HHS Public Access

Author manuscript

J Immunotoxicol. Author manuscript; available in PMC 2015 June 13.

Published in final edited form as:

J Immunotoxicol. 2014 October ; 11(4): 319–327. doi:10.3109/1547691X.2013.864736.

Modulation of Bone Morphogenic Protein Signaling in T-Cells for Cancer Immunotherapy

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Abstract

Immunotherapy is becoming an increasingly attractive therapeutic alternative for conventional cancer therapy. In recent years Foxp3$^+$ regulatory T-cells (T_R) were identified as the major obstacle to effective cancer immunotherapy. The abundance of these cells in peripheral blood is increased in patients with multiple types of cancer and their prevalence among tumor-infiltrating lymphocytes correlated with poor clinical prognosis. In contrast, removal or inactivation of T_R cells led to enhanced antitumor immune response and better efficacy of cancer vaccines. We report that Bone Morphogenic Protein Receptor 1α (BMPR1α, Alk-3), is expressed by activated effector CD4$^+$ and T_R cells and modulates functions of both cell types. Bone Morphogenic Proteins (BMPs) belong to the transforming growth factor (TGF)-β family of cytokines that also include TGFβ and activins. BMPs play crucial roles in- embryonic development, tissue differentiation and homeostasis and development of cancer. It was demonstrated that BMPs and activins synergize with TGFβ to regulate thymic T-cell development, maintain T_R cells and control peripheral tolerance. Inactivation of BMPR1α in T-cells results in impaired thymic and peripheral generation of T_R cells. BMPR1α -deficient activated T-cells produced higher level of interferon (IFN)-γ than BMPR1α-sufficient T-cells. Moreover, transplanted B16 melanoma tumors grew smaller in mice lacking expression of BMPR1α in T-cells and tumors had few infiltrating T_R cells and a higher proportion of CD8$^+$ T-cells than wild-type mice.

Keywords

regulatory T-cells; cancer; Bone Morphogenic Protein; immunotherapy; gene expression

Introduction

The immune system not only protects organisms from many types of cancer but also shapes tumor immunogenicity (Vesely et al., 2011). This mutual relationship of the developing tumor and the immune system has been conceptualized as cancer immunoediting. Cancer immunoediting is viewed as a dynamic process where the immune system eliminates most immunogenic tumor cells and tumor cell survival depends on creating an environment that compromises the functions of immune system. The process of altering the functions of the

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content of this manuscript.

immune system relies, to large extent, on the recruitment of cells of the innate and adaptive immune system which acquire immunosuppressive functions (Hanahan and Coussens, 2012).

Myeloid and dendritic cells and various subsets of regulatory T-cells are among the cell types that compromise adaptive immune responses and prevent eradication of tumors (Zou, 2006; Gabrilovich et al., 2012; Ma et al., 2012). The generation, maintenance and functions of immune cells in tumor environment depend on complex interactions between tumor infiltrating cells, neoplastic cancer cells and tumor stroma. Unraveling those interactions may lead to novel cancer therapies, especially immunotherapies that rely on activation of effector cytotoxic and helper T-cells to eliminate tumors. Increased understanding how adaptive T-cell functions are regulated in tumors has already led to effective cancer immunotherapies (Pardoll, 2012). It has also become apparent that not only cancer immunotherapy but also traditional cancer treatments, relying on surgical tumor removal, chemo- and/or radiation therapy depend on effective stimulation of the immune system to completely eradicate tumor cells (Arlen et al., 2009; Gray et al., 2009; Muranski and Restifo, 2009). Immune response against tumor antigens, that frequently are self-antigens, depends on overcoming mechanisms of peripheral tolerance and is often compromised by inefficient T-cell activation or anergy induction. In recent years, $Foxp3^+$ regulatory T-cells (T_R) that mediate immune tolerance by an active, and largely unknown, mechanism were identified as the major obstacle to effective cancer immunotherapy and their increased number in patients' blood was correlated with poor prognosis (Sakaguchi et al., 2001; Curiel et al., 2004; Nishikawa et al., 2005; Josefowicz et al., 2012). The success of current immunotherapy protocols depends on designing new strategies to control the generation and suppressor function of T_R cells (Quezada et al., 2010; Topalian et al., 2012).

To identify molecules that control suppressor functions of T_R cells, we compared gene expression profiles of resting and activated, conventional and T_R cells. We found that Bone Morphogenic Protein Receptor 1α (BMPR1α, Alk-3) was up-regulated by activated conventional $CD4^+$ and T_R cells. Gene chip studies did not show expression of BMPR1β or BMPR2, two other BMP receptors, to be changed.

Bone Morphogenic Proteins (BMP) belong to the TGFβ family of cytokines that also includes TGFβ and activins (Hinck, 2012; Poorgholi et al., 2012). BMP play crucial roles in embryonic development, tissue differentiation and homeostasis and development of cancer (Guo and Wang, 2009). They regulate various biological processes including cell proliferation, differentiation, apoptosis, migration, and adhesion and are involved in the development of multiple tissues and organs, including thymocytes (Graf et al., 2002). BMP are required for normal thymus development and BMPR1α expressed by thymocytes and $CD4^+$ T-cells is essential for BMP2/4 signaling during thymic development and in the periphery (Licona-Limon and Solde-vila, 2007; Sivertsen et al., 2007). In T_R cells, signaling through BMPR1α synergizes with TGFβ to sustain T_R cell phenotype and suppressor function (Fantini et al., 2004; Marie et al., 2005). BMP, as well as activins, enhance TGFβ-induced up-regulation of Foxp3 in conventional $CD4^+$ T-cells and generation of adaptive T_R cells (aT_R) cells (Huber et al. 2009; Lu et al., 2010).

In these studies, we sought to find new molecules that could regulate suppressor function of T_R cells. The data obtain showed that mice whose BMPR1α gene was removed in T-cells at the double-positive stage in the thymus (BMPR1α^{T-} mice) produced less T_R cells in the thymus and their remaining T_R cells expressed lower levels of Foxp3. Further, we saw that BMPR1α-deficient T-cells produced more interferon (IFN)-γ when activated *in vitro*. Lastly, it was noted that transplantable melanoma tumors grew slower in BMPR1α^{T-} mice and these were largely devoid of T_R cell infiltrates. In summary, these results suggested to us that BMPR1α controls activation of effector and T_R cells and that blocking of its function may potentiate immune responses during cancer.

