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## IL-12 Stimulates Interferon-gamma Mediated Inhibition of Tumor-Induced Regulatory T Cell Proliferation and Enhances Tumor Clearance

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### Abstract

To define the factors that modulate regulatory T (Treg) cells in the tumor setting, we co-cultured various tumor cells with either purified Treg cells, or with unfractionated splenocytes. We found that Treg expansion occurred only with unfractionated splenocytes, suggesting that accessory cells and/or factors produced by them play an essential role in tumor-induced Treg expansion. We performed gene expression profiling on tumor-associated Treg cells to identify candidate signaling molecules and studied their effects on tumor-induced Treg expansion. We inadvertently discovered that IL-12 treatment blocked Treg expansion in an IL-12 receptor-dependent fashion. Additional studies showed that IL-12 acts by stimulating Interferon-gamma mediated inhibition of Treg cell proliferation, which may partially account for the anti-tumor effects of IL-12. Furthermore, IL-12 treatment was found to decrease IL-2 production, which may lead to interferon-gamma independent inhibition of Treg cells, as IL-2 is required for their survival and expansion. Mechanistic studies revealed that Interferon-gamma signaling directly causes cell cycle arrest in Treg cells. This study demonstrates that an IL-12-Interferon-gamma axis can suppress tumor-induced Treg proliferation. This mechanism may counteract the ability of Treg cells to promote tumor growth *in vivo*.

### Keywords

Regulatory T cells; Cytokine; Interleukin 12; Interferon-gamma; Tumor clearance

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## Introduction

Clinical observations and animal models have established that the immune system responds to tumors, even though they are derived from the host. Despite the observations that activated, tumor-specific immune cells exist in cancer patients and animal models, the immune system often fails to prevent tumor formation and metastasis (1). Thus, established tumors must have developed strategies to inhibit or evade the immune system.

Sakaguchi and colleagues first established the essential role of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T (Treg) cells in the maintenance of peripheral tolerance to self-antigens (2,3). More recent studies have implicated Treg cells in inducing tolerance to tumors (4,5). Mounting evidence indicates that tumors can promote the expansion, recruitment, and activation of Treg cells (6). Increased numbers of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells have been found either in the circulation or in the tumors of patients with various cancers (lung, breast, colorectal, esophageal, gastric, ovarian, pancreatic, melanoma, hepatocellular, leukemia, and lymphoma) (7). In addition, large numbers of Treg cells in tumors and malignant ascites were shown to be correlated with worse clinical outcomes in several types of cancer (8). Likewise, studies using *in vivo* mouse models have shown that Treg cell proliferation/activation induced by tumors or self-antigens can help tumors to escape immunosurveillance (9,10). Furthermore, antigen-based tumor vaccines have also been found to activate Treg cells, which can considerably dampen antitumor immune responses (11).

Extensive investigations have improved our understanding of the molecular and cellular basis for the development, activation, and function of Treg cells. Antigen-TCR signals are not only determinants for thymic selection and differentiation of Treg cells (12,13), but they also control their peripheral activation and suppressive efficacy (14,15). Although Treg cells predominantly recognize self-antigens and suppress self-reactive T cells (12,13,16), it has also been shown that they can respond to non-self antigens, such as viral antigens, allo-antigens, and foreign antigens in transgenic mouse models (17-19). Cytokine signals also regulate Treg cell development, function, and homeostasis. IL-2 has been shown to be indispensable for Treg expansion, activation, and maintenance (20,21). Other gamma-chain cytokines (IL-4, IL-7, and IL-15) can also support Treg cell development and function (22,23). IL-10 has been reported to be a soluble mediator of Treg suppressive function (5,7). TGF- $\beta$  has been shown to promote the proliferation of natural Treg cells, and also the “conversion” of non-regulatory T cells into induced Treg cells (5,7).

