Cell PRESS
Immunity
Article

Tumor-Infiltrating γδ T Cells Suppress T and Dendritic Cell Function via Mechanisms Controlled by a Unique Toll-like Receptor Signaling Pathway

Guangyong Peng,[1,2,3] Helen Y. Wang,[1,2,3] Weiyi Peng,[1,2,3] Yukiko Kiniwa,[1,2,3] Kook Heon Seo,[1,2,3] and Rong-Fu Wang[1,2,3,*]
[1]The Center for Cell and Gene Therapy
[2]Department of Pathology
[3]Department of Immunology
Baylor College of Medicine, Houston, TX 77030, USA
*Correspondence: rongfuw@bcm.tmc.edu
DOI 10.1016/j.immuni.2007.05.020

SUMMARY

γδ T cells are important contributors to innate immunity against cancer, but their regulatory role in controlling immune responses remains largely unknown. Here we report that a dominant γδ1 T cell population among lymphocytes infiltrating breast tumors possessed a potent ability to suppress naive and effector T cell responses and to block the maturation and function of dendritic cells. Adoptive cotransfer experiments demonstrated their in vivo suppressive activity. However, their immunosuppressive activity could be reversed by human Toll-like receptor (TLR) 8 ligands both in vitro and in vivo. siRNA-mediated knockdown experiments revealed that MyD88, TRAF6, IKKα IKKβ, and p38α molecules in γδ1 cells were required for these cells to respond to TLR8 ligands, whereas TAK1, JNK, and ERK molecules did not appear to be involved in functional regulation. These results provide new insights into the regulatory mechanisms of tumor-specific γδ T cells and identify a unique TLR8 signaling pathway linking to their functional regulation.

INTRODUCTION

T cells play an essential role in the immunosurveillance and destruction of tumor cells, but attempts to translate this knowledge into clinically effective immunotherapies have met with only limited success (Dunn et al., 2004; Rosenberg et al., 2004). One of the major impediments to this goal is the activity of regulatory T (Treg) cells at tumor sites, which can markedly suppress immune responses and induce immune tolerance (Curiel et al., 2004; Liyanage et al., 2002; Wang et al., 2004, 2005; Woo et al., 2001). Although $CD4^+$ Treg cells have been extensively studied, much less is known about other subsets of Treg cells, some of which have the potential to suppress immune responses (Hayday and Tigelaar, 2003; Sakaguchi, 2004; Shevach, 2002).

T cells expressing γ and δ T cell receptor (TCR) chains with limited usage represent only a small subset (2%–3%) within the total T cell population. In contrast to recognition of antigens by αβ T cells, γδ T cells recognize antigens directly without any requirement for antigen processing and presentation or major histocompatibility complex (MHC) molecules (Brenner et al., 1986; Shin et al., 2005). Of the two major subsets of human γδ T cells, Vγ2Vδ2 (also known as Vγ9Vδ2, collectively designated Vδ2) T cells predominate in the peripheral blood and respond to microbial infections by recognizing small nonpeptide molecules (Bukowski et al., 1999; Constant et al., 1994; Eberl et al., 2004; Modlin et al., 1989). The other major subset, Vδ1 T cells (also called intraepithelial lymphocytes, or IELs), comprises 70%–90% of the γδ T cells in epithelial tissues and may recognize either MHC class I-related chain A or B (MICA or MICB), which are induced on epithelial cells and tumor cells by stress or structural damage (Groh et al., 1998; Hayday, 2000). However, MICA and MICB, as well as distantly related ULBP proteins, may be recognized via the activating receptor, NKG2D, which is expressed not only on Vδ1 T cells, but also on Vδ2 T cells, NK cells, and some αβ T cells (Bauer et al., 1999). In contrast to human γδ1 T cells, murine dendritic epidermal γδ T cells (DETCs) do not recognize bacterial phosphoantigens, but they share the capacity to respond via NKG2D to the MICA and ULBP ortholog, called Rae1 or H60, expressed by tumor cells, thus stimulating antitumor immunity (Diefenbach et al., 2001; Groh et al., 1999). Additionally, some murine γδ TCRs engage another MHC Class Ib gene product, T10 and T22, that may be overexpressed by activated cells (Shin et al., 2005). Indeed, murine γδ T cells were shown to mediate resistance to squamous cell carcinoma (Girardi et al., 2001). Collectively, these findings have raised the intriguing possibility that γδ T cell recognition of MICA and MICB molecules on tumor cells might be exploited to devise new strategies of cancer immunotherapy (Boismenu and Havran, 1994; Girardi et al., 2001; Hayday and Tigelaar, 2003; Jameson et al., 2002).

Despite the important roles of γδ T cells as a natural component of host innate immunity in response to tissue stress or damage, malignancy, or infectious pathogens (Havran, 2000; Hayday and Tigelaar, 2003; Jameson et al., 2003), the function of γδ T cells may be extremely pleiotropic, harboring regulatory as well as effector potential (Hayday and Tigelaar, 2003; Pennington et al., 2005). The possibility therefore exists that these γδ T cells may suppress immune responses.

In our previous study, we demonstrate that the suppressive function of human naturally occurring $CD4^+$ $CD25^+$ Treg cells and tumor-derived antigen-specific $CD4^+$ Treg cells can be reversed by TLR8 signaling pathway (Peng et al., 2005). This reversal effect does not require the involvement of dendritic cells (DCs), but requires the activation of TLR8-MyD88-IRAK4 signaling pathway in Treg cells for their functional reversal (Peng et al., 2005). Several other studies show that TLR2 plays a critical role in regulating the suppressive function of murine $CD4^+$ $CD25^+$ Treg cells (Liu et al., 2006; Sutmuller et al., 2006a). Expression of various TLRs has been documented in both human and murine Treg cells (Sutmuller et al., 2006b). However, it is not clear whether human γδ T cells also express TLRs. More importantly, whether the suppressive function of γδ T cells can be regulated through TLR signaling remains largely unknown.

In our recent efforts to establish tumor-specific T cells from breast cancers, we unexpectedly identified a dominant γδ1 T cell population in the total population of tumor-infiltrating lymphocytes (TILs). This prompted us to characterize these tumor-specific γδ1 T cells with regard to their function and regulatory mechanisms. Here we report that tumor-derived γδ1 T cells possess the potent capacity to suppress immune responses. Their immune-suppressive function could be controlled via the TLR8 signaling pathway. Further studies revealed that MyD88, TRAF6, IKKα, IKKβ, or p38α molecules in γδ1 T cells were required for their functional reversal in response to TLR8 ligands, whereas TAK1, JNK, and ERK molecules were not. These findings identify a natural pathophysiologic involvement of human γδ1 T cells in tumor immunity and provide new insights into their regulatory mechanisms for controlling their suppressive function.

RESULTS

γδ1 T Cells Are a Dominant Tumor-Infiltrating T Cell Population

We generated breast-tumor-derived tumor-infiltrating lymphocytes (TILs) with the intent of identifying new breast cancer antigens. Breast-tumor-derived TIL31 (BTIL31) cells specifically recognized autologous breast cancer cells (BC31) as detected by IFN-γ secretion but did not respond to allogeneic breast cancer cells (MCF-7, BC29, BC30, and BC36), prostate cancer cells (PC263 and PC267), melanoma cells (1363mel and 1359mel), 586 EBV-transformed B cells, or 293T cells (Figure 1A). Tumor reactivity and specificity were observed for several T cell clones established from the bulk BTIL31 population (Figure 1B), suggesting that BTIL31 and its clones are T cells specific for breast tumors.

To determine the requirement for MHC molecule-restricted T cell recognition by BTIL31, we performed a T cell functional assay in the presence or absence of specific antibodies against MHC molecules. None of these blocking antibodies could inhibit T cell recognition (Figures S1A and S1B in Supplemental Data available online), indicating that unlike conventional $CD4^+$ or $CD8^+$ T cells, these BTIL31 T cells do not require MHC-class I or II molecules for tumor recognition. FACS analysis revealed that these cells were positive for CD3, CD8, CD56, and γδ TCR molecules, but negative for the αβ TCR marker (Figure 1C), suggesting that they were tumor-specific γδ TCR-expressing T cells. The γ9δ2 subset is generally considered a dominant population representing 3%–5% of the total peripheral T cells, whereas Vδ1 T cells reside mainly in epithelial tissues and skin (Kabelitz et al., 2005). To determine the subtype of the BTIL31 T cells, we stained the bulk BTIL31 line and its clones with a Vδ1 or Vδ2 antibody. More than 95% of the T cells were positive for Vδ1 but negative for Vδ2 antibody staining (Figure 1D), indicating that the BTIL31 T cells are breast-tumor-specific γδ1 T cells that accumulate predominantly in breast-tumor tissues. To determine whether these $CD8^+$ T cells express CD8αα or CDαβ molecules, we stained γδ1 T cells as well as two conventional $CD8^+$ T cell lines (TIL1359-20 and BTIL29-C1, which recognize antigens in the context of MHC class I molecules) with CD8α and CD8β antibodies. These human $CD8^+$ γδ1 T cells expressed CD8αα homodimer, but not the CD8αβ heterodimer usually expressed by conventional T cells such as TIL1359-20 and BTIL29-C1 (Figure 1E), suggesting that human $CD8\alpha\alpha^+$ γδ1 T cells phenotypically resemble murine $CD8\alpha\alpha^+$ γδ T cells (Hayday, 2000).

Prevalence of γδ1 T Cells in Breast and Prostate Cancer

To substatiate the high percentage of γδ1 T cells observed in Figure 1D, we analyzed TILs derived from breast tumors established from an additional 10 breast cancer patients and observed a high percentages of γδ1 T cells among TILs (7.2%–75.7%; mean 33.2%) (Figure 2A). To exclude the possibility that the high percentage of γδ1 T cells observed in breast cancer-derived TILs was due to in vitro cell culture, we first tested the prevalence of γδ1 T cells in other tumors such as prostate cancer and melanoma. With the use of an identical culture condition and method, we found that prostate-tumor-derived TILs also contained high percentages of γδ1 T cells in the total T cell population (21.7%–96.3%; mean 40.1%), whereas among melanoma-derived TILs, the percentage was low (2.8%–7.5%; mean 5.7%) (Figure 2B). Thus, elevated proportions of γδ1 T cells are prevalent in tumors originating from epithelium, including breast and prostate cancers, but not in melanoma, consistent with our previous extensive work confirming a low percentage of γδ T cells in melanoma-derived TILs.