Materials and Methods

Mice

Foxp3^{GFP} reporter mice on the C57BL6 genetic background were generated in our laboratory (Kuczma et al., 2009b). Foxp3^{GFP} reporter mice were crossed with CD4-cre (Taconic, Germantown, NY) and BMPR1α^{loxP} (gift of Dr. Thistlethwaite, UCSD, San Diego, CA) conditional knockout mice to produce mice where expression of BMPR1α was abrogated specifically in T-cells (BMPR1α^{T-} mice) (Lee et al., 2001; Mishina et al., 2002). Full details of the study and all procedures performed on animals were approved by the Institutional Animal Care and Use Committee of the Georgia Regents University (approval #09-06-213) and complied with all state, federal, and NIH regulations.

All mice were maintained in specific pathogen-free facilities maintained at 29°C with a 65% relative humidity and a 12-hr light: dark cycle. All mice had *ad libitum* access to standard rodent chow and filtered water throughout the studies. In all cases, to obtain tissues/cells from the various hosts, CO_2 asphyxiation was used as the method of euthanasia.

Cell purification, flow cytometry and cell sorting

Single-cell suspensions were prepared from thymi, spleens, and lymph nodes by mechanical disruption and cells were stained with antibodies available commercially (eBioscience [San Diego, CA], BioLegend [San Diego], or BD Biosciences [San Jose, CA]). Tumor-infiltrating lymphocytes (TIL) were prepared from tumor lesions by scrubbing tumor tissue into phosphate-buffered saline (PBS, pH 7.4) containing 0.1 M EDTA. B16 cell suspension (10^7 cells/ml) was then overlaid atop 5 ml of a Lympholyte-M (Cederlane, Burlington, NC) gradient and spun at 2300 × g for 20 min at 24°C. The cells at the interphase were then collected and, after washing with Hanks' Balanced Salt Solution (HBSS; Cellgro, Manassas, VA), 3×10^5 cells were stained on ice in the dark for 30 min with monoclonal antibodies (0.02 μg each) for flow cytometry analysis and sorting. Cells were analyzed using a FACSCanto flow cytometer (Becton Dickinson, San Jose) and associated FACSDiva software. Cells were also sorted on a MoFlo cell sorter (Cytomation, Fort Collins, CO). A minimum of 100,000 events per sample was acquired. Purity of sorted populations routinely exceeded 98.5%.

Proliferation assay and Th cell generation

Lymph node proliferation assays were performed with 3-5 × 10^4 cells isolated from Foxp3^{GFP} or BMPR1α$^{T-/-}$ mice. Cells were sorted using the MoFlo sorter and then cultured in complete Minimal Essential Medium (MEM; Cellgro) containing 10% fetal bovine serum (FBS, Hyclone, Rockford, IL), penicillin/streptomycin and β-mercaptoethanol) at 37°C for 3 days in the wells of 96-well plates that had been coated overnight with anti-CD3 (10 μg/ml, eBioscience, San Diego) and anti-CD28 (1 μg/ml, eBioscience, San Diego) antibodies using standard protocols (Kuczma et al., 2009b). Proliferation responses were subsequently measured by adding [^{3}H]-thymidine (1 μCi/well; Moravek Biochemicals, Brea, CA) on Day 3 of the 4-day culture. Cells were then harvested on glass fiber filters (Perkin Elmer, Waltham, MA) and incorporated [^{3}H] assessed using a MicroBeta Liquid scintillation counter (Perkin-Elmer, Waltham, MA).

For Th1 differentiation cells were stimulated as above in the presence of anti-IL-4 antibody (10 μg/ml, eBioscience, San Diego) and IL-12 (10 ng/ml, Peprotech, Rocky Hill, NJ). For Th2 differentiation cells were stimulated in the presence of IL-4 (1000 U/ml, Peprotech, Rocky Hill, NJ), anti-IFN-γ (10 μg/ml, eBioscience, San Diego) and anti-IL-12 (10 μg/ml, eBioscience, San Diego) antibodies. Finally, for Th17 priming cells were stimulated in the presence of TGF-β (3 ng/ml, Peprotech, Rocky Hill, NJ) and IL-6 (20 ng/ml, Peprotech, Rocky Hill, NJ). Cells were cultured for 4 days.

Proliferation inhibition assay

Sorted CD4$^+$Foxp3^{GFP-} cells (5 × 10^4/well) were incubated in a 96-well plate with irradiated splenocytes from T-cell-deficient mice (TCRα chain knockout mice)(5 × 10^4/well, 3000 Rad) and soluble anti-CD3 (5 μg/ml). Sorted CD4$^+$Foxp3^{GFP+} cells (2.5 × 10^4/well) were added to each culture. After 3-day of culturing, proliferation among the cells was measured by adding 1 μCi [^{3}H]-thymidine to each well and then processing the cultures as outlined above.

RT-PCR

RNA was isolated from sorted cells using an RNeasy Mini Kit (Qiagen, Valencia, CA) and reverse transcribed using a Superscript kit (Invitrogen, Grand Island, NY) according to manufacturer instructions. Quantities of cDNA were normalized for β-actin. The primers used for amplification were: BMPR1α: fwd: GCCCAGATGATGCTATTAATAACAC, rev: GGATGCTGCCATCAAAGAACGGAC; BMP2: fwd: TTGAGGCTGCTCAGCATGTTTGGC, rev: TGGTGTCCAATAGTCTGGTCACAGG; BMP4: fwd: ACACTTCTACAGATGTTTG-GGCTGC, rev: ACCTCATTCTCTGGGATGCTGCTGAG; BMP7:fwd: ACCTCTTCTTGCTG-GACAGCCGCACC, rev: TTGGTGGCGTTCATGTAGGAGTTCAG; and, β-actin: fwd: CTA-GGCACCAGGGTGTGATGGT, rev: CTCTTTGATGTCACGCACGATTTC. All primers were obtained from Invitrogen.

Cytokine Detection

Cytokine production (specifically IFNγ) by CD4$^+$ T-cells from wild-type Foxp3^{GFP} reporter and BMPR1α$^{T-/-}$ mice was measured by using commercial ELISA kits (eBioscience),

following manufacturer instructions. For this, 2×10^5 cells were placed into 96-well plates and stimulated with anti-CD3 and anti-CD28 antibodies that were coating the well walls (as outlined above). Samples supernatants were collected after 30 hr of culture and then assessed (in triplicate) for IFNγ. Level of sensitivity of the kit was 15 pg IFNγ/ml.

