratumoral T<sub>reg</sub> cells, the CD8<sup>+</sup> T-cell cytolytic activity was similar to the cytolytic activity seen in unstimulated CD8<sup>+</sup> T cells.

Next, we examined the influence of intratumoral T<sub>reg</sub> cell on the cytotoxic activity of infiltrating CD8<sup>+</sup> T cells toward autologous lymphoma B cells. As shown in Fig. 5C, coculture of infiltrating activated CD8<sup>+</sup> T cells with intratumoral T<sub>reg</sub> cells attenuated the cytotoxic activity against autologous lymphoma B cells (9.6 ± 1.62% at E/T ratio 25:1, *n* = 6) compared with activation-induced CD8<sup>+</sup> T cells alone (18 ± 5% at E/T ratio 25:1, *P* = 0.015). Coculture of infiltrating CD8<sup>+</sup> T cells with CD4<sup>+</sup>CD25<sup>-</sup> T cells had minimal effect of CTL activity compared with activated CD8<sup>+</sup> T cells alone (18 ± 4.2% at E/T ratio 25:1, *P* = 0.497). These results indicate that intratumoral T<sub>reg</sub> cells strongly suppress the cytotoxic function of infiltrating CD8<sup>+</sup> T cells and suggest that infiltrating CD8<sup>+</sup> T cells could successfully destroy autologous lymphoma B cells were it not for the protective suppressive effect of intratumoral T<sub>reg</sub> cells present at the site of B-cell NHL.

**Increased numbers of intratumoral T<sub>reg</sub> cells are associated with decreased infiltrating CD8<sup>+</sup> T-cell numbers in B-cell NHL.** As shown above using an *in vitro* assay, we observed significant attenuation of infiltrating CD8<sup>+</sup> T-cell function by intratumoral T<sub>reg</sub>



**Figure 5.** Intratumoral T<sub>reg</sub> cells inhibit the cytotoxicity of infiltrating CD8<sup>+</sup> T cells to lymphoma B cells. **A**, cytotoxic activity, measured by specific lysis of targets cells, was analyzed in activated (solid line) or resting (dashed line) CD8<sup>+</sup> T cells at the indicated E/T ratio. Target cells include DoHH2, Karpas, and Raji cells (*n* = 3). Cytotoxic activity of resting or activated infiltrating CD8<sup>+</sup> T cells cocultured with CD4<sup>+</sup>CD25<sup>-</sup> or CD4<sup>+</sup>CD25<sup>+</sup> T cells toward Raji (*n* = 3; **B**) or autologous lymphoma B-cells (*n* = 6; **C**) at the indicated E/T ratios. The percentage of 7-AAD<sup>+</sup> cells was used to calculate the % lysis of target cells by CD8<sup>+</sup> T cells. Points, mean; bars, SD. \*, *P* < 0.05.