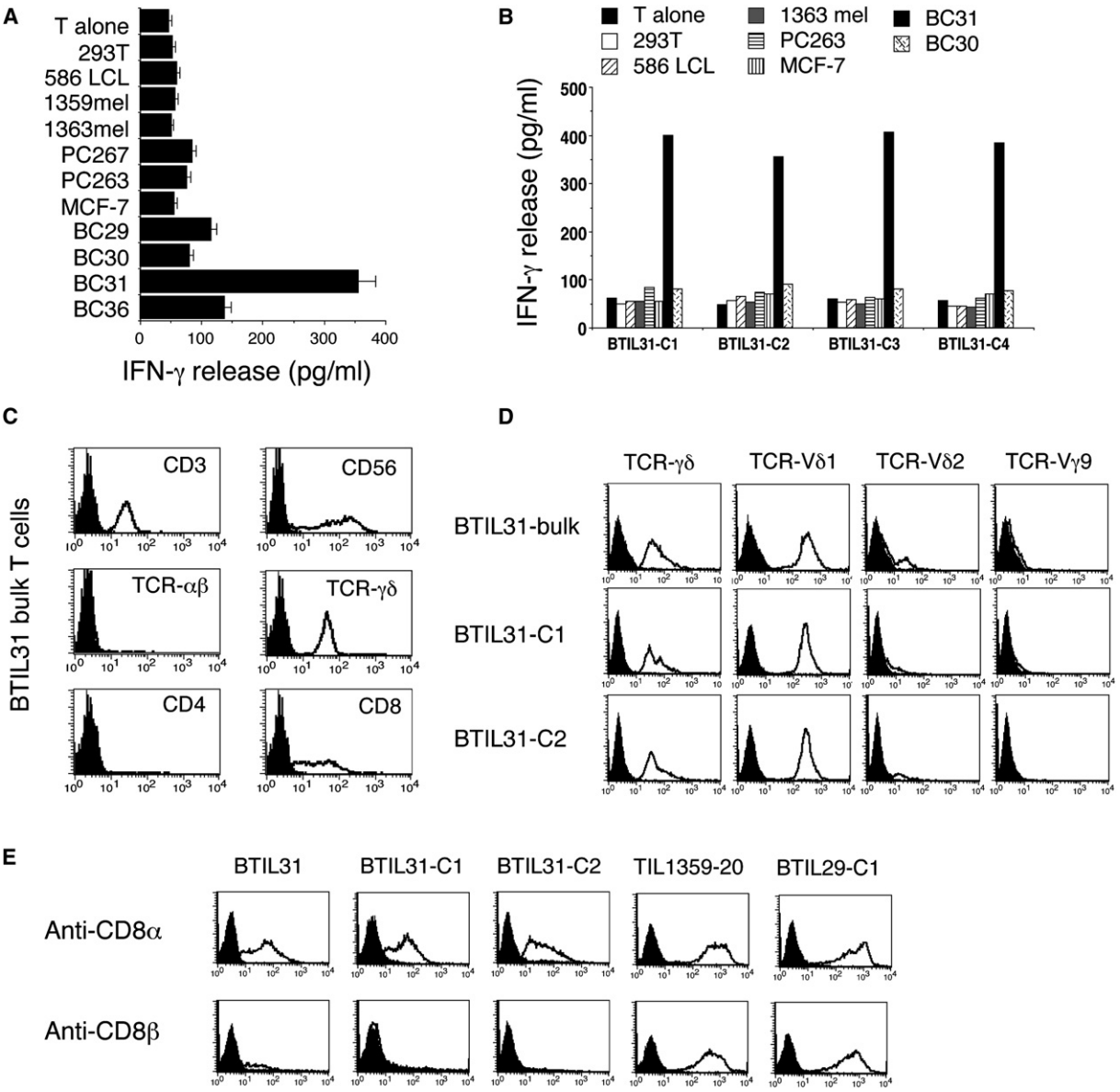

Figure 1. Generation and Characterization of Tumor-Specific γδ1 T Cells

(A) Recognition of autologous breast-tumor cells by breast cancer-derived BTIL31 cells. BTIL31 T cells isolated from breast cancer tissues were cocultured with autologous BC31 tumor cells as well as other tumor cell lines. After 18–24 hr culture, T cell reactivity was determined by measuring IFN-γ secretion in culture supernatants.

(B) Antigen specificity of BTIL31-derived T cell clones. T cell clones were established from the BTIL31 bulk cell line, and we tested their ability to recognize BC31 tumor cells. Other cell lines served as controls.

(C) FACS analysis of surface markers of BTIL31 cells. BTIL31 cells were stained with mAb to CD3, CD4, CD8, CD56, CD161, TCR-αβ, and TCR-γδ molecules. Isotype control antibodies served as negative controls.

(D) BTIL31 bulk and clones cells were predominantly γδ1 T cells. BTIL31 cells and clones were stained with mAb to TCR-γδ, TCR-Vδ1, TCR-Vδ2, and TCR-Vγ9.

(E) Expression of CD8αα homodimer by BTIL31 cells and its clones. BTIL31 cells and their clones were stained with CD8α and CD8β mAb. The stained cells were then analyzed by FACS. Conventional $CD8^+$ T cells (TIL1359-20 and BTIL 29-C1) served as positive controls for CD8β mAb.

Data are one of three experiments yielding similar results.

We also isolated single-suspension cells from breast-tumor (BT) samples and directly stained them with CD3 and γδ TCR antibodies. FACS analysis gating on $CD3^+$ T cell population revealed high percentages (ranging from 12% to 69%) of γδ T cells in the total T cell population from five breast-tumor samples analyzed (Figure 2C). These data further suggest that an elevated percentage of γδ T cells in breast tumors is an intrinsic property of

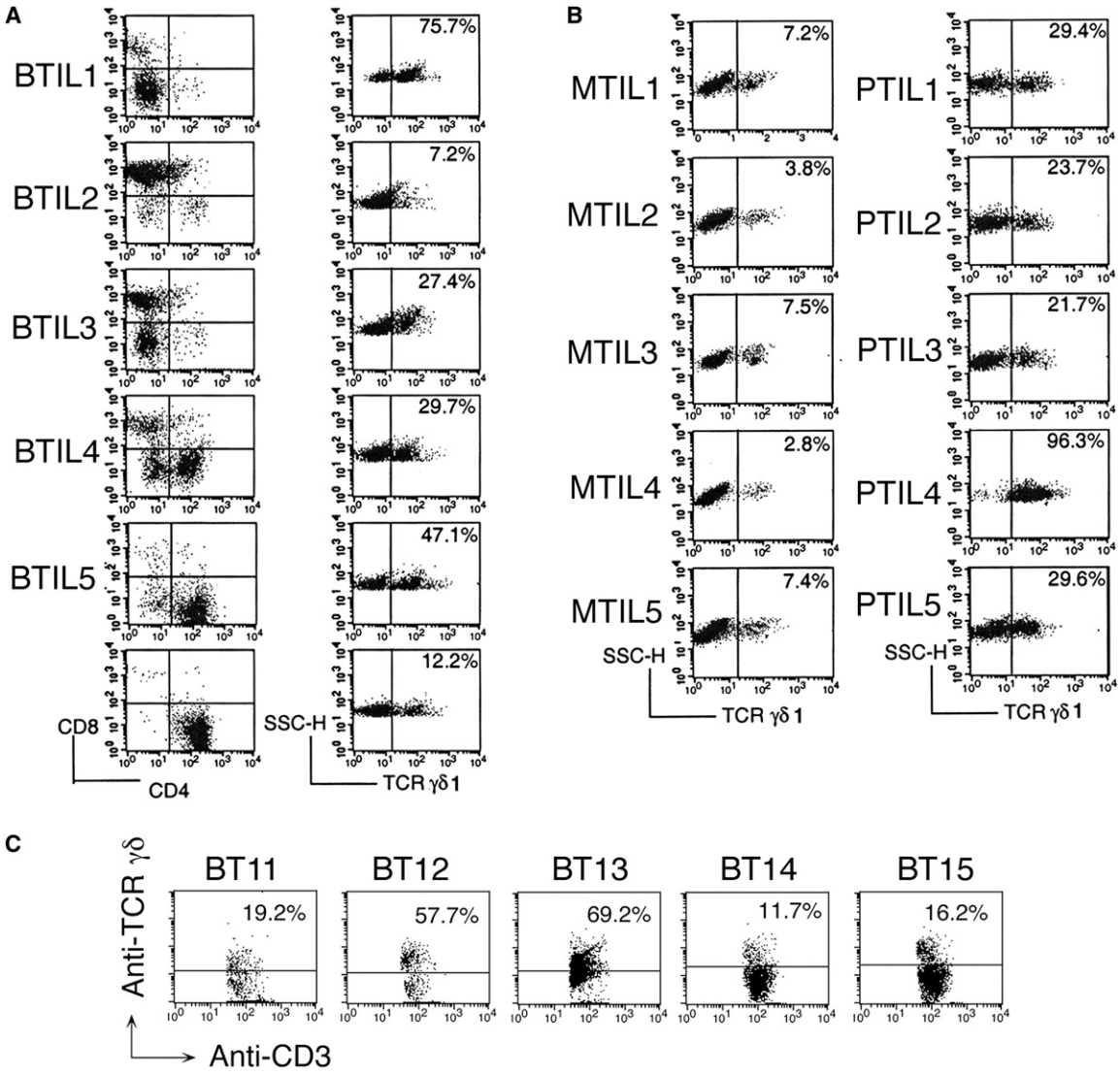

Figure 2. Prevalence of γδ1 T Cells Derived from Breast and Prostate Tumors

(A) High proportions of γδ T cells in BTILs derived from breast tumors. The numbers are the percentage of $\gamma\delta1^+$ T cells in the total T cell population established from breast cancer tissues.

(B) Percentages of $\gamma\delta1^+$ T cells in the total T cell population established from prostate cancer and melanoma.

(C) High proportions of γδ T cells present in the T cell population directly isolated from breast tumors. FACS analysis was conducted after gating on the $CD3^+$ T population.

Data are representatives of three experiments yielding similar results.

epithelium-derived tumors, rather than a result of cell culturing in vitro.

TCR-Mediated Signal Is Critical for Tumor Recognition by γδ1 T Cells

Although γδ1 T cells can recognize tumor cells without the requirement for MHC class I and II molecules, we thought it important to determine whether the recognition of tumor cells by γδ1 T cells requires TCR-mediated antigen stimulation. To test this possibility, we cultured the γδ1 T cells with BC31 tumor cells in the presence or absence of antibodies against MHC class I, class II, NKG2D, MICA, MICB, γδ, or αβ TCR molecules. The recognition of tumor cells by BTIL31 γδ1 T cells was completely blocked by a TCR γδ antibody, but only partially blocked by a NKG2D antibody, either alone or in combination with MICA and MICB antibody. In contrast, little or no inhibition was observed with anti-MICA, anti-MICB, anti-CD1d, anti-MHC class I, anti-MHC class II, or control antibodies (Figure 3A). These results suggest that tumor recognition by BTIL31 γδ1 T cells requires interaction between TCR and antigens expressed by tumor cells, whereas NKG2D may enhance T cell recognition by interacting with MICA. To test whether interaction between NKG2D and MICA and MICB may