Tumors

The mouse melanoma cell line B16F1 was obtained from ATCC (Manassas, VA) and maintained in culture in complete MEM medium. To produce tumors, B16 melanoma cells (5×10^4) were injected subcutaneously into the upper inside portion of both thighs of 6-8-wk-old Foxp3^{GFP} or BMPR1α$^{T-/-}$ mice. Tumor size was assessed every second day staring at Day 8 after injection; the animals were euthanized at Day 17 and tumors and lymph nodes from each were recovered for analyses.

Microarray analysis

T_R and conventional CD4$^+$ T-cells from *scurfy* mice (S*f*Foxp3^{GFP}) and Foxp3^{GFP} mice were analyzed in triplicate (Kuczma et al., 2009b). RNA was prepared from sorted cell subsets using RNeasy kit (Qiagen). RNA was amplified using TargetAmp kit (Epicentre, Madison, WI). The resulting cRNA was hybridized to Affymetrix GeneChip M430 2.0 Plus (Santa Clara, CA).

Microarray data were first normalized using RMA and subsequently analyzed using LIMMA (Bolstad et al., 2003; Smyth, 2004). We analyzed all arrays as a factorial experiment in which cell type (conventional of T_R cell) was one factor and cell status (resting vs. activated) was a second factor, along with the interaction of cell type and cell status. Genes differentially-expressed in activated conventional and T_R cells were those found significant for the interaction, regardless of significance for the main effects. Genes with no significant interaction and no significant response to activation, but having a significant difference between conventional and T_R cell, were those that are cell type specific regardless of activation status. Genes with no significant interaction and no significant difference between cell types, but having a significant difference between resting and activated cells were those that responded to activation equally in both conventional and T_R cell, with no differences between cell types. The advantage to LIMMA is that the B statistic (log posterior odds of differential expression) used in this analysis quantified the evidence for the alternate hypothesis vs. the evidence for the null hypothesis. Since B is on a log scale, a B of 0 indicated both the alternate and null hypotheses were equally likely. If the B statistics was positive, then the evidence supported the alternative hypothesis of some difference; a negative B supported the null hypothesis. The advantage of the B-statistic was that it accurately ranked the genes in order of likelihood of being differentially expressed. Choosing a cutoff for B, however, was just as challenging as using any other statistic. We called all genes with a $B \geq 1.5$ significant, since the evidence for the alternative would no longer be considered weak. This choice of cutoff also seemed reasonable as the q-values (expected false discovery rates) for those genes we called significant were ≈ 0.01.

Statistical analysis

The significance of differences between samples or groups of mice was determined using paired, one-tailed Student t test. Differences between samples with p values ≤ 0.05 were considered significant.

Results

Identification of genes preferentially up-regulated in activated T_R cells

T_R cells have to be activated through the TCR in an antigen-specific way to suppress immune responses. We reasoned that T-cell activation, which changes expression of genes controlling proliferation and phenotypic markers, also incites a genetic program that directs acquisition of suppressor functions. To identify genes that control T_R cell suppressor functions, we compared global gene expression profiles of resting and activated conventional $CD4^+$ T-cells, *in vitro* generated adaptive regulatory T-cells (aT_R) cells, and resting and activated natural T_R (nT_R) cells. Pure populations of resting $CD4^+CD44^-CD62L^+$ $Foxp3^{GFP-}$ conventional and nT_R $Foxp3^{GFPhi}$ cells were sorted on a flow cell sorter and RNA was isolated. To generate aT_R cells, sorted naive $CD4^+$ T-cells were stimulated with plate-bound anti-CD3/anti-CD28 antibodies in the presence of TGFβ and interleukin (IL)-2. After 4 days, aT_R cells (cells that up-regulated GFP) were re-sorted and used for RNA isolation. To examine gene expression in activated conventional T-cells, a population of $CD4^+CD44^+CD62L^-Foxp3^{GFP-}$ cells was sorted from $Foxp3^{GFP}$ *scurfy* mice with advanced autoimmune disease (Kuczma et al., 2009b). Sorted $CD4^+Foxp3^{GFPhi}$ cells were stimulated *in vitro* with plate-bound anti-CD3/anti-CD28 antibodies and 11-2 for 4 days and used as activated T_R cells. RNA was prepared from sorted cell subsets and gene expression was analyzed on Affymetrix GeneChip.

We analyzed resting and activated conventional and nT_R cells as a factorial experiment in which cell status (resting vs. activated) was one factor and cell type (conventional $CD4^+$ vs. nT_R) was a second factor, along with the interaction of cell status and cell type. Genes whose response was activation dependent were those found significant for the interaction, regardless of significance for the main effects. Genes with no significant interaction and no significant response to activation, but having a significant difference between cell types, are those that were cell type-specific regardless of activation status. Genes with no significant interaction and no significant difference between resting and activated cells, but having a significant difference between T_R and conventional $CD4^+$ T-cells, were those that respond to activation equally in both cell types. Genes with statistically significant differences in expression levels were identified. In addition to a factorial analysis described above, we conducted a 3-way comparison of differentially-expressed genes in resting and activated conventional $CD4^+$ T-cells and aT_R cells. Figure 1 show the result of the factorial and 3-way analyses and includes genes with >3-fold difference of expression. Venn diagrams show how many genes were differentially expressed depending on the cell populations, activation status, and possible interaction between experimental factors. Diffentially-expressed genes are listed in Supplemental data 1.

The number of differentially expressed genes (>3 fold) between activated nT_R and aT_R cells was 617. Factorial analysis (Figure 1A) shows that 155 genes were differentially expressed between conventional and nT_R cells regardless of activation status. Expression of 190 genes changed in the same manner between conventional and nT_R cells when they were activated and for 362 genes, expression depended on cell type (shows interaction between cell type and status). Expression of 430 genes was different between activated and resting cells regardless of cell type (conventional and T_R). Comparison of gene expression in resting, activated, and aT_R cells identified 419 genes differentially-expressed between resting and aT_R cells that were not differentially-expressed when activated and aT_R cells were compared, and when activated and resting T-cells were compared (Figure 1B). This set of genes was combined with genes identified in the factorial analysis. One of the genes, i.e., BMPR1α, was selected for further analysis based on the reports indicating the importance of BMPs in controlling cellular differentiation.