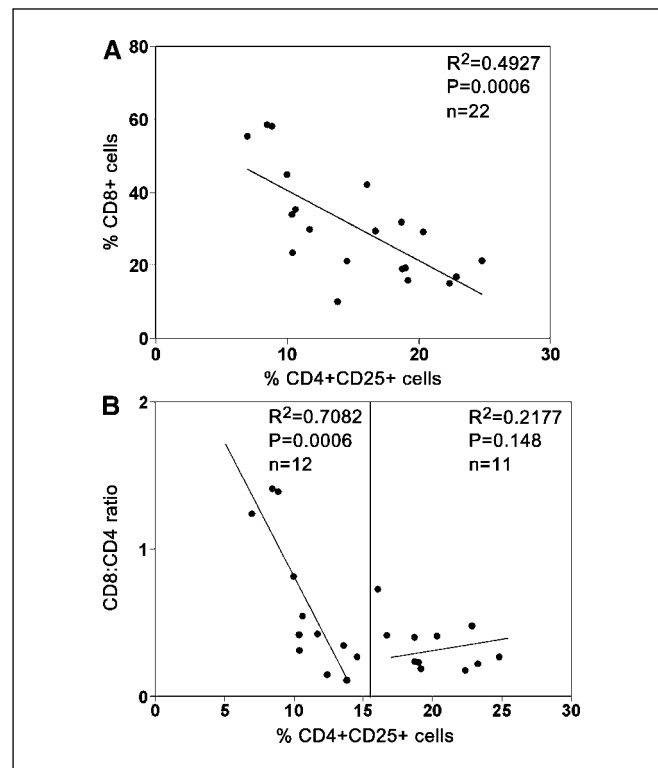
cells. Dampening of immune function within the tumor microenvironment may have clinical significance, and measurement of T<sub>reg</sub> numbers may provide us with an indirect measure of T<sub>reg</sub> activity. Therefore, we measured the percentage of CD8<sup>+</sup> T cells, the CD8/CD4 ratio, as well as the frequency of intratumoral T<sub>reg</sub> cells, CD4<sup>+</sup>CD25<sup>+</sup>, in 22 freshly isolated biopsy specimens from patients with B-cell NHL and examined the relationship between intratumoral T<sub>reg</sub> cells and infiltrating CD8<sup>+</sup> T-cell population as well as the value of CD8/CD4 ratio. We found that there was a strong inverse relationship between the frequency of intratumoral T<sub>reg</sub> cells and the percentage of CD8 cells in biopsy specimens (*n* = 22, *R*<sup>2</sup> = 0.4927, *P* = 0.0006; Fig. 6A). The samples that had a high percentage of intratumoral T<sub>reg</sub> cells had a low percentage of infiltrating CD8<sup>+</sup> T cells. Conversely, the samples that had a low percentage of intratumoral T<sub>reg</sub> cells showed a high percentage of infiltrating CD8<sup>+</sup> T cells. Because the CD8/CD4 ratio has been used

as a clinical prognostic index for patients with cancer (32), we also compared the frequency of intratumoral T<sub>reg</sub> cells with the CD8/CD4 ratio in our samples (Fig. 6B). Interestingly, a linear inverse relationship could be seen between intratumoral T<sub>reg</sub> cells and the CD8/CD4 ratio when the frequency of intratumoral T<sub>reg</sub> cells was <15% (*n* = 12, *R*<sup>2</sup> = 0.7082, *P* = 0.0006). However, we found that the CD8/CD4 ratio was uniformly low when the frequency of intratumoral T<sub>reg</sub> cells was >15% (*n* = 11, *R*<sup>2</sup> = 0.2177, *P* = 0.148). These findings suggest that increased numbers of T<sub>reg</sub> cells in the tumor microenvironment suppress the relative number of CD8<sup>+</sup> T cells.

**Discussion**

Orchestration of an effective immune response against tumor cells is a complex process that involves numerous branches of the immune system. Subtle alterations in costimulatory molecule expression, cytokine secretion, and ineffective migration of immune cells to the inflammatory milieu can vastly alter the clearance of antigen. Recent studies by our group, as well of others, have highlighted the significance of T<sub>reg</sub> cells in this process and suggest that T<sub>reg</sub> cells contribute to the ineffective immune response observed against tumors (3, 10, 11, 33, 34). Much of this work has focused on CD4<sup>+</sup> CD4<sup>+</sup> T cells or has been accomplished using murine models of solid tumors. However, there is increasing literature suggesting a role for T<sub>reg</sub> cells in hematologic malignancies as well (12, 35–38).

In previous work, we have shown that intratumoral T<sub>reg</sub> cells significantly suppress the functional capabilities of autologous



**Figure 6.** Increased numbers of intratumoral T<sub>reg</sub> cells are associated with decreased numbers of infiltrating CD8<sup>+</sup> T cells in B-cell NHL. **A**, relationship between the percentage of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells and the percentage of CD8<sup>+</sup> of CD3<sup>+</sup> T cells in 22 fresh biopsy samples of B-cell NHL. Liner line was drawn to calculate *R*<sup>2</sup>. **B**, relationship between the percentage CD4<sup>+</sup>CD25<sup>+</sup> T cells and the ratio of CD8<sup>+</sup> to CD4<sup>+</sup> T cells in 23 fresh biopsy samples of B-cell NHL. The liner lines were drawn based on percentage of CD4<sup>+</sup>CD25<sup>+</sup> T cells. The value of 15% makes two populations.