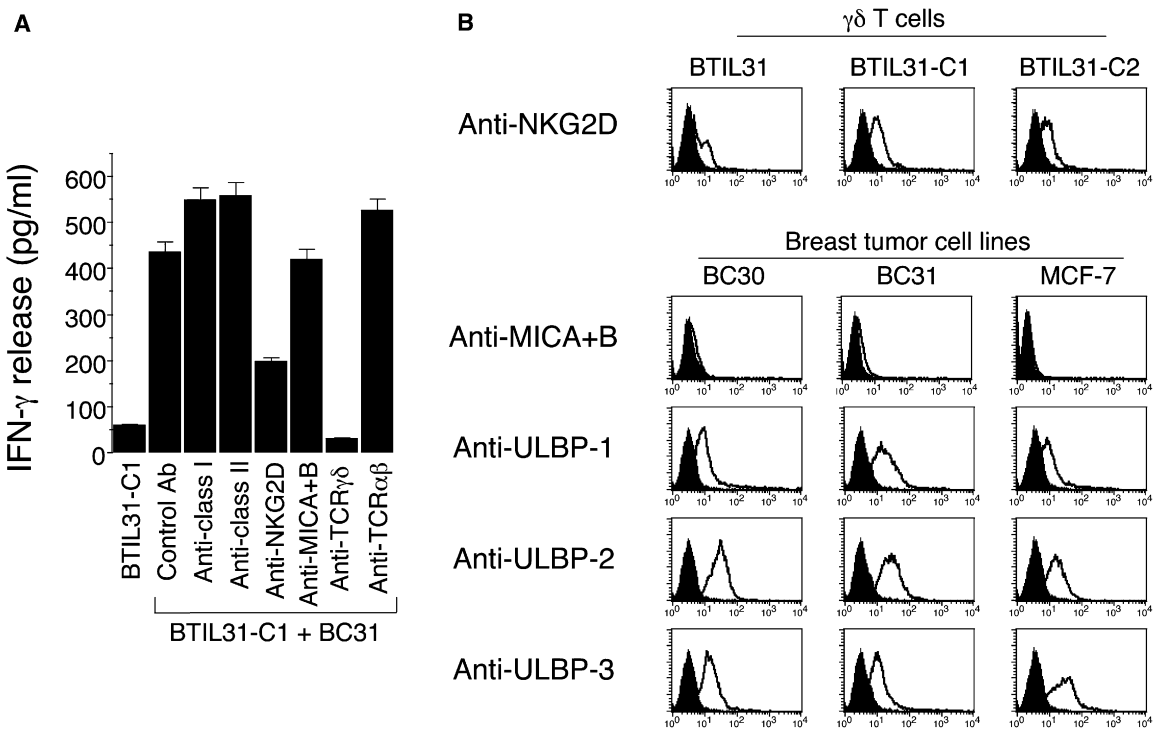

Figure 3. Recognition of Tumor Cells by BTIL31 T Cells

(A) Requirement of TCR-mediated signal in T cell recognition of tumor cells. IFN-γ release from T cells was determined from the supernatants of BTIL31 and BC31 tumor cell coculture in the presence of control antibody or antibodies against TCR, NK-G2D, MHC class I, class II, MICA, and MICB molecules.

(B) FACS analysis for expression of NKG2D and ULBP molecules by T cells and tumor cells. BTIL31 T cells were stained with anti-NK-G2D, and breast-tumor cell lines (BC30, BC31, and MCF-7) were stained with antibodies against MICA, MICB, ULBP-1, ULBP2, or ULBP3 molecules. The stained cells were then analyzed by FACS.

Data are one of three experiments yielding similar results.

function as a costimulatory signal, we evaluated the expression of NKG2D on T cells and MICA and MICB on tumor cells by FACS analysis and showed that although γδ1 T cells expressed NKG2D molecules, three breast-tumor cell lines were negative for MICA or MICB (Figure 3B). Because NKG2D may interact with the ULBP molecules of MICA and MICB paralog, we stained breast-tumor cells with anti-ULBP1, anti-ULBP2, and anti-ULBP3, and we found that indeed these breast-tumor cell lines expressed ULBP1, ULBP2, and ULBP3 molecules. Taken together, these results indicate that the interaction between the TCR and antigens expressed on tumor cells is necessary and sufficient for T cell activation, whereas the interaction between NKG2D and ULBP enhances tumor-cell recognition by T cells.

Tumor-Specific γδ1 T Cells Suppress Naive and Effector T Cell Function

Given that tumor cells can grow even in the presence of up to 95% tumor-specific γδ1 T cells, it is clear that γδ1 T cells fail to eliminate these tumor cells. Instead, the growing tumor may drive the expansion of γδ1 T cells through tumor antigen-mediated activation, raising the possibility of a negative regulatory function for tumor-derived γδ1 T cells. Thus, we reasoned that these tumor-derived γδ1 T cells might play a role in inhibiting immune responses against tumor cells. To test this possibility, we performed a proliferation assay by using anti-CD3-(OKT3)-coated plates without APCs to determine their function (Peng et al., 2005). We found that the bulk BTIL31 cell line and its clones strongly inhibited the proliferation of naive T cells (Figure 4A). In contrast, γδ2 T cells isolated from normal PBMCs enhanced, rather than inhibited, the proliferation of naive T cells in response to anti-CD3 stimulation. γδ1 T cell-mediated inhibition was also observed for naive $CD8^+$ T cells as well as γδ2 effector T cells isolated from PBMCs (Figures S2A and S2C). To test whether other γδ1 T cells derived from breast tumors possess suppressive function, we purified γδ1 T cells from bulk TILs by FACS sorting after staining with γδ1 antibody and assessed their ability to suppress naive T cell proliferation (Figure 4B). Like BTIL31 γδ1 T cells, most breast-tumor-derived γδ1 T cells possessed suppressive activity (Figure 4B). These results strongly suggest that the majority of breast-tumor-derived γδ1 T cells possess a potent suppressive function.

We next tested whether these tumor-specific BTIL31 γδ1 T cells could inhibit IL-2 secretion from $CD4^+$ or

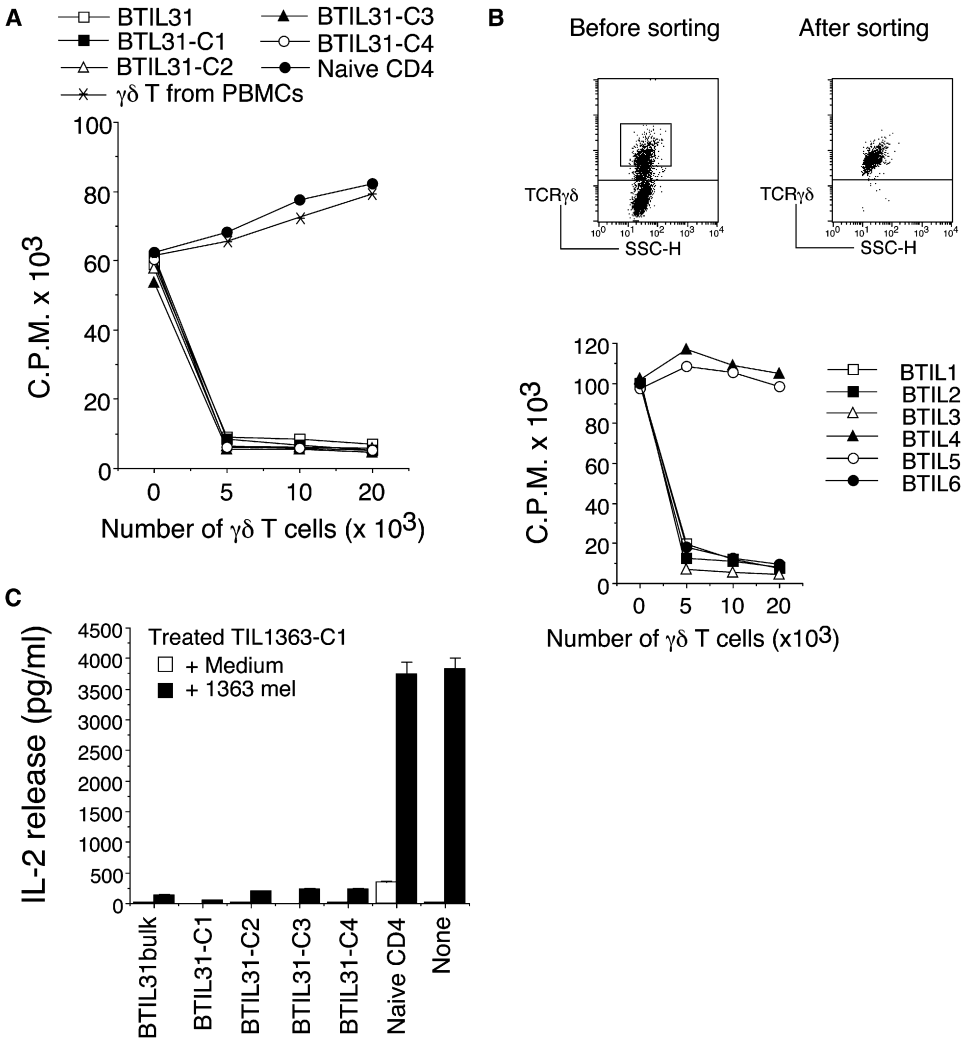

Figure 4. BTIL31 γδ1 T Cells Function as Treg Cells

(A) Suppression of naive T cell proliferation by BTIL31 γδ1 T cells in a functional assay. Proliferation assays were conducted as described in Experimental Procedures.

(B) Functional analysis of γδ1 T cells purified from bulk BTILs. γδ1 T cells were purified by FACS sorting and used to test for their ability to inhibit naive T cell proliferation.

(C) BTIL31 γδ1 T cells inhibit IL-2 release from $CD4^+$ effector T cells. Anti-CD3 antibody-activated BTIL31 γδ1 T cells, T cell clones, or control T cells were cocultured with $CD4^+$ TIL1363 T helper cells for 24 hr. After washing, all T cells were mixed with 1363 mel tumor cells. IL-2 secretion by $CD4^+$ TIL1363 T helper cells was determined by ELISA after 18 hr incubation. Data from one of three independent experiments with similar results are shown.

$CD8^+$ effector T cells in response to TCR stimulation. As previously demonstrated (Wang and Wang, 2005), $CD4^+$ T helper TIL1363 cells secreted large amounts of IL-2 after stimulation with 1363mel tumor cells (Figure 4C). Coculturing with OKT3-pretreated γδ1 T cells inhibited IL-2 secretion from the $CD4^+$ T helper TIL1363 cells (Figure 4C), in contrast to control $CD4^+$ T cells and PBMC-derived γδ T cells, which lacked any inhibitory effect on IL-2 release by $CD4^+$ effector cells. We also found that γδ1 T cells inhibited IL-2 secretion by $CD8^+$ TIL1359 effector T cells after stimulation with target 1359mel cells (Figure S2B). These results suggest that γδ1 T cells have a potent ability to suppress IL-2 production by both $CD4^+$ and $CD8^+$ effector T cells (hereafter termed γδ1 Treg cells).