We recently demonstrated that Treg cells in the tumor environment (but not Treg cells in the peripheral lymphoid tissues) utilize granzyme B to suppress anti-tumor immunity (24), which suggests that Treg cells are subject to regulation by local factors during anti-tumor immune responses. To identify such factors, we utilized the Foxp3-GFP reporter mice in this study, and developed an *in vitro* co-culture system to study Treg cells in the tumor setting. We found that tumor cells indirectly drive Treg cell proliferation via accessory immune cells. We also discovered, inadvertently, that IL-12 profoundly inhibits tumor-induced Treg cell proliferation; this inhibition is partially dependent on Interferon-gamma. This study provides definitive evidence that an IL-12-Interferon-gamma axis can inhibit Treg cell proliferation. These findings demonstrate that Treg cells are subject to cytokine-mediated inhibition, and support a notion that cytokines with such a property may have the potential to serve as an adjuvant for tumor vaccine-based immunotherapy.

## Materials and Methods

### Animals and tumor cells

Wild type (WT) 129/SvJ, and WT and *IFN $\gamma$ RI*<sup>-/-</sup> mice in the C57Bl/6J strain were obtained from the Jackson Laboratory. WT 129/SvPas mice were obtained from the Charles River Laboratory. The *IL12R $\beta$ 2*<sup>-/-</sup> mice in the C57Bl/6J strain were maintained as described (25). The *IFN $\gamma$ RI*<sup>-/-</sup> mice in the 129/SvPas mice were obtained from Dr. Herbert Virgin. The RMAS lymphoma cells, originally derived from C57BL/6 mice (H-2<sup>b</sup>, I-A<sup>b</sup>), were obtained from Dr. Wayne Yokoyama and maintained in complete K10 medium (24). The primary APL samples are cryopreserved cells harvested from the spleens of 3 independent leukemic mCG-PML-RAR $\alpha$  transgenic mice in pure C57BL/6 strain (26,27). All mice were bred and maintained in SPF housing, and all experiments were conducted in accordance with Roswell Park Cancer Institute and Washington University School of Medicine animal care guidelines, using protocols approved by the animal studies committee.

### Reagents and antibodies

The purified CD25 antibody (PC61) and rat IgG1 isotype control (HRPN) were purchased from Bio Express. The CD4<sup>+</sup>CD25<sup>+</sup> Treg isolation and Pan T cell isolation kits, and T cell depletion (CD90) microbeads were purchased from Miltenyi Biotec, and T cell isolation or depletion was performed by following the manufacturer's instruction manuals. Antibodies used include anti-mouse CD3 (145-2C11), CD4 (RM4-5), CD8 (53-6.7), CD25 (7D4, PC61), CD16/32 (2.4G2) (BD Biosciences), Foxp3 (eBioscience, FJK-16s).

### *In vitro* tumor-splenocytes co-cultures

$4 \times 10^6$  unfractionated splenocytes were harvested from naïve mice and mixed with  $1.2 \times 10^5$  RMAS tumor cells (3000RAD-irradiated) in 2 ml of K10 medium in tissue culture treated 12-well plates for 4-5 days. To study exogenous cytokine effect, various doses of cytokines were added in the beginning of the co-culture experiments. At the beginning and the end of co-cultures, absolute numbers of various T cell compartments were obtained by multiplying the percentages (determined by flow cytometry as described below, and represented in Figure S1) by the total numbers of live cells harvested from the cultures. T cell expansion is presented as fold change by the equation below:

$$\text{Fold change} = (\text{Absolute numbers at the end of co-culture}) / (\text{Absolute numbers at the beginning of co-culture})$$

### Intracellular staining and flow cytometry

$1 \times 10^6$  cells were washed and resuspended in staining buffer (PBS, 0.5% bovine serum albumin, 0.5 mM EDTA). Samples were labeled with primary-conjugated antibodies against cell surface markers (anti-mouse CD3, CD4, CD8, CD25) (BD Biosciences). Samples were fixed, permeabilized (Foxp3 staining kit, eBioscience), and stained with primary-conjugated anti-Foxp3 antibody (FJK-16s; eBioscience) diluted at 1:400 in staining buffer. Sample data were acquired on a Cytex-modified FACScan (Becton Dickinson) flow cytometer, and analyzed using FlowJo (TreeStar) software.