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infiltrating CD4<sup>+</sup>CD25<sup>-</sup> T cells in NHL, and that T<sub>reg</sub> cells migrate to areas involved by lymphoma in response to factors secreted by the malignant B cells (3). The ability of tumor cells to attract T<sub>reg</sub> cells, and thereby dampen the immune response, is intriguing and suggests that a dynamic relationship exists between tumor cells and those of the immune system. However, one question that still remained was whether or not T<sub>reg</sub> cells had a direct effect on CTL activity, and in turn, the viability of human tumor cells.

Classically, T<sub>reg</sub> cells are phenotypically characterized by expression of CD4, CD25<sup>high</sup>, and Foxp3 (39–41). Currently, the tools do not exist to isolate T cells for experimental use based on intracellular Foxp3 expression; thus, we are only able to use CD25 as a marker for purification. In other human systems, this may be problematic; however, in our studies on NHL tumor specimens, we have found that irrespective of the CD25 expression level (high versus low), all CD4<sup>+</sup>CD25<sup>+</sup> cells expressed Foxp3 (3). Additionally, the staining pattern of Foxp3<sup>+</sup> cells correlates well with the staining of CD4<sup>+</sup> and CD25<sup>+</sup> cells (Fig. 1). However, in other systems, Foxp3 expression may not necessarily confer regulatory activity. Recent work by Gavin et al. (42) found that small amounts of Foxp3 expression could be detected in *in vitro* activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Unlike classic regulatory T cells, these cells were unable to suppress Th1 cytokine synthesis. However, they were not able to directly determine whether or not these cells had suppressive capabilities. In our experiments, Foxp3 expression was confined to the CD4<sup>+</sup> population and was not detected in CD8<sup>+</sup> T cells (data not shown). Further characterization of Foxp3 expression and regulatory activity of human T cells in both the normal and malignant scenerio will hopefully shed light recent findings.

Although it has been reported that T<sub>reg</sub> cells inhibit CD8<sup>+</sup> T-cell proliferation under several pathologic conditions, such as infection (18, 19) and transplantation (20, 21), it was less clear how T<sub>reg</sub> cells influence CD8<sup>+</sup> T cells in a human tumor model. We found that intratumoral T<sub>reg</sub> cells isolated from tissue biopsy specimens from patients could completely inhibit proliferation of patient-matched autologous CD8<sup>+</sup> T cells (Fig. 2). The inability of CD8<sup>+</sup> T cells to proliferate in response to antigen within the tumor microenvironment may be one mechanism by which T<sub>reg</sub> cells hinder the initial steps of a normal immune response. This is in contrast to what has been seen in mouse tumor models where experiments found that T<sub>reg</sub> cells did not interfere with accumulation or division of tumor-specific CD8<sup>+</sup> T cells (23). The exact mechanisms that T<sub>reg</sub> cells use to inhibit proliferation of CD8<sup>+</sup> T in a human model may therefore be unique.

Intratumoral T<sub>reg</sub> cells suppress not only the proliferation of infiltrating CD8<sup>+</sup> T cells but also production and release of PFN and GzmB. Our *in vitro* experiments indicate that intratumoral T<sub>reg</sub> cells can dramatically inhibit activation-induced intracellular expression of PFN and GzmB by CD8<sup>+</sup> T cells (Fig. 3). We also found that a majority of intratumoral CD8<sup>+</sup> T cells express minimal amounts of PFN and GzmB, suggesting that they are relatively inactive. Production of PFN and GzmB is essential for CD8<sup>+</sup> T cells to function as effector cells, and it has been shown that the perforin/granzyme pathway is critical for CTL-mediated lymphoma B-cell lysis in B-cell NHL (43). In parallel experiments, we found that expression of CD107a, a measure of degranulation, on infiltrating CD8<sup>+</sup> T cells was also inhibited by intratumoral T<sub>reg</sub> cells (Fig. 4). Degranulation is one of the key steps in the cytolytic process and is required for lysis of tumor cells. Our studies show that expression of CD107a on infiltrating CD8<sup>+</sup> T cells was up-regulated upon activation by PHA or exposure to tumor cells. We

also found that expression of CD107a on CD8<sup>+</sup> T cells, upon exposure to lymphoma B cells, was suppressed by intratumoral T<sub>reg</sub> cells. Inhibition of perforin and GzmB expression by infiltrating CD8<sup>+</sup> T, as well as suppression of granule release, may therefore be an additional mechanism by which by T<sub>reg</sub> cells suppress normal CTL responses. These results are in accordance with murine studies, which have also shown that T<sub>reg</sub> cells abolish tumor-specific CD8<sup>+</sup> T-cell cytotoxic activity (23).