To determine whether BTIL31 γδ1 Treg cells share any phenotypic properties with $CD4^+$ Treg cells, we examined their cytokine profiles and surface markers. Cytokine profiling analysis showed that these γδ1 Treg cells secreted IFN-γ and GM-CSF, but not other cytokines, such as IL-2, IL-4, IL-10, or TGF-β, when they were stimulated with either autologous tumor cells or a CD3 antibody (Figure S3A). To determine whether BTIL31 γδ1 Treg cells express surface markers typically found on $CD4^+$ Treg cells, we performed both FACS and real-time PCR analyses for

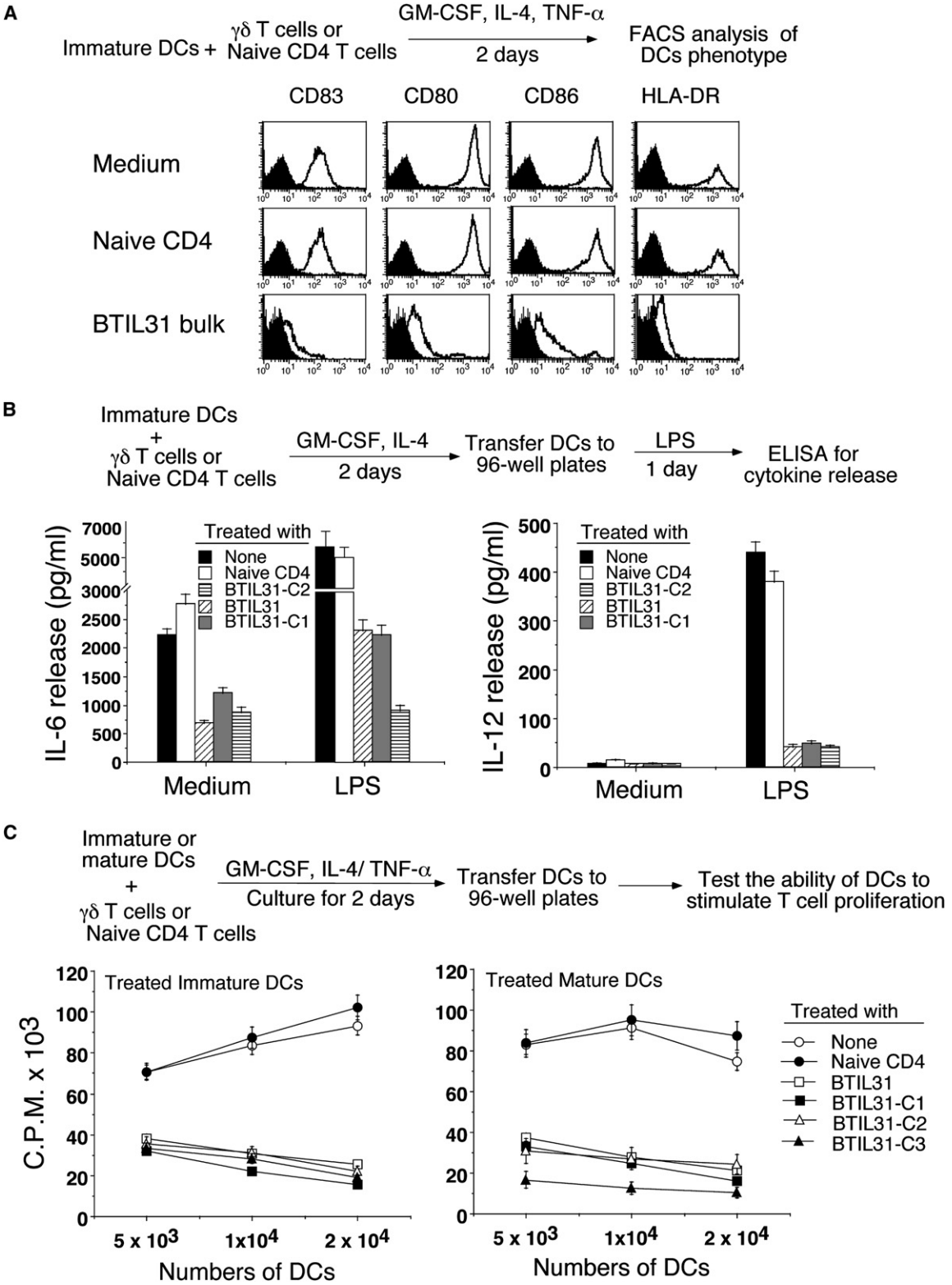

Figure 5. Suppression of DC Maturation and Function by BTIL31 γδ1 T Cells

(A) Inhibition of DC maturation by BTIL31 γδ1 T cells. The treated and untreated DCs were stained with CD83, CD80, CD86, and HLA-DR antibodies and analyzed by FACScan.

(B) Inhibition of DC function to secrete IL-6 and IL-12 by BTIL31 γδ1 T cells in response to LPS stimulation. Cytokine release is plotted as mean ± SD.

CD25, GITR, and Foxp3. BTIL31 γδ1 Treg cells were negative for CD25 and GITR (Figure S3B) and expressed little or no Foxp3 (Figure S3C), compared with results for previously characterized $CD4^+$ Treg cells. Thus, BTIL31 γδ1 T cells do not share the relatively specific markers of $CD4^+$ Treg cells.

In addition, we found that BTIL31 γδ1 Treg cells could mediate immune suppression even in a transwell, suggesting that soluble factors are involved in immune suppression (Figure S4A). Indeed, a small amount of cell supernatant was capable of inhibiting naive T cell proliferation (Figure S4B). However, neither anti-TGF-β nor IL-10 antibody added in assays affected their suppressive function (Figure S4C), consistent with their inability to secrete TGF-β or IL-10. To test whether other cytokines are involved in the suppressive function of BTIL31 γδ1 Treg cells, we found that they could secrete IL-6, but not IL-1β, TNF-α, and IL-12 (Figure S5A). Neither anti-IL-6 nor anti-TNF-α in a functional assay affected the suppressive function of γδ1 Treg cells (Figure S5B). Thus, these results excluded the possibility that TGF-β, IL-10, or IL-6 are involved in the γδ1 Treg cell-suppressive function. By using molecular weight cut-off columns, we found that the suppressive activity was present in fractions with a molecular mass larger than 100 kDa and could be inactivated by heat treatment at 56°C for 30 min, but not by DNase or RANase treatment (data not shown). Taken together, these results indicate that γδ1 Treg cells are functionally distinct from $CD4^+$ Treg cells.

Tumor-Specific γδ1 Treg Cells Impair DC Maturation and Function

We next asked whether BTIL31 γδ1 Treg cells could inhibit the maturation and function of DCs. Because they mediated immune suppression through soluble factors, we cultured BTIL31 γδ1 Treg or control cells in the inner well and DCs in the outer wells of a transwell plate and then tested the ability of DCs to mature in the presence of IL-4, GM-CSF, and TNF-α on the basis of the DC maturation markers CD83, CD80, CD86, and HLA-DR molecules. DCs treated with or without naive $CD4^+$ T cells expressed high amounts of CD83, CD80, CD86, and HLA-DR after being incubated with the maturation-inducing cytokines (Figure 5A). In sharp contrast, treatment with BTIL31 γδ1 Treg cells blocked DC maturation, in that expression of all four maturation markers were markedly inhibited. To test whether BTIL31 γδ1 Treg cells could impair the function of DCs, we treated the DCs with BTIL31 γδ1 Treg cells or naive $CD4^+$ T cells in a transwell plate for 48 hr and then assessed their ability to respond to LPS. The untreated mature DCs secreted large amounts of IL-6 and IL-12 in response to LPS stimulation. In contrast, the release of both cytokines from DCs markedly decreased after treatment with BTIL31 γδ1 Treg cells, whereas treatment with naive CD4 T cells had no effect on cytokine release by DCs (Figure 5B).

The ability of DCs to stimulate the proliferation of naive T cells in response to soluble CD3 antibody is well recognized (Shevach, 2002). To test whether BTIL31 γδ1 Treg cells can inhibit DC stimulation of naive T cell proliferation, we cocultured naive $CD4^+$ T cells with different numbers of immature or mature DCs that had been treated with BTIL31 γδ1 Treg cells or left untreated. The untreated DCs strongly stimulated the proliferation of naive $CD4^+$ T cells, regardless of their maturation status (Figure 5C). However, neither immature nor mature DCs treated with BTIL31 γδ1 Treg cell culture supernatants could stimulate naive T cell proliferation; the stimulating ability of DCs treated with naive $CD4^+$ T cells was not impaired. We conclude from these results that BTIL31 γδ1 Treg cells not only can block the maturation of DCs but also can suppress their ability to secrete cytokines in response to LPS.

Reversal of γδ1 Treg Cell-Suppressive Function by TLR8 Ligands

Although poly-guanosine (Poly-G) oligonucleotides can directly reverse the suppressive function of $CD4^+CD25^+$ Treg cells (Peng et al., 2005), it was not clear whether TLR8 ligands could reverse the suppressive function of γδ1 Treg cells. To test this possibility, we treated γδ1 Treg cells with a panel of TLR ligands and tested for their ability to suppress naive T cell proliferation. The TLR8 ligands Poly-G3 and ssRNA40, but not ligands for other TLRs, reversed the suppressive function of BTIL31 γδ1 Treg cells and restored the proliferation of naive $CD4^+$ T cells (Figure 6A). To confirm that Poly-G3 or ssRNA40 treatment restored the cell division of naive T cells, but not γδ1 Treg cells, we cultured BTIL31 γδ1 Treg cells with CFSE-labeled naive $CD4^+$ T cells in the presence or absence of Poly-G3. After 48 hr, the CFSE-labeled cells were gated for FACS analysis. Both BTIL31 γδ1 Treg and clones strongly inhibited the cell division of naive $CD4^+$ T cells compared with naive T cells not exposed to γδ1 Treg cells, whereas treatment with Poly-G3 oligonucleotides completely restored the cell division of naive T cells (Figure 6B). Similarly, we also found that treatment of γδ1 Treg cells with Poly-G3 oligonucleotides abrogated their suppressive effects on the maturation and function of DCs (Figure S6). Thus, these results indicate that Poly-G3 oligonucleotide treatment not only abolishes the ability of BTIL31 γδ1 Treg cells to suppress T cell proliferation and function but also abrogates their ability to inhibit DC maturation and function.

Control of $CD8^+$ T Cell-Mediated Antitumor Immunity by Tumor-Specific γδ1 Treg Cells In Vivo

We next sought to determine whether the γδ1 Treg cells could inhibit antitumor immune responses in a previously

(C) Inhibition of the ability of DCs to stimulate naive T cell proliferation in the presence of soluble OKT3 antibody after treatment with BTIL31 γδ1 T cells. Proliferation is plotted as mean ± SD.
Results are representative data from three independent experiments.