Expression pattern of BMPR1α and its ligands in peripheral T-cells

To corroborate microarray data, we investigated BMPR1α expression in resting and *in vitro* activated conventional and T_R cells using RT-PCR (Figure 2). In normal, healthy mice low levels of BMPR1α are found only in nT_R cells expressing high level of Foxp3. This subset of T_R cells was earlier found to have stable suppressor phenotype (Kuczma et al., 2009a). BMPR1α is up-regulated upon *in vitro* activation in helper $CD4^+$ T-cells but not in cytotoxic cells, is further up-regulated in nT_R cells and T-cells converted to become aT_R cells. To determine which T-cell populations expressed BMPR1α in mice undergoing immune responses, $Foxp3^{GFP}$ reporter mice were immunized with *Staphylococcal* enterotoxin B. Analysis of T-cell subsets sorted from the experimental mice showed that BMPR1α expression pattern was consistent with $CD4^+$ T-cell subsets activated *in vitro*. Low-level BMPR1α was expressed in activated helper T-cells and much higher levels in T_R cells. BMPR1α was also expressed in a population of activated human $CD4^+$ T-cells, especially in the presence of TGFβ. In conclusion, expression pattern of BMPR1α suggested to us that its main function was to regulate functions of activated T_R cells and generation of aT_R cells.

BMPs are involved in regulating cellular differentiation and functions by establishing local gradients. Therefore, we examined if activated T-cells could produce BMP that could be involved in autocrine signaling loops. We saw that BMPR1α ligands, i.e., BMP2, 4 and 7, were not produced by naive T-cells (Figure 2). Low levels of BMP2 were produced by $CD4^+$ T-cells activated to become T-helper (T_H)-1 cells and high levels produced by cells activated to become T_H2 cells. Low levels of BMP7 were produced by $CD4^+$ T-cells activated to become T_H2 cells. We did not detect BMP4 in activated $CD4^+$ T-cells. These results suggested that expression of BMPs strongly depended on activation status of helper T-cells.

BMPR1α was necessary for thymic generation of T_R cells, but not conventional T-cells

To further evaluate possible role of BMPR1α in T-cells, we generated mice with a T-cell-specific deletion of this gene. BMPR1α conditional knockout mice, prepared by flanking the kinase domain of the receptor with loxP sites ($BMPR1α^{loxP}$ mouse), were crossed to CD4-cre mice expressing cre recombinase in T-cells starting at the stage of double positive

thymocytes (Lee et al., 2001; Mishina et al., 2002). In addition, we introduced Foxp3^{GFP} reporter transgene into CD4-cre/BMPR1α$^{loxP/loxP}$ mice to tag T_R cells with the GFP (Kuczma et al., 2009b). Mice transgenic for CD4-cre and Foxp3^{GFP} and homozygous for BMPR1α$^{loxP/loxP}$ allele (BMPR1α^{T-}mice) had normal numbers/proportions of double- and single-positive thymocytes and normal or only slightly lower numbers of conventional peripheral CD4$^+$ and CD8$^+$ T-cells (Supplemental data 2). In contrast to conventional T-cells, thymic generation of T_R cells was impaired and BMPR1α^{T-} mice had only small population of peripheral T_R cells (Figure 3). BMPR1α^{T-} mice had moderately expanded population of activated/memory T-cells.

Lack of BMPR1α expression affected proliferation\functions of peripheral conventional and T_R cells

To test how BMPR1α deficiency affects peripheral, mature T cells we sorted naive CD4$^+$ T-cells from BMPR1α^{T-} mice and stimulated them *in vitro*. The results indicated that BMPR1α-deficient T-cells proliferated equally or slightly better than cells expressing BMPR1α (Figure 4A).

Expression of BMPR1α in activated cells indicates that it may regulate generation of effector CD4$^+$ T-cells. In fact, when T-cells were activated, BMPR1α-deficient T-cells produced more IFNγ (Figure 4B). In contrast, when cells that were BMPR1α deficient were stimulated in the presence of TGFβ and Il-2, the generation of aT_R cells was impaired (Figure 5). This result demonstrated that lack of signaling through the BMPR1α receptor differentially affected T-cell activation, promoting effector T_H1 cells and inhibiting generation of aT_R cells.

To test how Foxp3$^+$ T-cells from BMPR1α^{T-} mice control T-cells activation, a proliferation inhibition assay was performed. As can be seen, T_R cells isolated from BMPR1α^{T-}mice were less effective in controlling proliferation of responder T-cells than corresponding cells from wild-type mice (Figure 6). Using responder cells from BMPR1α-deficient and -sufficient mice, it was demonstrated that decreased inhibition of cell proliferation depended on reduced T_R suppression and not on resistance of BMPR1α-deficient responder cells to T_R cell-mediated suppression. Thus, these experiments revealed that BMPR1α signaling differentially affected proliferation of effector cells and regulated CD4$^+$ T-cell activation by promoting T_R cell generation.

BMPR1α expression modulated immune responses in tumor-bearing mice

Data obtained so far, i.e., reduced numbers of T_R cells and biased production of T_H1 cells, led us to examine how expression of BMPR1α modulated immune responses during/to cancer. Specifically, we investigated tumor growth in BMPR1α^{T-} mice inoculated with B16 melanoma cells. We noted that melanoma tumors grew slower in BMPR1α^{T-} mice compared to wild-type counterparts (Figure 7). Tumor infiltrates in BMPR1α^{T-} mice had higher proportions of CD8$^+$ T-cells and more T-cells with up-regulated CD44 expression (Figure 8). In addition, the infiltrates in BMPR1α^{T-} mice were devoid of T_R cells that were still present in the draining lymph nodes of the same mice. This finding suggested that BMPR1α might regulate generation and/or migration of T_R cells into tumors. Since CD8$^+$

T-cells do not express BMPR1α, it is likely the better expansion of these cells into the tumor tissue and the higher activation marker expression resulted from a lack of inhibition by T_R cells and not a lack of direct influence of BMPs (data not shown).