The ability of T<sub>reg</sub> cells the shutdown various aspect of CTL mediated immunity is clear; however, it was still unknown whether or not intratumoral CD8<sup>+</sup> T cells could kill autologous tumor cells and what effect T<sub>reg</sub> cells would have on this activity. As shown in Fig. 5, our results show that CD8<sup>+</sup> T cells from NHL tumors are functional, and that they can kill both tumor cell lines as well as autologous lymphoma B cells. When we further characterized the CD8<sup>+</sup> T cell population, we found that the vast majority (>90%, *n* = 3) of infiltrating CD8<sup>+</sup> T cells lack expression of CD45RA and CCR7, suggesting that they are effector memory cells (data not shown) and should therefore be capable of effective immune response (44). Of note, when we phenotyped the CD8<sup>+</sup> T-cell population in a nonmalignant hyperplastic lymph node, we found that a vast majority (>90%, *n* = 1) of infiltrating CD8<sup>+</sup> T cells expressed CD45RA and CCR7, suggesting a naive phenotype (data not shown). The significance of these data and what effect it may have on the immune response to NHL B cells is currently under investigation, but it may indicate that the malignant scenario or the increased number of T<sub>reg</sub> cells in the tumor microenvironment may influence the development or attraction of CD8<sup>+</sup> effector memory cells.

Throughout the experiments we noted that infiltrating CD4<sup>+</sup>CD25<sup>-</sup> had the ability to reduce CTL function, albeit to a lesser extent than CD4<sup>+</sup>CD25<sup>+</sup> T cells. In light of these data, we have done preliminary FACS experiments that suggest that a small subpopulation of CD4<sup>+</sup>CD25<sup>-</sup> T cells that express Foxp3 can be found in NHL tumors, and they may therefore be responsible for the suppressive activity seen in our studies (data not shown). In support of these initial findings, Nishioka et al. have identified a population of CD4<sup>+</sup>CD25<sup>-</sup>Foxp3<sup>+</sup> suppressive T cells in aged mice (45). Throughout our experiments, we have found no significant difference in the function and frequency of the various T-cell subpopulations, as a percentage of total T cells, among the different NHL histologic subtypes studied thus far. However, a formal analysis has not been done due to the small number of samples used in this study.

The ability of T<sub>reg</sub> cells to shutdown antitumor activity suggests that their presence in the tumor microenvironment may have significant effect on CD8<sup>+</sup> T-cell function as well as the progression of NHL. Therefore, we next wanted to determine if the occurrence of T<sub>reg</sub> cells in NHL was related to the number of CD8<sup>+</sup> T cells present. When we measured the percentage of CD8<sup>+</sup> T cells and intratumoral T<sub>reg</sub> cells in biopsy specimens from patients with B-cell NHL, we found that there was a strong inverse relationship between the frequency of intratumoral T<sub>reg</sub> cells and the percentage of CD8<sup>+</sup> cells in biopsy specimens. Taken together, these data suggest that T<sub>reg</sub> cells influence the function of CD8<sup>+</sup>, and that increased numbers of T<sub>reg</sub> cells in the tumor microenvironment may suppress the relative number of CD8<sup>+</sup> T cells.

In summary, our data provide evidence indicating that T<sub>reg</sub> cells contribute in part to the ineffective antitumor response seen in B-cell NHL by suppressing numerous facets of the CTL response. Our findings are in accordance with recent finding in mice where regulatory T cells have been shown to suppress CTL activity

(22–24). Taken together, these data highlight the importance of the T<sub>reg</sub> cell within the tumor microenvironment and suggest that CTLs could potentially eradicate tumor cells in the absence of T<sub>reg</sub> cells. Therefore, we contend that use of novel therapies that would inhibit the function of intratumoral T<sub>reg</sub> cells will result in significant clinical benefit for patients with B-cell NHL.

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