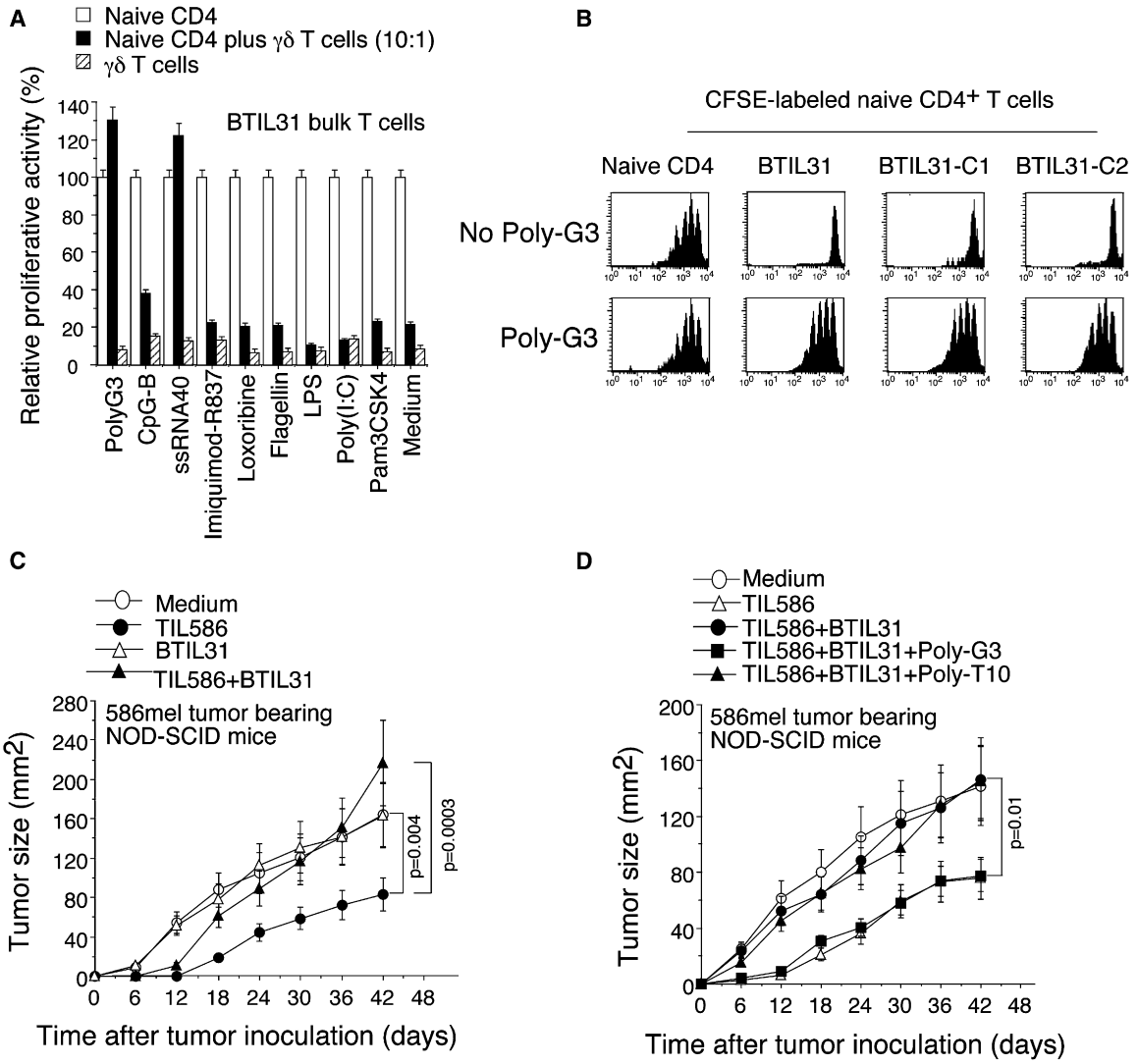

Figure 6. TLR8 Ligands Reverse the Suppressive Function of BTIL31 γδ1 Treg Cells In Vitro and In Vivo

(A) Reversal of suppressive function of γδ1 T cells by TLR8 ligands. Relative proliferative activity (%) is plotted as mean ± SD. Proliferative activity of naive T cells without γδ1 T cells serves as a basis (100%) to calculate relative proliferation of naive T cells plus γδ1 T cells in the presence of various TLR ligands. Results are one of three independent experiments.

(B) Restoration of CFSE-labeled naive $CD4^+$ T cell division by Poly-G3 oligonucleotides. CFSE-labeled naive $CD4^+$ T cells were cocultured with BTIL31 γδ1 Treg cells or their clones in the absence or presence of poly-G3 oligonucleotides in OKT3-coated 24-well plates. After 3 days of culture, cells were harvested and analyzed for cell divisions by FACS gated on the CFSE-labeled cells. CFSE-labeled naive $CD4^+$ T cells alone served as a control. Data are one of two independent experiments.

(C) Inhibition of antitumor responses by BTIL31 γδ1 T cells in NOD-SCID mice. Human 586mel tumor cells were subcutaneously injected into NOD-SCID on day 0. Tumor-specific $CD8^+$ TIL586 cells were injected i.v. on day 3 with or without OKT3-preactivated BTIL31 γδ1 T cells. Tumor volumes were measured and presented as means ± SD (n = 5 mice per group).

(D) Poly-G reverses the suppressive function of BTIL31 and enhances antitumor immunity in NOD-SCID mice. Experimental procedures and tumor cell injection were same as in (C) with the exception that OKT3-preactivated γδ1 T cells were treated with Poly-G3 or Poly-T10. Tumor volumes were measured and presented as means ± SD (n = 5 mice per group). p values in (C) and (D) were determined by the one-way analysis of variance (ANOVA). Similar results were obtained in three repeat experiments.

established tumor model (Peng et al., 2005), because Treg cells can suppress any effector T cells in antigen-nonspecific way once they are activated. When injected alone into NOD-SCID (NK, T, and B cell-deficient) mice, 586mel tumor cells showed progressive growth. Tumor growth was inhibited when tumor-bearing mice were treated with autologous tumor-specific $CD8^+$ TIL586 cells, which can kill 586mel cells (Figure 6C). However, when $CD8^+$ TIL586 cells were coinjected with anti-CD3-preactivated γδ1 Treg cells, γδ1 Treg cells inhibited the ability of $CD8^+$ TIL586

cells to kill tumor cells and tumor cells grew progressively ($p = 0.004$), suggesting that γδ1 Treg cells have the potent ability to suppress tumor-specific $CD8^+$ T cells in vivo.

To further demonstrate that treatment of γδ Treg cells with Poly-G oligonucleotides can reverse their suppressive function and restore the antitumor immune response, we adoptively transferred TIL586 cells and Poly-G3-treated γδ1 Treg cells into tumor-bearing mice. Poly-G treatment restored the ability of $CD8^+$ TIL586 cells to inhibit tumor growth ($p = 0.01$) (Figure 6D). Thus, TLR8 ligand Poly-G oligonucleotides can reverse the suppressive function of γδ1 Treg cells both in vitro and in vivo.

Critical Molecules Required for the Reversal of γδ Treg Cell Function

We next sought to determine whether TLR8 is expressed in γδ Treg cells by real-time PCR. TLR8 mRNA expression in γδ Treg cells was at least 15-fold higher than that in 293 cells (Figure S7A). Further experiments showed that these γδ Treg cells also expressed TLR1, TLR7, and TLR9, but weakly or did not express other TLRs (Figure S7B). Because most TLRs use the common adaptor MyD88 molecule to transduce the signaling from TLR receptors to the downstream pathway, the critical question is why TLR8 ligands, but not ligands for other TLRs, can reverse the suppressive function of γδ Treg cells. To address this issue, we constructed a GFP-expressing lentivirus encoding a cDNA for hTLR7 and transduced them into γδ Treg cells. GFP^+ (transduced) γδ Treg cells were purified by FACS sorting and then used in a functional assay to determine their ability to respond to TLR7 ligand loxoribine treatment. Neither TLR7-transduced GFP^+ γδ Treg cells nor untransduced cells could respond to loxoribine treatment (Figure 7A), suggesting that activation of TLR7 signaling pathway does not control the suppressive function of γδ Treg cells. To demonstrate that lenti-hTLR7 construct was functional, we showed that lenti-hTLR7 constructs were capable of activating the NF-κB-luc reporter gene in response to the TLR7 ligand loxoribine (Figure S8A). Treatment of endogenous TLR7-expressing γδ Treg cells with TLR7 ligand loxoribine also resulted in the translocation of phosphorylated p65 (NF-κB) into the nucleus (Figure S8B). Furthermore, we found that the endogenous TLR7-expressing γδ Treg cells did not respond to ligands for TLR7 and TLR9, but did respond to TLR8 ligands for their functional reversal (Figure S8C). Taken together, we conclude that TLR7 signaling is capable of activating NF-κB pathway but does not control the suppressive function of γδ1 Treg cells.

Based on these results, we reasoned that hTLR8 likely uses a unique downstream signaling pathway linked to the control of γδ Treg cell function. To test this hypothesis, we used an siRNA-mediated knockdown approach to identify critical molecules required for the reversal of Treg cell function. A panel of GFP-expressing lentivirus-based U6 promoter-driven shRNA (lenti-shRNA) constructs (four constructs per gene) was made against IRAK4, TRAF6, TAK1, IKKα, IKKβ, JNK1, JNK2, ERK1, ERK2, and p38α molecules. By using a previously described approach (Peng et al., 2005), we screened and identified the lenti-shRNA constructs with the highest knockdown efficiency for their corresponding target genes by western blot analysis (Figure S9). The specificity and knockdown efficiency of the lenti-shRNA constructs were also confirmed in T cells. Specific knockdown of JNK1, TAK1, and IKKβ at both mRNA and protein levels was observed in T cells transduced with relevant shRNA, but there was no effect on irrelevant genes, as shown in Figures S10A and S10B. We did not observe any effect on target genes when scrambled shRNAs were used (Figure S10C).

The lenti-shRNAs with the highest knockdown efficiency were selected for the transduction of γδ1 Treg cells. GFP^+ (tranduced) and GFP^- (untransduced) Treg cells were purified by FACS sorting and used to test for their ability to respond to Poly-G10 or Poly-T10 oligonucleotides (a control). We found that the knockdown of TLR8, IRAK4, and TRAF6 in γδ Treg cells markedly diminished their ability to respond to Poly-G10 treatment (Figure 7B). In contrast, the TAK1 knockdown in GFP^+ T cells did not have any effect, although TAK1 has been implicated to play a critical role in the TLR signaling pathway (Akira et al., 2006). Further knockdown experiments with lenti-shRNA against IKKα, IKKβ, JNK1, JNK2, ERK1, ERK2, and p38α molecules revealed that the knockdown of either IKKα or IKKβ molecule resulted in the partial inhibition of Treg cells to respond to TLR8 ligand treatment, whereas the RNAi-mediated p38α knockdown completely blocked Poly-G10-mediated reversal effect on γδ Treg cells (Figure 7C). In contrast, the knockdown of JNK1, JNK2, ERK1, or ERK2 molecule did not have any effect (Figure 7C). These results identify a distinct TLR8 signaling pathway requiring the participation of MyD88, IRAK4, TRAF6, p38, and IKK molecules, whereas the TAK1, JNK, and ERK pathways do not appear to be involved in the reversal of γδ Treg cell function.