### CFSE labeling of naive CD4<sup>+</sup>CD25<sup>+</sup> Treg cells and mixing co-cultures

CD4<sup>+</sup>CD25<sup>+</sup> Treg cells were isolated from the resting spleens of WT mice, *IL12R $\beta$ 2*<sup>-/-</sup> mice, and *IFN $\gamma$ RI*<sup>-/-</sup> mice using the CD4<sup>+</sup>CD25<sup>+</sup> Treg isolation kit, according to the manufacturer's instructions (Miltenyi Biotec). The purified Treg cells were labeled at 37°C in PBS with 150 nM of CFSE for 15 minutes and mixed into WT splenocytes, or *IL12R $\beta$ 2*<sup>-/-</sup> splenocytes, or *IFN $\gamma$ RI*<sup>-/-</sup> splenocytes cultured with irradiated RMAS cells for 4-5 days.

### **Bioluminescence imaging of *in vivo* tumor clearance**

RMAS cells were transduced with a  $\Delta U3$  retroviral construct that drives the expression of a fusion cDNA encoding click beetle red luciferase (CBR) and green fluorescence protein (GFP) as previously described (24). CBR luciferase-expressing RMAS clones were injected IP into WT mice in 200  $\mu$ l RPMI 1640 on day 0, and bioluminescence imaging was performed 2 hours later and variously through 10 after injection. For imaging, mice were injected IP with D-luciferin (150  $\mu$ g/g body weight), anesthetized (2% isoflurane) and placed in an IVIS 50 imaging system (Caliper) (exposure time, 1-30 sec; binning, 2-8; no filter; f/stop, 1; FOV, 12 cm) (28). Regions of interest (ROI) were defined manually over the whole body using Living-Image software (Igor Wavemetrics) for determining tumor burden signal intensities. Data were expressed as photon flux (photons/sec) across all mice receiving the same treatment.

### **Flow-based *in vitro* cell death assay**

Naïve, unfractionated splenocytes from Foxp3-GFP knock-in mice were co-cultured with irradiated RMAS cells as described above for 4 days. Lymphocytes were harvested from the co-cultures and stained with primary-conjugated antibodies against cell surface markers (CD3, CD4, and CD8) and 7-AAD to assess cell death in Treg and other T cell compartments by flow cytometry.

## **Results**

### **Tumor cells induce Treg cell expansion in the presence of accessory splenocytes**

To identify factors that may modulate Treg cell function, we explored several approaches to study Treg cells *in vitro* in a tumor relevant setting. First, both purified Foxp3-GFP<sup>+</sup> cells (from Foxp3-GFP reporter mice) and CD4<sup>+</sup>CD25<sup>+</sup> cells failed to proliferate, and the majority of them died when co-cultured with RMAS lymphoma cells (live or lethally irradiated) for 4-5 days (Figure 1A). Secondly, when unfractionated splenocytes (which contain lymphocytes and accessory cells) were co-cultured with live tumor cells, the tumor cells outgrew the lymphocytes (data not shown). Finally, we found that co-culturing irradiated RMAS cells with unfractionated splenocytes induces significant expansion of CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells, but not of CD4<sup>+</sup>Foxp3<sup>-</sup> or CD8<sup>+</sup> T cells (Figures 1A and 2); this expansion occurs in K10 medium without any exogenous growth factors. To rule out an RMAS-specific effect, and to determine whether primary tumor cells can induce Treg expansion in the cultures, we used cryopreserved primary tumor cells derived from the splenic tumors of mice with acute promyelocytic leukemia (APL) (26, 27). Similar amounts of Treg expansion occurred with 3 independent primary APL tumor samples in the co-cultures (Figure 1B).

Notably, RMAS cells and the primary APL cells used in this study (derived from B6 mice, and sharing MHC I and II with 129 mice, but expressing different minor histocompatibility antigens) induced greater Treg expansion when co-cultured with 129/Sv splenocytes (up to 4-fold expansion), compared with B6 splenocytes (~2 fold expansion, Figure 1A). Both 129/SvJ and 129/SvPas splenocytes supported similar amounts of Treg expansion.