Discussion

T_R cells were found to inhibit effector cells in many cancers including melanoma and depletion of T_R cells slowed down tumor growth (Tanaka et al., 2002; Jones et al., 2004; Viguier et al., 2004). To devise strategies to modify T_R suppressor function, we compared global gene expression profiles of naive and activated conventional T-cells, aT_R cells, and both resting and activated nT_R cells. The goal was to identify molecules expressed in activated T_R cells and also involved in T_R cell suppressor functions that could represent targets for anti-tumor therapy. We found that ~ 300 genes were differentially expressed between activated conventional and T_R cells making this gene set a potential target for selective manipulation of only one cell type. One of these genes was BMPR1α, a receptor for BMP 2, 4, and 7. BMP are multi-functional regulatory proteins that belong to the TGFβ superfamily and control a diverse array of normal cellular and physiological processes including growth arrest, apoptosis and cell differentiation (Chen et al., 2004). BMPs are involved in embryogenesis, angiogenesis, and cancer, and have been investigated for their use as potential therapeutics (He et al., 2004; Guo and Wang, 2009; Senta et al., 2009). BMPs are produced by many cell types, including tumor stromal cells, neoplastic cells, and various cells of the innate and adaptive immune system. Dysregulation of BMP signaling leads to developmental defects and has been seen in multiple cancers including juvenile polyposis, melanoma and prostate cancer (Rothhammer et al., 2005; Doak et al., 2007; Shola et al., 2012). In the immune system, BMP signaling is involved in development of the thymus and T-cells (Hager-Theodorides et al. 2002; Bleul and Boehm, 2005).

BMPs bind to the extracellular domain of BMP Type I receptors that are serine/threonine kinase receptors (also called Alkl, Alk2, Alk3, or BMPR1α and Alk6 or BMPR1β). Type I receptors interact with Type II receptors (BMPR2) or activin receptors (ActR-IIA or ActR-IIB) to form heteromeric receptor-ligand complexes. Ligand binding to Type I receptors leads to transphosphorylation by Type II receptors, receptor oligomerization, and signal transduction. Activated BMPRI, in turn, phosphorylate transcription factor Smads 1, 5 and 8, promoting their translocation to the nucleus where they form a complex with Smad4 and regulate gene expression via interactions with multiple transcription factors. In many cell types, signaling pathways initiated by ligand binding to BMPR1α intersect with multiple other pathways including those important for T_R cell generation and function (like Wnt, Akt/mTOR, Notch); however, the extent of these interactions in T-cells is not well known (Tian et al., 2005; Itasaki and Hoppler, 2010; Poorgholi et al., 2012). In mature T-cells, inhibition of signaling through BMP was found to produce complex effects that included inhibition of T_H17 cell differentiation and IL-2 production (Yoshioka et al., 2012). BMP and activins augment T_R cells generation, acting syner-gistically with TGFβ (Huber et al., 2009; Itasaki and Hoppler, 2010; Lu et al., 2010).

Using phenotypic and functional analyses we further defined the role of BMP and BMPR1α in T cell ontogeny and in regulating functions of mature T cells. Using novel conditional

knockout mouse model we showed that inhibition of BMPR1α in double-positive thymocytes selectively inhibited T_R cell development in the thymus. While development of conventional T-cells in BMPR1α^{T-} mice was not affected, functional status of activated cells changed since only activated cells express BMPR1α and become sensitive to BMPs. Peripheral CD4$^+$ T cells from BMPR1α^{T-} mice preferentially generated Th1 effector cells and only poorly differentiated into aT_R cells. This last result is consistent with a report showing synergy between TGFβ and BMP2/4, two ligands of BMPR1α, in cells up-regulating Foxp3 and producing aT_R cells (Lu et al., 2010). Considering that BMPR1α is expressed in CD4$^+$ T-cells on antigen stimulation, one may postulate this molecule is involved in lineage choices between generating a suppressor vs. a helper T-cell. This might be relevant in tumors where many cell types produce BMP. Among T-cells, T_H2 cells - generally considered detrimental for effective immune responses during/to cancer - were found to produce the highest levels of BMP. Thus, BMPR1α might be an element of the immunosuppressory signaling circuit that operates in tumors (Biggs and Eiselein, 2001; Muranski and Restifo, 2009). In conclusion, this and previous reports support the role of BMPR1α in regulating a balance between immune responses and their suppression. Collectively, our data show that blocking BMPR1α will skew immune response towards cell populations favored for effective anti-tumor response.

While development of T_R cells in BMPR1α^{T-} mice was impaired, these mice did not suffer from acute autoimmune diseases; this indicated at least some T_R cells function was preserved. Proliferation inhibition assays prepared using various combinations of T_R and responder cells demonstrated that while BMPR1α-deficient T_R cells have decreased suppressor capacity BMPR1α-deficient effector CD4$^+$ T cells remained susceptible to inhibition by T_R cells. This finding suggests that by eliminating signaling through the BMPR1α in early stages of CD4$^+$ T cell activation no aberrant effector cells are produced which could escape immune regulation.

To examine what was the significance of eliminating BMPR1α for an immune response to/during cancer, we investigated tumor growth in BMPR1α^{T-} mice. B16 melanoma tumors grew slower in BMPR1α mice and tumor infiltrating CD4$^+$ and CD8$^+$ T-cells had higher expression of activation marker CD44. A surprising result was very low proportion or lack of Foxp3^{GFP+} T-cells in tumor infiltrate. This suggested that BMPR1α controlled migration of T_R cells into tumors and/or expansion of these cells. Signaling circuits in T-cells downstream of BMPR1α are currently not known, but BMPR1α may control expression of cytokine receptors that mediate recruitment and migration of hematopoietic cells into tumors (Curiel et al., 2004). Smad transcription factors were found to interact with factors of the forkhead family that control expression of CD62L and CCR7 that regulate T-cell homing (Arden, 2004; Kerdiles et al., 2009). Alternatively, BMPR1α may regulate Foxp3 expression in T_R cells. We have identified multiple Smad1/5/8 binding sites in the promoter and enhancer regions of the Foxp3 gene. One of these sites is adjacent to Smad 2/3 site in the CNS2 region of the enhancer that is responsible for the stabilization of Foxp3 expression in T_R cells (Tone et al., 2008; Zheng et al., 2010).

The expression pattern and the role of BMPR1α suggest that this molecule may be targeted to skew immune response towards effector T cell subsets known to inhibit tumor growth.

The potential risk associated such modulation of immune system for therapy of cancer is development of autoimmunity. The best studied autoimmune reactions occurred in humans and experimental mice treated for melanoma but the symptoms were rather mild – vitiligo, and limited to tumor cell precursors, melanocytes (Engelhard et al., 2002; Overwijk et al., 1999). The limited insight into BMPR1α function makes it difficult to assess the potential of severe/generalized autoimmunity as a result of targeting this molecule. Since the BMPR1α is not expressed in resting T cells inhibiting its signaling would be limited to activated T cells and possible immunosuppression would not be general and reversible but the possibility of adverse effects needs to be considered in the design of any form of immune therapy.