DISCUSSION

Negative immune regulatory activity at tumor sites has typically been attributed to $CD4^+$ $CD25^+$ Treg cells, although recent findings suggest that other T cell subsets can function as suppressors of antitumor immune responses (Shevach, 2002; Wang, 2006). In contrast to an earlier study showing a high percentage of $CD4^+$ $CD25^+$ Treg cells in the case of breast cancer (Liyanage et al., 2002), we did not find an increased percentage of $CD4^+$ $CD25^+$ Treg cells in our TILs derived from breast tumors (data not shown), which is consistent with a recent report of comparable numbers of $CD4^+$ $CD25^+$ Treg cells in cancer patients versus healthy donors (Okita et al., 2005). These observations, together with data of a dominant $CD8\alpha\alpha^+$ γδ1 T cell population among breast TILs, led us to postulate a suppressive role of this T cell subset at tumor sites. In the present study, we demonstrate that breast- as well as prostate-tumor-derived TILs contain a dominant γδ1 T cell population. Because currently available γδ TCR antibodies do not work for immunohistochemistry staining, we could not evaluate the percentage of γδ1 T cells in tumor tissues. However,

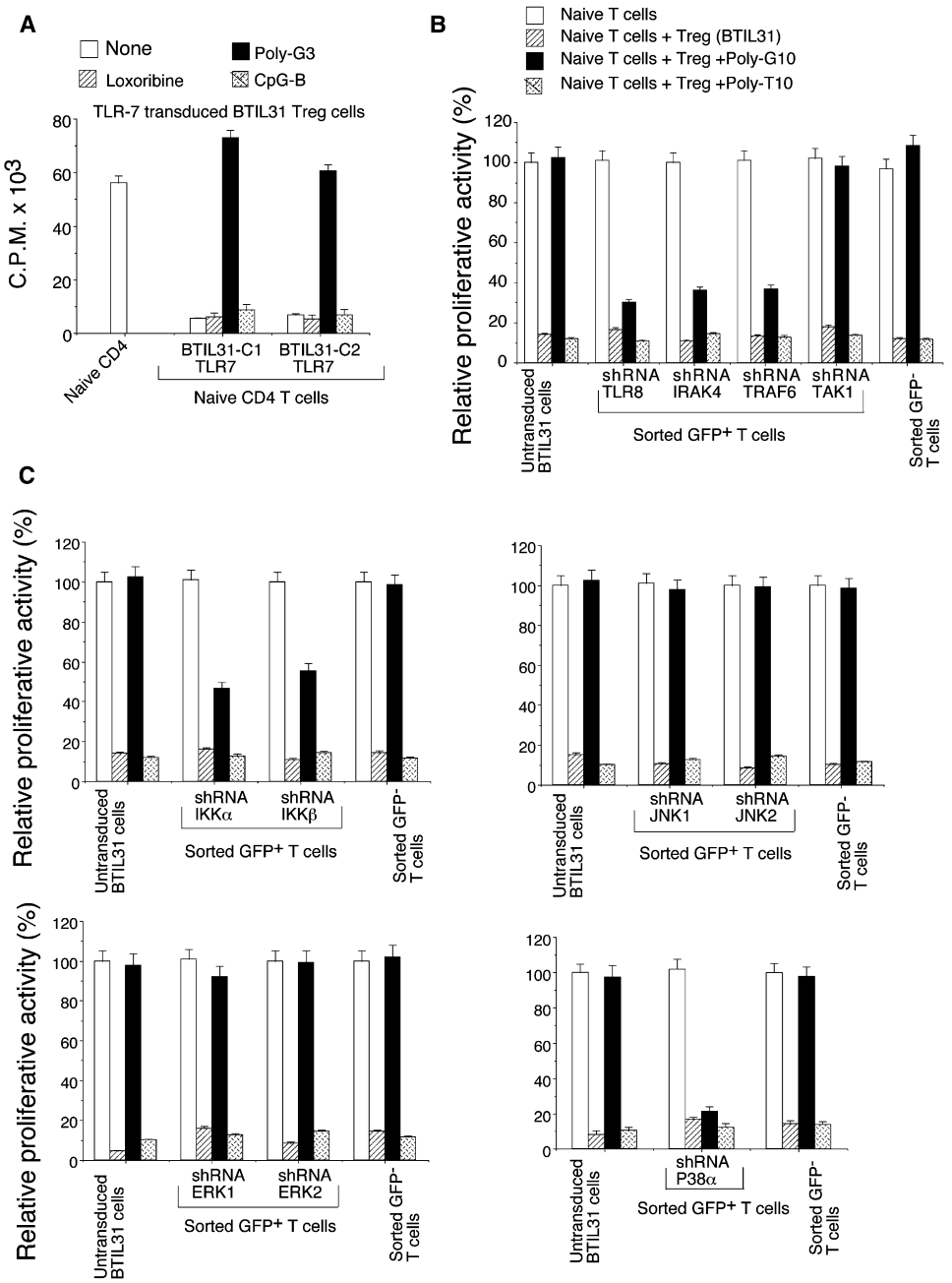

Figure 7. Identification of TLR8 Signaling Pathways and Key Molecules Required for the Functional Control of γδ1 Treg Cells by Poly-G10 Oligonucleotides

(A) Specificity of TLR8-mediated functional reversal of γδ1 Treg cells. TLR7-expressing BTIL31 T cell clones were generated and purified by FACS sorting. The GFP$^+$ T cells were used to evaluate their ability to respond to different ligands for TLR7 (Loxoribine), TLR8 (Poly-G3), and TLR9 (CpG-B) in a functional proliferation assay. Data are plotted as means ± SD and are one of three experiments.

(B) Knockdown of TLR8, IRAK4, and TRAF6, but not TAK1, blocked the ability of γδ1 Treg cells to respond to Poly-G10 treatment. BTIL31 γδ1 Treg cells were infected with lenti-shRNAs specific for TLR8, IRAK4, TRAF6, and TAK1 molecules, respectively. GFP$^+$ (transduced) and GFP$^-$ (untransduced) BTIL31 γδ1 Treg cells were obtained by FACS sorting and then used to determine their ability to respond to Poly-G10 in functional proliferation assays. Untransduced BTIL31 γδ Treg cells and Poly-T10 served as controls. Proliferative activity of naive T cells without γδ1 T cells serves as a basis (100%) to calculate relative proliferation of naive T cells in the presence of γδ1 T cells and various TLR ligands.

(C) Determination of downstream pathways required for TLR8-mediated functional reversal of γδ1 Treg cells. Experimental procedures were identical to those in (B). Untransduced BTIL31 γδ1 Treg cells and Poly-T10 served as controls.

Results are representative of three experiments.

our conclusion is supported by a previous study showing an increased γδ1 T cell population (27%–74%) in renal carcinoma (Choudhary et al., 1995), even though the suppressive function of these γδ1 T cells was not reported. In contrast, by using the same short-term culture condition, we could not demonstrate a high percentage of γδ1 T cells in melanoma-derived TILs, which are generally dominated by conventional $CD4^+$ and $CD8^+$ T cell populations. Thus, we suggest that tumor-infiltrating T cells from epithelium-derived tumor cells contain a higher proportion of γδ1 T cells than do TILs originating in other tissues.

Our findings raise the intriguing question of how γδ1 Treg cells are recruited and then expanded in breast tumors. It has been suggested that tumor cells and immune cells recruit $CD4^+$ Treg cells to tumor sites through cytokine and chemokine attraction (Curiel et al., 2004; Huang et al., 2006). γδ1 Treg cells do not express the CCR4 or CCR5 receptor (data not shown), so they probably do not use the same mechanism as $CD4^+$ Treg cells to infiltrate into tumor sites. Because they naturally reside in epithelial tissues, γδ1 Treg cells can readily traffic in both normal and malignant epithelium. A plausible mechanism for the expansion of γδ1 Treg cells at tumor sites may be through direct presentation of antigens by tumor cells. In our previous studies, we showed that tumor cells express tumor-specific antigens such as LAGE1 and ARTC1 and directly stimulate antigen-specific Treg cells (Wang et al., 2004, 2005). However, our preliminary data (not shown) indicate that these breast-tumor-derived γδ1 Treg cells cannot recognize the previously identified small nonpeptide molecules (Eberl et al., 2004; Hayday, 2000), suggesting that they recognize new and distinct antigens expressed on autologous tumor cells. Identification of such tumor antigens may help us to understand how γδ1 Treg cells are activated and maintained at tumor sites.

Although a regulatory role has been suggested for γδ T cells (Pennington et al., 2005), the suppressive function of human γδ1 cells has not been demonstrated thus far. Our studies with both breast-tumor-derived bulk γδ T cell lines and clones clearly demonstrate a role for γδ1 T cells in the negative regulation of antitumor immunity. They potently suppress the proliferation and IL-2 secretion of naive/effector T cells and inhibit DC maturation and function. More importantly, we found that these breast cancer-derived γδ1 Treg cells inhibit antitumor immune responses in animal models. Although they share some suppressive properties with $CD4^+$ $CD25^+$ Treg cells, these tumor-derived γδ Treg cells also possess unique or distinctive phenotypic and functional features. For example, the tumor-derived γδ1 Treg cells do not express CD25 and Foxp3 markers, which are typically expressed by $CD4^+$ Treg cells, and they suppress immune responses through a soluble factor-dependent mechanism independent of IL-10 and/or TGF-β, in contrast to the cell-cell contact-dependent suppressive mechanism of $CD4^+$ $CD25^+$ Treg cells. The clear demonstration that murine γδ T cells can "cross-suppress" conventional TCRαβ T cells was recently reported by Pennington et al. (2006), where they also showed that the suppression by γδ T cells does not require Foxp3. Hence, epithelial tumor-derived γδ1 T cells represent a unique subset of Treg cells. We are aware that identification of soluble factors responsible for immune suppression is critical to a full understanding of the molecular mechanisms by which γδ1 Treg cells suppress naive T cells. Unfortunately, attempts to define such molecules have so far proved unsuccessful, not only for γδ1 Treg cells but also for more widely studied naturally occurring $CD4^+$ $CD25^+$ Treg cells. This gap in knowledge underscores the need to continue our efforts to identify the molecular mechaisms of immune suppression mediated by Treg cells.

The ability of γδ1 Treg cells to suppress the function of both T cells and DCs suggests that their depletion or the reversal of their suppressive function could enhance antitumor immune responses against breast cancer. Because γδ1 Treg cells do not express a high amount of CD25 molecules, they could not be eliminated with CD25 antibody or the IL-2-toxin fusion protein (Ontak). An alternative would be to manipulate TLR signaling in these Treg cells, which was shown to reverse the suppressive function of naturally occurring $CD4^+$ Treg as well as tumor-specific Treg cells (Peng et al., 2005). Indeed, we found that tumor-derived γδ1 T cells express TLR8 ligands and that treatment with TLR8 ligands can reverse their suppressive function both in vivo and in vitro, implying that such cells share a common TLR8 signaling-mediated mechanism with previously characterized $CD4^+$ Treg cell subsets. In contrast to our present and previous demonstration of the unique function of TLR8 signaling (Peng et al., 2005), human TLR5 has recently been reported to enhance rather than reverse the suppressive function of $CD4^+$ $CD25^+$ Treg cells (Crellin et al., 2005). Furthermore, activation of TLR2 with its ligand (Pam3Cys) directly increases the proliferation of murine Treg cells and temporally reverses their suppressive function (Liu et al., 2006; Sutmuller et al., 2006a). It should be noted that TLR8 is not functional in mice (Jurk et al., 2002). Other TLRs such as TLR4 and TLR9 have been reported to be involved in the control of murine Treg cell function, mainly through activating DCs by TLR ligands (Pasare and Medzhitov, 2003), in contrast to human TLR8 signaling, which directly controls the suppressive function of human $CD4^+$ Treg cells as well as $CD8\alpha\alpha^+$ γδ1 Treg cells without DC involvement. These studies indicate that TLR signaling is critically important in controlling the suppressive function of Treg cells, but that the mechanisms of TLR-mediated regulation of Treg cell suppression in humans likely differ from those in mice.

Our functional studies via an RNAi-mediated knockdown approach revealed that IRAK4 and TRAF6 are critical for TLR8-mediated reversal of γδ1 Treg cells; however, we unexpectedly found that TAK1 is not required for such an effect. This hypothesis is supported by an overexpression study of TLR8 in HEK293 cells, showing that hTLR8 mediates TAK1-independent NF-κB and JNK activation (Qin et al., 2006), even though such experiments did not provide information on the functional consequence of this effect. TAK1 has been demonstrated to play an essential role in T cell development and survival and is a key

molecule downstream of TRAF6 in response to TLR- and antigen-mediated stimulation in T cells (Sato et al., 2006; Wan et al., 2006) as well as in B cells (Sato et al., 2005). Thus, although TAK1 is essential for T cell proliferation and development, it does not appear to be involved in the TLR8-mediated signaling pathway. These results together with the published reports suggest unique features of the TLR8 versus the common TLR signaling pathway. Because MEKK3 has been suggested to transduce signaling from TRAF6 to p38 and NF-κB activation (Huang et al., 2004), we are currently evaluating this possibility. In addition, it is likely that this unique TLR8 signaling pathway also operates in $CD4^+$ Treg cells. If so, manipulation of TLR8 signaling pathway through its ligands or drug inhibitors of key molecules in this pathway may allow us to shut off the suppressive function of different subsets of Treg cells, thus increasing the likelihood to improve therapeutic potential of cancer vaccines.