### **Non-T accessory cells and IL-2 are required for tumor-induced Treg expansion**

Because tumor-induced Treg expansion did not occur with purified Treg cells, we performed a series of experiments to define the cells and/or factors that promote tumor-induced Treg expansion. First, we used MACS separation techniques to fractionate total splenocytes into Pan T cells vs. T cell-depleted (TcD) accessory cells. Treg expansion occurred only when both non-T splenocytes and irradiated tumor cells were added to the cultures (Figures S2A and S2B). Secondly, since IL-2 and TGF- $\beta$  have been shown to be relevant for Treg expansion, we used monoclonal antibodies to neutralize IL-2 and TGF- $\beta$  in the tumor-splenocyte co-cultures. IL-2

neutralization completely blocked Treg expansion (Figures S2C and S2D), whereas TGF- $\beta$  neutralization had no effect. These results suggest that the accessory non-T splenocytes and IL-2 are both required for tumor-induced Treg expansion *in vitro*.

### Profiling of Treg gene expression *in vivo* to identify candidate cytokines that may modulate Treg expansion

From the co-culture experiments, we hypothesized that tumor cells may indirectly drive the expansion of Treg cells via antigen presentation and cytokine production by other splenocytes. To identify factors that may modulate Treg cell function in tumor-bearing mice *in vivo*, we utilized an array-based approach. We performed gene expression profiling on Treg cells using Affymetrix MOE430v2 arrays. Naïve Treg cells were purified from the resting spleens of Foxp3-GFP reporter mice using high speed flow sorting on the GFP signal. Tumor-associated Treg cells were purified from the tumor ascites of Foxp3-GFP reporter mice injected 5 days earlier with RMAS cells, while Spleen<sup>T</sup> Treg cells were purified from the spleens of the same tumor-bearing mice. RMA normalization was performed for all microarray data. 2-group comparison (t-test) of Ascites vs. Naïve samples, with multiple test corrections (adjusted p-value / FDR (false discovery rate) = q-value) was performed. Using these approaches, we have identified 419 probesets with  $q < 0.005$  and linear fold change  $\geq 2$  (Ascites:Naïve) and 114 probesets with  $q < 0.005$  and linear fold change  $\leq 0.5$  (Ascites:Naïve) (Figure S8 and Supplemental Table). By comparing cytokine receptor expressions of tumor-associated Treg cells to that of naïve or Spleen<sup>T</sup> Treg cells, we identified several candidate cytokines, as shown in Figure S3. IL-2 has been shown to be essential for Treg homeostasis and function (15, 29, 30); as expected, IL-2 receptor  $\alpha$  (CD25) is upregulated in tumor-associated Treg cells. IL-7 receptor is also up-regulated, while IL-10 receptor mRNA abundance in Treg cells is unchanged. Neuropilin-1, a known VEGF receptor that has been proposed as a Treg cell surface marker, is down-regulated in the tumor-associated Treg cells (31). In addition, other pathways involved in Treg function are also differentially regulated. For example, granzyme B is the third most up-regulated gene (39-fold), consistent with our previous report that tumor-associated Treg cells activate granzyme B to suppress anti-tumor immune response (24). Multiple cell division genes (*Cdc6*, *Cdca5*, *Ccna2*, *Cdca8*) are among the top 30 up-regulated genes (Supplemental Table), suggesting that tumor-associated Treg cells may undergo active proliferation. These profiles suggest a complex network in which multiple pathways may influence the expansion and/or function of tumor-associated Tregs cells.

### IL-12 treatment blocks tumor-induced Treg cell expansion

We tested the candidate cytokines for their activity in the tumor-splenocyte co-culture system. IL-2 treatment, at doses between 0.1-20 ng/ml, mildly increases Treg expansion (data not shown). Though VEGF blockade was shown to reduce intratumoral Treg cells and enhance GM-CSF stimulated tumor rejection (32), VEGF treatment revealed no effect on Treg expansion at doses ranging from 1ng/ml to 1000 ng/ml. Likewise, most of the other candidates, such as IL-10 and TNF $\alpha$ , show no effect on Treg expansion, even though multiple concentrations (directed by their levels in the tumor ascites) were tested individually and in combination with 2 to 3 candidates (data not shown).