Conclusions

Large-scale gene expression analysis of naive and activated conventional and T_R cells has made it possible to identify genes expressed specifically in T_R cells upon activation. This set of genes controls suppressor functions of T_R cells that depend on stimulation through the TCR. One gene, *BMPR1α*, was shown to control the thymic development of T_R cells and conversion of conventional $CD4^+$ T-cells into aT_R cells. The pattern of BMPR1α expression revealed it was up-regulated in response to TCR stimulation and, in conventional T-cells, inhibited generation of T_H1 cells and promoted T_R cell generation. In conclusion, this gene may be important in regulating bias during an immune response to cancer. This interpretation was supported by the finding of slower growth of B16 melanoma tumors in $BMPR1\alpha^{T-}$ mice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank L. Ignatowicz and members of his laboratory for reading the manuscript and helpful discussions and Jeanene Pihkala for cell sorting. This research was funded by NIH grant R21 AI097600 to P.K.

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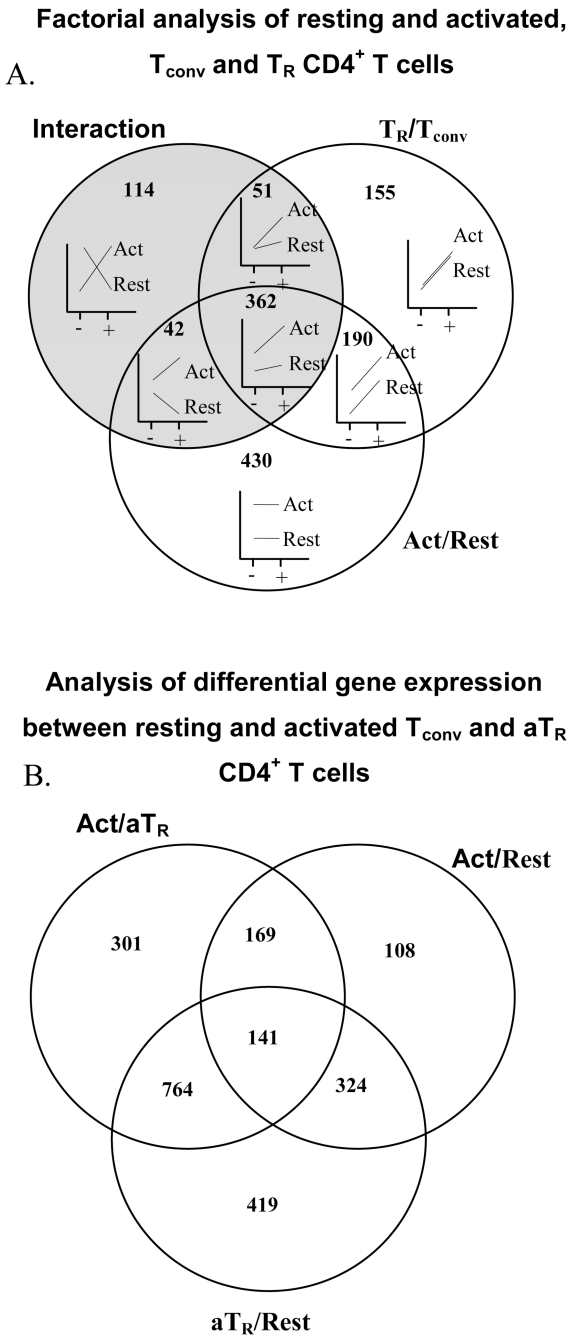

Figure 1.

Transcriptional profile of resting and activated conventional (T_{conv}) and T_R $CD4^+$ T cells. **(A)** Venn diagram of genes differentially-expressed in activated vs. resting cells and T_R vs. T_{conv} cells. Shaded circle includes genes showing interaction effect suggesting that expression of genes in T_{conv} and T_R cells depends on activation status and cell type. Plots inside Venn diagram show examples of possible gene expression profiles in each section of the diagram. "+" denotes $Foxp3^{GFP+}$ (T_R cells), "-" denotes $Foxp3^{GFP-}$ (T_{conv} cells), "Act" denotes activated and "Rest" denotes resting T cells. **(B)** Venn diagram of genes

differentially-expressed in adaptive aT_R and resting and activated T_{conv} cells. Right upper circle = genes differentially-expressed between activated and resting T_{conv} cells; lower circle = genes differentially-expressed between aT_R and resting T_{conv} cells; left upper circle = genes differentially-expressed between activated T_{conv} and aT_R cells.

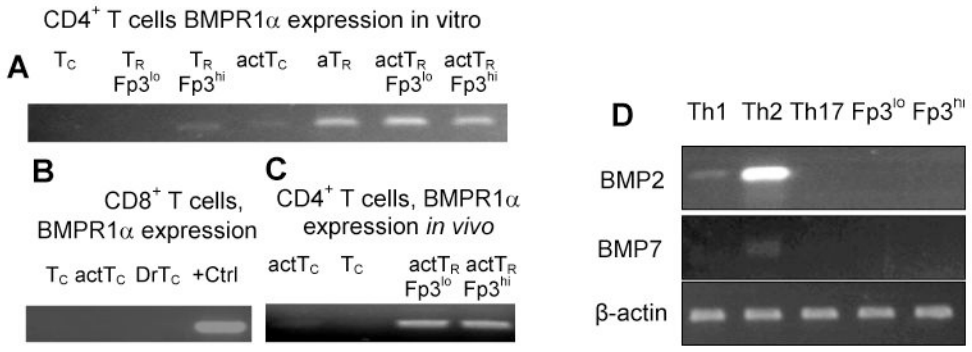

Figure 2.

BMPR1α expression in T-cell subsets. **(A)** BMPR1α expression in resting conventional $CD4^+$ T-cells (Tc), T_R cells expressing low (T_R $Fp3^{lo}$) and high (T_R $Fp3^{hi}$) levels of Foxp3, cells activated with anti-CD3/anti-CD28 Ab ($actT_c$), adaptive T_R cells (aT_R), and activated T_R cells expressing low ($actT_R$ $Fp3^{lo}$) and high ($actT_R$ $Fp3^{hi}$) level of Foxp3. **(B)** BMPR1α expression *in vivo* in resting $CD8^+$ T-cells (Tc), activated $CD8^+$ T-cells (actTc) and in activated $CD8^+$ T-cells isolated from tumor draining lymph nodes (DrTc). Positive control (+Ctrl) is BMPR1α expression in RM-1 cells. **(C)** BMPR1α expression in $CD4^+$ T-cells activated *in vivo* by injecting $Foxp3^{GFP}$ mice with SEB. RT-PCR analysis was done on activated $CD4^+CD44^+CD62L^-Foxp3^{GFP-}$ (actTc), naive $CD4^+CD44^-CD62L^+Foxp3^{GFP-}$ T-cells (Tc), and T_R cells expressing low ($actT_R$ $Fp3^{lo}$) and high ($actT_R$ $Fp3^{hi}$) levels of Foxp3. **(D)** Expression of BMPR1α ligands BMP2 and BMP7 in T-cells activated to generate T_H1, T_H2, and T_H17 cells and in *in vitro* activated T_R cells expressing low and high levels of Foxp3.