EXPERIMENTAL PROCEDURES

Cell Lines and Antibodies

MCF-7 breast-tumor cells and HEK293T cells were obtained from the American Tissue Culture Collection (ATCC). Melanoma 1359 mel, 1363 mel, and Epstein-Barr virus (EBV)-transformed B cell line 586LCL were obtained from the Surgery Branch, NCI. These lines were maintained in RPMI 1640 medium containing 10% fetal calf serum (FCS). Breast carcinoma cell lines (BC36, BC31, BC30, and BC29) and prostate cancer cell lines (PC263 and PC267) were established in our laboratory and maintained in keratinocyte medium containing 25 μg/ml bovine pituitary extract, 5 ng/ml epidermal growth factor, 2 mM L-glutamine, 10 mM HEPES buffer, 2% heat-inactivated FBS, and penicillin-streptomycin (Invitrogen, Inc. San Diego, CA). TIL1359-C20 and BTIL-C1 were conventional $CD8^+$ T cell clones capable of recognizing the corresponding tumor cells in the context of MHC class I molecules, whereas TIL1358-4B8 comprised $CD4^+$ T cell clones that recognize an antigen in the context of HLA-DR4 molecules.

Specific anti-human antibodies including anti-CD4, anti-CD8, anti-CD56, anti-CD16, anti-CD19, anti-CD161, anti-CD25, anti-CTLA-4, anti-TCRαβ, anti-TCRγδ, anti-CD80, anti-CD83, anti-CD86, and anti-HLA-DR conjugated FITC or PE, anti-JNK1 were purchased from R&D Systems and BD Biosciences. TAK1 antibody was purchased from Cell Signaling Technology. Anti-Vδ1 (R9.2), anti-Vδ2 (IMMU 389), and anti-Vδ9 (IMMU 360) were purchased from Beckman Coulter. Anti-GITR mAb were obtained from R&D Systems. Anti-MHC class I (HLA-A, B, C; HB95) and anti-class II (HLA-DR, DP, DQ; HB145) were purified from hybridoma supernatants.

Generation of Tumor-Infiltrating Lymphocytes and T Cell Cloning

Breast and prostate cancer tissues were minced into small pieces followed by digestion with triple enzymes mixture containing collagenase type IV, hyaronidase, and deoxyribonuclease for 2 hr at room temperature. After digestion, the cells were washed twice in RPMI1640 and cultured in RPMI1640 containing 10% human serum supplemented with L-glutamine and 2-mercaptethanol and 1000 U/ml of IL-2 for the generation of T cells. Once T cells were released from tumor tissues, they were grown in high-dose IL-2 medium for 1 week. These T cells were then transferred to a fresh well and grown in low-dose IL-2 (50 U/ml)-containing medium. T cell clones were generated from TILs by the limiting dilution cloning method, as previously described (Wang et al., 2004). Tumor-reactive T cell clones were expanded and selected for further analysis. γδ2 T cells were purified from human PBMCs by sorting after being stained with Vδ2 antibody.

FACS Analysis and Sorting

The expression of CD25 and GITR on γδ T cells was determined by FACS analysis after staining with specific antibodies (purchased from R&D Systems and BD Biosciences). To purify the γδ T cells, we stained T cells with a γδ antibody conjugated to either PE or FITC. After washing, the cells were sorted by FACSARIA. For experiments with carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled cells, we stained naive $CD4^+$ T cells with CFSE (4.5 μM) from Molecular Probes at 37°C for 15 min. After washing, the labeled cells were cultured in RPMI 1640 containing 10% human AB serum and IL-2 (300 IU/ml). To determine suppression of cell division by BTIL31 γδ1 Treg cells, we added unlabeled γδ1 T cells to CFSE-labeled naive T cells at a 1:1 ratio in OKT3-coated 24-well plates in the presence or absence of Poly-G3 (3 μg/ml). After 2 days in culture, the cells were analyzed by FACS gating on the CFSE-labeled cells.

To determine the frequency of γδ T cells in the total T cell population (directly isolated from resected tumor samples without in vitro culture), we digested breast-tumor samples to obtain single-suspension cells and then stained them with CD3-FITC and TCRγδ-PE antibody. FACS analysis was conducted after gating on anti-CD3-positive T cell population.

Proliferation and Cytokine Assays

Proliferation assays were done as previously described (Peng et al., 2005; Wang et al., 2004). Transwell experiments were performed in 24-well plates with a pore size of 0.4 μm (Corning Costar, Cambridge, MA) as described previously (Wang et al., 2004). 2×10^5 freshly purified naive $CD4^+$ T cells were cultured in the outer wells of 24-well plates in medium containing 0.1 μg/ml anti-CD3 (OKT3) antibody and 2×10^5 APCs. Equal numbers of γδ T cells or naive $CD4^+$ T cells were added into the inner wells in the same medium containing 0.1 μg/ml CD3 antibody and 2×10^5 APCs. After 56 hr of culture, the cells in the outer and inner wells were harvested separately and transferred to 96-well plates. [^{3}H]thymidine was added, and the cells were cultured for an additional 16 hr before being harvested for counting the radioactivity with a liquid scintillation counter. All experiments were performed in triplicate. To determine whether the T cell recognition of autologous tumor cells could be blocked by specific Abs, we assessed T cell activity in the absence or presence of various Abs as previously described (Wang et al., 1999).

Suppression Assay for IL-2 Release from $CD4^+$ and $CD8^+$ Effector T Cells

CD3 mAb-activated BTIL31 cells and their clones were cocultured with $CD4^+$ TIL1363 helper T cells at a 1:1 ratio in RPMI 1640 growth medium containing 30 IU/ml IL-2 supplemented with 10% human serum for 24 hr. After washing, the treated T cells were cultured with 1363 mel for another 24 hr. IL-2 secretion in the culture supernatants was determined by ELISA after 18 hr incubation. Naive $CD4^+$ T cells purified from human PBMCs were used as controls. Similar experiments were performed for $CD8^+$ TIL1359 effector cells and 1359 mel tumor cells.

Inhibition of DC Maturation and Function by γδ1 T Cells

Immature and mature dendritic cells (DCs) were derived from the monocytes of healthy donors in culture with IL-4 and GM-CSF with or without TNF-α. The immature DCs or mature DCs were treated with γδ T cells in the transwell system. In brief, 5×10^5 BTIL31 or its clones were put into the inner wells, and 1×10^6 immature or mature DCs were cultured in the outer wells of 24-well plates in medium containing IL-4 and GM-CSF with or without TNF-α. After 48 hr, the treated and untreated DCs were harvested and divided into different groups. For phenotypic analysis, the surface markers of CD83, CD80, CD86, and HLA-DR of DCs were analyzed by FACScan (Becton Dickinson). For cytokine secretion, the treated and untreated DCs were stimulated with LPS (5 μg/ml) for 24 hr. Release of IL-12 and IL-6 by DCs was measured by ELISA Kit (R&D System). To determine the ability of DCs to stimulate the proliferation of allogeneic $CD4^+$ T cells, we cocultured the γδ T cell-treated or untreated immature and mature DCs with naive

$CD4^+$ T cells at different ratios and determined the proliferation of naive $CD4^+$ T cells.

Toll-like Receptor Ligands and Proliferation Assays

Naive $CD4^+$ T cells were purified from PBMCs by microbeads (Miltenyi Biotec). Naive $CD4^+$ T cells (10^5/well) were cultured with regulatory T cells at a ratio of 10:1 in OKT3 (2 μg/ml)-coated, U-bottomed 96-well plates containing the following TLR ligands: LPS (100 ng/ml), imiquimod (10 μg/ml), loxoribine (500 μM), poly (I:C) (25 μg/ml), ssRNA40/LyoVec (3 μg/ml), ssRNA33/LyoVec (3 μg/ml), pam3CSK4 (200 ng/ml), and flagellin (10 μg/ml), all purchased from Invivogen (San Diego, CA). CpG-A (3 μg/ml), CpG-B (3 μg/ml), and poly-G3 oligonucleotides (3 μg/ml) were synthesized by Integrated DNA Technologies (Coralville, IA). All experiments were performed in triplicate.

Real-Time Quantitative PCR Analysis

Foxp3 mRNA level on γδ T cells or $CD4^+$ Treg cells was quantified by real-time PCR via ABI/PRISM7000 sequence detection system (PE Applied Biosystems, Foster City, CA) as previously described (Peng et al., 2005). The PCR reaction was performed with specific primers; an internal fluorescent TaqMan probe specific to Foxp3 or HPRT was purchased from PE Applied Biosystems. Foxp3 mRNA level in each sample was normalized with the relative quantity of HPRT. All samples were run in triplicate.

Lentivirus-shRNA Constructs and Screening

Three or four shRNA constructs per gene were made. shRNA design, cloning, and screening were identical to those previously described (Peng et al., 2005). shRNA constructs with the highest knockdown efficiency were selected to transduce T cells. To determine the specificity and knockdown efficiency of lenti-shRNA, we performed similar experiments for JNK1, TAK1, and IKKβ, as previously described (Peng et al., 2005).

In Vivo Tumor Growth and Antitumor Immunity

Human 586 mel tumor cells (1×10^6) in 100 ml of buffered saline were subcutaneously injected into NOD-SCID (lacking T and B cells) on day 0. Tumor-specific $CD8^+$ TIL586 cells (5×10^6), which recognized and kill 586mel cells, were i.v. injected on day 3 with or without BTIL31 cells (1×10^6) pretreated with Poly-G3 or Poly-T10. Tumor size was measured with calipers every 2–3 days. Tumor volume was calculated on the basis of two-dimension measurements. All mice were maintained under specific pathogen-free conditions according to institutional guidelines and animal study protocols approved by the institutional animal care and use committees.

Statistical Analysis

Unless indicated otherwise, data are expressed as mean ± SD. The significance of difference between groups was determined by a one-tailed Student's t test or the one-way analysis of variance (ANOVA).

Supplemental Data

Ten figures are available at http://www.immunity.com/cgi/content/full/27/2/334/DC1/.

ACKNOWLEDGMENTS

We thank staff in pathology core of Baylor Breast SPORE, Baylor Prostate SPORE, and M.D. Anderson Skin SPORE for providing tumor samples. We also thank E. Shevach for his critical reading and discussion, B. Su for providing JNK1 and JNK2 plasmid, J. Hong and J. Zhang for experimental assistant, and L. Old for his support and collaboration. This work is in part supported by grants from American Cancer Society and Cancer Research Institute and National Institutes of Health (NIH). G.P. is partially supported by NIH T32 training grant. The authors declare that they have no competing financial interests.

Received: December 30, 2006
Revised: April 6, 2007
Accepted: May 25, 2007
Published online: July 26, 2007

REFERENCES

Akira, S., Uematsu, S., and Takeuchi, O. (2006). Pathogen recognition and innate immunity. Cell *124*, 783–801.