Remarkably, addition of IL-12 to the culture, at doses between 0.1-2 ng/ml, significantly inhibited the expansion of CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells in a dose-dependent fashion (Figure 2A), but had no significant effect on the numbers of CD4<sup>+</sup>Foxp3<sup>-</sup> or CD8<sup>+</sup> T cells (Figure 2). Similar IL-12 effects were observed when the primary APL tumor cells were used in the co-culture assay (data not shown), ruling out a tumor cell type-specific effect. Notably, this finding is consistent with a previous report that IL-12 treatment reduced CD4<sup>+</sup>CD25<sup>+</sup> T cell infiltration in a lung tumor model (33).

### IL-12 signaling inhibits Treg expansion indirectly

To further validate the IL-12 phenotype, we analyzed Treg expansion in IL-12 receptor  $\beta$ 2-deficient (*IL-12R $\beta$ 2*<sup>-/-</sup>) mice. Treg cell numbers in the resting spleens of *IL-12R $\beta$ 2*<sup>-/-</sup> mice are comparable to that of strain-matched wild-type (WT) mice (Figure 3A). Using *IL-12R $\beta$ 2*<sup>-/-</sup> splenocytes, we found that these cells are completely resistant to IL-12 mediated suppression (Figure 3B). However, the lack of detectable IL-12 receptor mRNA in Treg cells suggested an indirect mechanism of IL-12 action (Figure S3). To test this hypothesis, we used CFSE to label CD4<sup>+</sup>CD25<sup>+</sup> Treg cells purified from WT and *IL-12R $\beta$ 2*<sup>-/-</sup> mice, and measured the expansion of CFSE<sup>+</sup> Treg cells mixed with WT splenocytes or *IL-12R $\beta$ 2*<sup>-/-</sup> splenocytes in co-culture assays. In WT co-cultures, WT Treg cells and *IL-12R $\beta$ 2*<sup>-/-</sup> Treg cells are equally susceptible to IL-12 mediated inhibition (Figure 3C); in *IL-12R $\beta$ 2*<sup>-/-</sup> co-cultures, both WT Treg cells and *IL-12R $\beta$ 2*<sup>-/-</sup> Treg cells are equally resistant to IL-12 mediated inhibition (Figure 3D). These data suggest an indirect mechanism, in which certain IL-12 responsive cell types and/or cytokines mediate the inhibitory effect on Treg cells.

Interestingly, a subset of Treg cells were found to retain high levels of CFSE, while other subpopulations of Treg cells appeared to be more tumor-reactive and diluted CFSE signal to lower levels by proliferating more extensively (Figure S4).

### Interferon-gamma signaling partially accounts for IL-12 mediated Treg inhibition

It has previously been shown that Interferon-gamma (IFN $\gamma$ ) produced by CD8<sup>+</sup> T cells may regulate the generation/activation of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells *in vivo* (34). IL-12 is known to induce IFN $\gamma$  production by other immune cells, including CD4<sup>+</sup> and CD8<sup>+</sup> T cells (35,36). As expected, we found that Treg cells do express IFN $\gamma$  receptor 1 mRNA and protein (Figures S3 and 4A). Therefore, we used an IFN $\gamma$  neutralizing antibody (H22) to determine whether IFN $\gamma$  is responsible for IL-12 mediated Treg inhibition. IFN $\gamma$  neutralization increased Treg cell expansion by more than 30% in WT co-cultures in the absence of exogenous IL-12 (Figure 4B), suggesting that a basal level of IFN $\gamma$  (produced by other immune cells in the cultures) inhibits Treg cell expansion. In addition, IFN $\gamma$  neutralization partially abolished IL-12 mediated Treg inhibition. Similarly, in the absence of exogenous IL-12, Treg cells in IFN $\gamma$  receptor 1-deficient (*IFN $\gamma$ RI*<sup>-/-</sup>) co-cultures proliferated >60% more efficiently than Treg cells in strain-matched WT co-cultures; Treg cells in the *IFN $\gamma$ RI*<sup>-/-</sup> co-cultures are more resistant to IL-12 mediated inhibition (Figure 4C). To determine the IFN $\gamma$  levels that inhibit Treg expansion, we performed ELISA using cell-free media harvested from the cultures. Splenocytes in the culture secreted a basal level of IFN $\gamma$  (0.2-0.9 ng/ml) in the absence or presence of tumor cells (Figure S5A). Exogenous IL-12 treatments enhanced IFN $\gamma$  secretion to 5-17 ng/ml. Furthermore, adding 1-100 ng/ml exogenous IFN $\gamma$  to WT co-cultures inhibits Treg expansion by 30-50% (Figure 4D). As expected, Treg cells in the *IFN $\gamma$ RI*<sup>-/-</sup> co-cultures are completely resistant to IFN $\gamma$  mediated inhibition (Figure 4D), but partially resistant to IL-12 inhibition (Figure 4C). Together, these data show that IFN $\gamma$  signaling partially accounts for IL-12 mediated inhibition of tumor-induced Treg expansion.