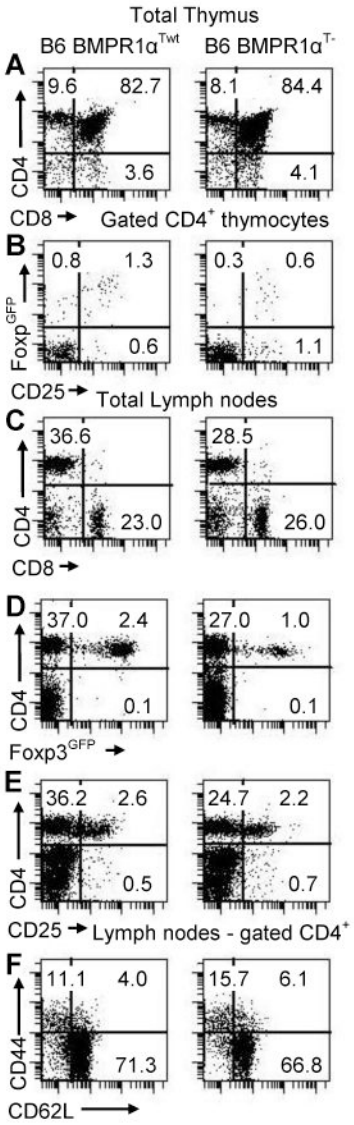

Figure 3.

Characterization of BMPR1α^{T-} mice. Panels in left column are normal Foxp3^{GFP} mice on C57BL6 genetic background (BMPR1α^{Twt}) and in right column are BMPR1α^{T-} mouse. **(A, C)** CD4 and CD8 expression on thymocytes and lymph node cells. **(B)** CD25 and Foxp3 expression on gated CD4$^+$ thymocytes. **(D, E)** Analysis of CD4 and Foxp3 or CD25 expression on lymph node cells. **(F)** Expression of activation markers CD44 and CD62L on gated CD4$^+$ lymph node cells. Cells ware stained with indicated antibodies and numbers show percentage of cells in each quadrant of dot-plot. A representative experiment of three (each experiment used 2-3 mice) each is shown.

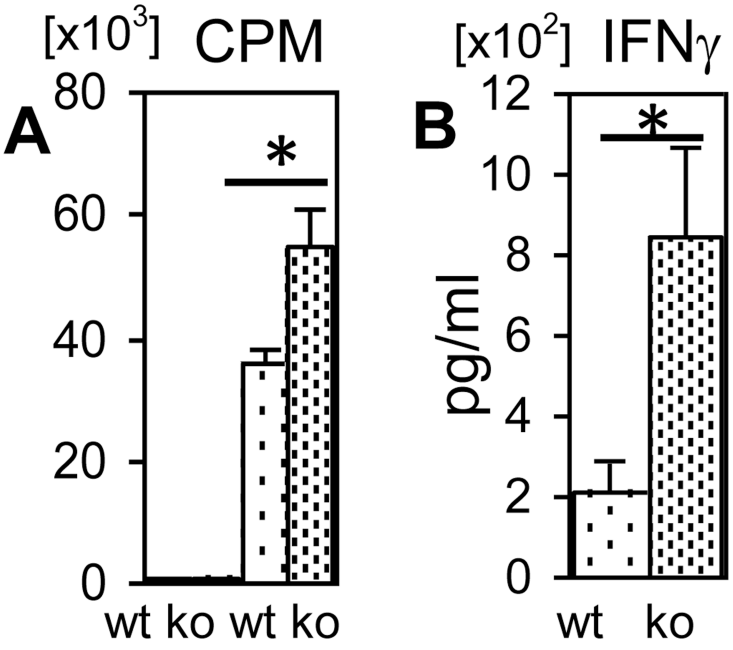

Figure 4.

Proliferation and IFNγ production by $CD4^+$ T-cells from $BMPR1\alpha^{Twt}$ (WT, rare dots) and $BMPR1\alpha^{T-}$ (KO, dense dots) mice. **(A)** Sorted naive $CD4^+$ T-cells were incubated in a 96-well plate without (Columns 1, 2) or with (Columns 3, 4) plate-bound anti-CD3/anti-CD28 antibodies for 4 days and proliferation was measured via ^{3}H-thymidine incorporation. **(B)** IFNγ production (ELISA) by conventional $CD4^+$ T-cells stimulated *in vitro* with plate-bound anti-CD3/anti-CD28 antibodies. All experiments were repeated four times. Columns represent mean values and error bars represent standard deviations. Samples were compared using Student t test, Asterisks denote statistically significant differences between samples.

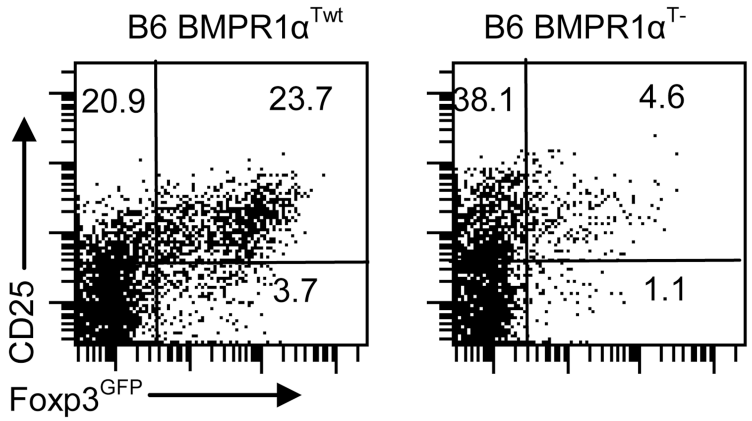

Figure 5.