Bauer, S., Groh, V., Wu, J., Steinle, A., Phillips, J.H., Lanier, L.L., and Spies, T. (1999). Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. Science *285*, 727–729.

Boismenu, R., and Havran, W.L. (1994). Modulation of epithelial cell growth by intraepithelial gamma delta T cells. Science *266*, 1253–1255.

Brenner, M.B., McLean, J., Dialynas, D.P., Strominger, J.L., Smith, J.A., Owen, F.L., Seidman, J.G., Ip, S., Rosen, F., and Krangel, M.S. (1986). Identification of a putative second T-cell receptor. Nature *322*, 145–149.

Bukowski, J.F., Morita, C.T., and Brenner, M.B. (1999). Human gamma delta T cells recognize alkylamines derived from microbes, edible plants, and tea: implications for innate immunity. Immunity *11*, 57–65.

Choudhary, A., Davodeau, F., Moreau, A., Peyrat, M.A., Bonneville, M., and Jotereau, F. (1995). Selective lysis of autologous tumor cells by recurrent gamma delta tumor-infiltrating lymphocytes from renal carcinoma. J. Immunol. *154*, 3932–3940.

Constant, P., Davodeau, F., Peyrat, M.A., Poquet, Y., Puzo, G., Bonneville, M., and Fournie, J.J. (1994). Stimulation of human gamma delta T cells by nonpeptidic mycobacterial ligands. Science *264*, 267–270.

Crellin, N.K., Garcia, R.V., Hadisfar, O., Allan, S.E., Steiner, T.S., and Levings, M.K. (2005). Human CD4+ T cells express TLR5 and its ligand flagellin enhances the suppressive capacity and expression of FOXP3 in CD4+CD25+ T regulatory cells. J. Immunol. *175*, 8051–8059.

Curiel, T.J., Coukos, G., Zou, L., Alvarez, X., Cheng, P., Mottram, P., Evdemon-Hogan, M., Conejo-Garcia, J.R., Zhang, L., Burow, M., et al. (2004). Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat. Med. *10*, 942–949.

Diefenbach, A., Jensen, E.R., Jamieson, A.M., and Raulet, D.H. (2001). Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity. Nature *413*, 165–171.

Dunn, G.P., Old, L.J., and Schreiber, R.D. (2004). The three Es of cancer immunoediting. Annu. Rev. Immunol. *22*, 329–360.

Eberl, M., Jomaa, H., and Hayday, A.C. (2004). Integrated immune responses to infection—cross-talk between human gammadelta T cells and dendritic cells. Immunology *112*, 364–368.

Girardi, M., Oppenheim, D.E., Steele, C.R., Lewis, J.M., Glusac, E., Filler, R., Hobby, P., Sutton, B., Tigelaar, R.E., and Hayday, A.C. (2001). Regulation of cutaneous malignancy by gammadelta T cells. Science *294*, 605–609.

Groh, V., Steinle, A., Bauer, S., and Spies, T. (1998). Recognition of stress-induced MHC molecules by intestinal epithelial gammadelta T cells. Science *279*, 1737–1740.

Groh, V., Rhinehart, R., Secrist, H., Bauer, S., Grabstein, K.H., and Spies, T. (1999). Broad tumor-associated expression and recognition by tumor-derived gamma delta T cells of MICA and MICB. Proc. Natl. Acad. Sci. USA *96*, 6879–6884.

Havran, W.L. (2000). A role for epithelial gammadelta T cells in tissue repair. Immunol. Res. *21*, 63–69.

Hayday, A.C. (2000). [gamma][delta] cells: a right time and a right place for a conserved third way of protection. Annu. Rev. Immunol. *18*, 975–1026.

Hayday, A., and Tigelaar, R. (2003). Immunoregulation in the tissues by gammadelta T cells. Nat. Rev. Immunol. *3*, 233–242.

Huang, Q., Yang, J., Lin, Y., Walker, C., Cheng, J., Liu, Z.G., and Su, B. (2004). Differential regulation of interleukin 1 receptor and Toll-like receptor signaling by MEKK3. Nat. Immunol. *5*, 98–103.

Huang, B., Pan, P.Y., Li, Q., Sato, A.I., Levy, D.E., Bromberg, J., Divino, C.M., and Chen, S.H. (2006). Gr-1+CD115+ immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell anergy in tumor-bearing host. Cancer Res. *66*, 1123–1131.

Jameson, J., Ugarte, K., Chen, N., Yachi, P., Fuchs, E., Boismenu, R., and Havran, W.L. (2002). A role for skin gammadelta T cells in wound repair. Science *296*, 747–749.

Jameson, J., Witherden, D., and Havran, W.L. (2003). T-cell effector mechanisms: gammadelta and CD1d-restricted subsets. Curr. Opin. Immunol. *15*, 349–353.

Jurk, M., Heil, F., Vollmer, J., Schetter, C., Krieg, A.M., Wagner, H., Lipford, G., and Bauer, S. (2002). Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848. Nat. Immunol. *3*, 499.

Kabelitz, D., Marischen, L., Oberg, H.H., Holtmeier, W., and Wesch, D. (2005). Epithelial defence by gamma delta T cells. Int. Arch. Allergy Immunol. *137*, 73–81.

Liu, H., Komai-Koma, M., Xu, D., and Liew, F.Y. (2006). Toll-like receptor 2 signaling modulates the functions of CD4+CD25+ regulatory T cells. Proc. Natl. Acad. Sci. USA *103*, 7048–7053.

Liyanage, U.K., Moore, T.T., Joo, H.G., Tanaka, Y., Herrmann, V., Doherty, G., Drebin, J.A., Strasberg, S.M., Eberlein, T.J., Goedegebuure, P.S., and Linehan, D.C. (2002). Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. J. Immunol. *169*, 2756–2761.

Modlin, R.L., Pirmez, C., Hofman, F.M., Torigian, V., Uyemura, K., Rea, T.H., Bloom, B.R., and Brenner, M.B. (1989). Lymphocytes bearing antigen-specific gamma delta T-cell receptors accumulate in human infectious disease lesions. Nature *339*, 544–548.

Okita, R., Saeki, T., Takashima, S., Yamaguchi, Y., and Toge, T. (2005). CD4+CD25+ regulatory T cells in the peripheral blood of patients with breast cancer and non-small cell lung cancer. Oncol. Rep. *14*, 1269–1273.

Pasare, C., and Medzhitov, R. (2003). Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. Science *299*, 1033–1036.

Peng, G., Guo, Z., Kiniwa, Y., Voo, K.S., Peng, W., Fu, T., Wang, D.Y., Li, Y., Wang, H.Y., and Wang, R.-F. (2005). Toll-like receptor 8 mediated-reversal of CD4+ regulatory T cell function. Science *309*, 1380–1384.

Pennington, D.J., Vermijlen, D., Wise, E.L., Clarke, S.L., Tigelaar, R.E., and Hayday, A.C. (2005). The integration of conventional and unconventional T cells that characterizes cell-mediated responses. Adv. Immunol. *87*, 27–59.

Pennington, D.J., Silva-Santos, B., Silberzahn, T., Escorcio-Correia, M., Woodward, M.J., Roberts, S.J., Smith, A.L., Dyson, P.J., and Hayday, A.C. (2006). Early events in the thymus affect the balance of effector and regulatory T cells. Nature *444*, 1073–1077.

Qin, J., Yao, J., Cui, G., Xiao, H., Kim, T.W., Fraczek, J., Wightman, P., Sato, S., Akira, S., Puel, A., et al. (2006). TLR8-mediated NF-kappaB and JNK activation are TAK1-independent and MEKK3-dependent. J. Biol. Chem. *281*, 21013–21021.

Rosenberg, S.A., Yang, J.C., and Restifo, N.P. (2004). Cancer immunotherapy: moving beyond current vaccines. Nat. Med. *10*, 909–915.

Sakaguchi, S. (2004). Naturally arising CD4+ regulatory T cells for immunologic self-tolerance and negative control of immune responses. Annu. Rev. Immunol. *22*, 531–562.

Sato, S., Sanjo, H., Takeda, K., Ninomiya-Tsuji, J., Yamamoto, M., Kawai, T., Matsumoto, K., Takeuchi, O., and Akira, S. (2005). Essential function for the kinase TAK1 in innate and adaptive immune responses. Nat. Immunol. *6*, 1087–1095.

Sato, S., Sanjo, H., Tsujimura, T., Ninomiya-Tsuji, J., Yamamoto, M., Kawai, T., Takeuchi, O., and Akira, S. (2006). TAK1 is indispensable for development of T cells and prevention of colitis by the generation of regulatory T cells. Int. Immunol. *18*, 1405–1411.

Shevach, E.M. (2002). CD4+ CD25+ suppressor T cells: more questions than answers. Nat. Rev. Immunol. *2*, 389–400.

Shin, S., El-Diwany, R., Schaffert, S., Adams, E.J., Garcia, K.C., Pereira, P., and Chien, Y.H. (2005). Antigen recognition determinants of gammadelta T cell receptors. Science *308*, 252–255.

Sutmuller, R.P., den Brok, M.H., Kramer, M., Bennink, E.J., Toonen, L.W., Kullberg, B.J., Joosten, L.A., Akira, S., Netea, M.G., and Adema, G.J. (2006a). Toll-like receptor 2 controls expansion and function of regulatory T cells. J. Clin. Invest. *116*, 485–494.

Sutmuller, R.P., Morgan, M.E., Netea, M.G., Grauer, O., and Adema, G.J. (2006b). Toll-like receptors on regulatory T cells: expanding immune regulation. Trends Immunol. *27*, 387–393.

Wan, Y.Y., Chi, H., Xie, M., Schneider, M.D., and Flavell, R.A. (2006). The kinase TAK1 integrates antigen and cytokine receptor signaling for T cell development, survival and function. Nat. Immunol. *7*, 851–858.

Wang, R.F. (2006). Functional control of regulatory T cells and cancer immunotherapy. Semin. Cancer Biol. *16*, 106–114.

Wang, H.Y., and Wang, R.F. (2005). Antigen-specific CD4+ regulatory T cells in cancer: implications for immunotherapy. Microbes Infect. *7*, 1056–1062.

Wang, R.-F., Wang, X., Atwood, A.C., Topalian, S.L., and Rosenberg, S.A. (1999). Cloning genes encoding MHC class II-restricted antigens: mutated CDC27 as a tumor antigen. Science *284*, 1351–1354.

Wang, H.Y., Lee, D.A., Peng, G., Guo, Z., Li, Y., Kiniwa, Y., Shevach, E.M., and Wang, R.-F. (2004). Tumor-specific human CD4+ regulatory T cells and their ligands: implication for immunotherapy. Immunity *20*, 107–118.

Wang, H.Y., Peng, G., Guo, Z., Shevach, E.M., and Wang, R.-F. (2005). Recognition of a new ARTC1 peptide ligand uniquely expressed in tumor cells by antigen-specific CD4+ gegulatory T cells. J. Immunol. *174*, 2661–2670.

Woo, E.Y., Chu, C.S., Goletz, T.J., Schlienger, K., Yeh, H., Coukos, G., Rubin, S.C., Kaiser, L.R., and June, C.H. (2001). Regulatory CD4(+)CD25(+) T cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer. Cancer Res. *61*, 4766–4772.