However, these data also suggest that there may be additional component(s) that mediate IL-12 inhibition of Treg expansion. Because neutralization of IL-2 completely blocks Treg expansion (Figure S2), we tested whether IL-12 treatment could influence IL-2 secretion. Interestingly, higher levels of IL-2 were detected in the splenocyte-only cultures (210-320 pg/ml) compared to the tumor-splenocyte co-cultures (30-70 pg/ml), where the higher levels of IL-12, in the absence of tumor cells, did not correlate with significant Treg expansion. Nevertheless, IL-12 treatment significantly reduced IL-2 secretion in the co-cultures (Figure S5B), which may account for the IFN $\gamma$ -independent inhibition of Treg cells.

## Interferon-gamma signaling inhibits Treg proliferation directly

To determine whether IFN $\gamma$  receptor-mediated signaling in Treg cells directly inhibits proliferation, we used CFSE to label CD4<sup>+</sup>CD25<sup>+</sup> Treg cells purified from WT or *IFN $\gamma$ RI*<sup>-/-</sup> mice, and compared their proliferative activity in the WT splenocyte-based co-cultures. *IFN $\gamma$ RI*<sup>-/-</sup> Treg cells proliferate significantly faster than WT Treg cells in the absence of exogenous IL-12 (Figure 5A), and are significantly more resistant to IL-12 treatment. These data suggest that IFN $\gamma$  receptor-dependent signaling in Treg cells directly inhibits proliferation, and is required for IL-12 mediated maximal inhibition of Treg cell expansion. In addition, cell cycle analyses revealed that IL-12 treatment significantly reduces proliferating Treg cells in the S-phase (Figures 5B and 5C). In contrast, *IFN $\gamma$ RI*<sup>-/-</sup> Treg cells display significantly more cells in S-phase in the absence of exogenous IL-12, and are more resistant to the cell cycle arrest caused by IL-12 treatment. Furthermore, IFN $\gamma$  treatment directly reduced proliferating Treg cells in the S-phase (Figure 5D), though to a lesser extent compared to IL-12 treatment that induced more intensive inhibition due to an additional mechanism, which may be caused by reduced secretion of IL-2 (Figure S5B). Interestingly, these treatments appear to 'trap' more Treg cells in the G2/M phase (Figure 5B). It has been reported that disruption of T cell receptor (TCR) signaling (37), or IL-2 deprivation (38) can cause G2/M arrest in CD4<sup>+</sup> T cells, and that IFN $\gamma$  treatment can also induce G2/M arrest in human cell lines (39). Our data suggest that lack of stimulation may account for G2/M accumulation in the splenocyte only cultures, whereas IL-12 treatment apparently resulted in an elevation of IFN $\gamma$  and a reduction of IL-2 (Figure S5) that may jointly lead to increased G2/M arrest.

## IL-12 treatment inhibits Treg expansion and enhances tumor clearance *in vivo* in an IFN $\gamma$ -dependent fashion

To study the impact of IL-12 on tumor growth *in vivo*, we first tested whether IL-12 could directly influence tumor cell proliferation or survival. We used various doses of IL-12 (0.1, 0.5, 1, 2, and 5 ng/ml) to treat RMAS cells for 2 weeks in culture. Fresh IL-12 was replenished at each passage of the culture. These treatments showed no effect on RMAS cell proliferation or survival, ruling out a direct effect of IL-12 on tumor growth.