Naive sorted $CD4^+$ T-cells from $BMPR1\alpha^{T-}$ mice do not efficiently convert into aT_R cells when stimulated with anti-CD3/anti-CD28 antibodies in presence of TGFβ (3 ng/ml) and Il-2 (50 U/ml) for 3 days. The experiment was repeated at least three times.

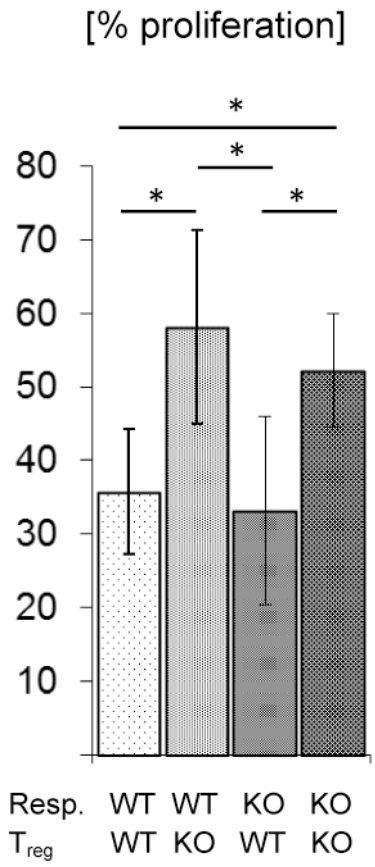

Figure 6.

Proliferation inhibition assay. $CD4^{+}Foxp3^{GFP-}$ responder cells (Resp)(4×10^4/well) and T_R cells (2×10^4/well) from $BMPR1\alpha^{Twt}$ (WT) or $BMPR1\alpha^{T-}$ (KO) mice cells were stimulated with soluble anti-CD3 antibody (5 μg/ml) in the presence of irradiated splenocytes (5×10^4/well, 3000 Rad). After culturing cells for 3 days, 1 μCi ^{3}H-thymidine/well was added and proliferation then measured. The percentage proliferation was calculated by comparing proliferation readings from a well with responder cells only to that from a well containing both responder and T_R cells. The plot shows a representative experiment (of four). Columns represent mean values and error bars represent standard deviations. Samples were compared using Student t test. The p values between samples of WT and KO mice marked with an asterisk were ≤ 0.05.

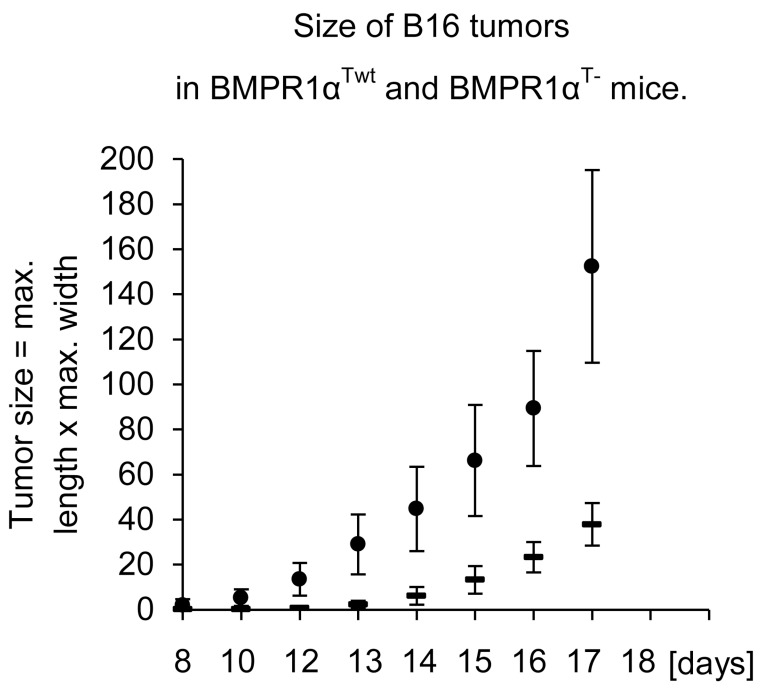

Figure 7.

Size of B16 tumors growing in BMPR1α^{Twt} (•) and BMPR1α^{T-} (—) mice inoculated with B16 melanoma cells (5×10^4 cells, subcutaneously). Nine mice were analyzed in each series. Two measurements [mm] of each tumor (at the largest dimensions, perpendicular to each other) were taken and multiplied (Y-axis). The duration of tumor growth is shown on the X-axis. The plot shows average values and standard deviations for each time point. The differences in the size of tumors between BMPR1α^{Twt} and BMPR1α^{T-} mice were statistically significant for all time points (t test, $p \leq 0.05$). One experiment of two is shown.

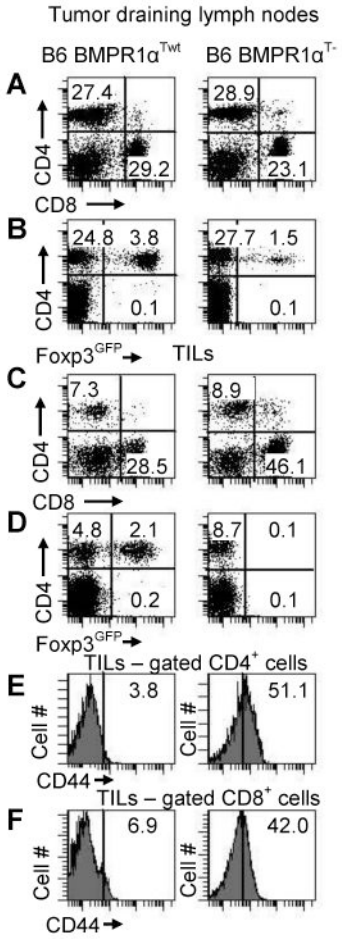

Figure 8.

T-Cell populations. T-cell levels in **(A-D)** tumor draining lymph nodes and **(E, F)** tumors of tumor-bearing BMPR1α^{Twt} (left column) and BMPR1α^{T-} (right column) mice. **(A)** CD4 and CD8 T-cell populations in tumor draining lymph nodes. **(B)** Foxp3 expression on CD4^{+} T-cells in tumor draining lymph nodes. **(C)** CD4 and CD8 T-cell populations in tumor-infiltrating cells. **(D)** Foxp3 expression on CD4^{+} T-cells in tumors. **(E, F)** CD44 expression on gated CD4^{+} and CD8^{+} T-cells in tumors. The plot shows a representative experiment (of three).