To test whether IL-12 mediated inhibition of Treg cell proliferation has an impact on tumor clearance *in vivo*, we compared the effect of IL-12 treatment with that of Treg cell depletion. We utilized luciferase-expressing RMAS cells and performed bioluminescence imaging to monitor mice injected intraperitoneally (IP) with these cells. Daily IL-12 treatment (100 ng per mouse) significantly enhances tumor clearance in (Figures S6 and 6A). As expected, Treg cell depletion also increases tumor clearance. Notably, IL-12 treatment enhances tumor clearance more efficiently than Treg cell depletion, perhaps because it also activates immune effector cells (33). Finally, the combination of Treg depletion with IL-12 treatment appears to have a similar effect as IL-12 treatment alone (Figure 6A), perhaps because IL-12 treatment alone has blocked Treg expansion *in vivo*. To test this hypothesis, we measured absolute T cell numbers in the peritoneum of naïve mice vs. mice IP-injected with RMAS cells. CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells are increased about 20-fold in the tumor-bearing 129/SvJ mice, and IL-12 treatment almost completely blocks the increase (Figure 6B). Similar tumor-induced increases in the populations of CD4<sup>+</sup>Foxp3<sup>-</sup> and CD8<sup>+</sup> effector T cells were also detected. IL-12 treatment also reduced the number of effector T cells, perhaps because it reduced tumor burden, which drove the increase of effector T cells in this model.

To define the effect of IL-12 and IFN $\gamma$  on tumor growth *in vivo*, we used RMAS cells to challenge *IL-12R $\beta$ 2*<sup>-/-</sup> and *IFN $\gamma$ RI*<sup>-/-</sup> mice that are in syngeneic C57BL/6J background. We injected mice with a tumor dose that caused about 50% death of WT mice. As shown in Figure 6C, all the WT mice treated by exogenous IL-12 were rescued from death caused by tumor growth; endogenous IL-12 was not sufficient to impact tumor growth since *IL-12R $\beta$ 2*<sup>-/-</sup> mice

showed a survival rate similar to that of WT mice. However, all the *IFN $\gamma$ R1*<sup>-/-</sup> mice succumbed to tumor growth, indicating that endogenous IFN $\gamma$  is required for tumor immunity in this system. Furthermore, IL-12 treatment did not improve the survival of the *IFN $\gamma$ R1*<sup>-/-</sup> mice, suggesting that IL-12's anti-tumor effect is dependent on IFN $\gamma$  signaling. We also examined the effect of IL-12 and IFN $\gamma$  on Treg expansion *in vivo*. As shown in Figure 6D, Treg expansion was observed in WT mice after tumor challenge, which was completely blocked by exogenous IL-12 treatment. Treg cells in *IL-12R $\beta$ 2*<sup>-/-</sup> mice expanded to levels comparable to that in WT animals, suggesting that endogenous IL-12 was not sufficient to control Treg expansion. As expected, significantly higher levels of Treg expansion were observed in *IFN $\gamma$ R1*<sup>-/-</sup> mice, which was partially inhibited by IL-12 treatment. However, this partial inhibition of Treg expansion by exogenous IL-12 did not improve the survival of *IFN $\gamma$ R1*<sup>-/-</sup> mice, perhaps because *IFN $\gamma$ R1* deficiency disabled effector lymphocytes, which require normal IFN $\gamma$  function to clear the tumor cells (Figure 6C).

In summary, these *in vivo* observations now confirm the *in vitro* data, and strengthen the role of the IL-12-IFN $\gamma$  axis in regulating Treg cells and tumor immunity.

## Discussion

In this study, we developed an *in vitro* system to study factors that influence Treg cell expansion in the tumor setting. Irradiated tumor cells preferentially induce Treg cells to expand, with little effect on T effector cells. Although we do not know the specific antigens that cause Treg expansion, our data suggest that both accessory cells and IL-2 are required for Treg expansion. We also tested the effects of candidate cytokines on Treg cell expansion; most cytokines had no effect, but IL-12 inhibited tumor-induced Treg expansion in an Interferon-gamma dependent fashion. Mechanistic studies revealed that Interferon-gamma signaling directly inhibits Treg cell proliferation. Our findings support a model in which IL-12 stimulates immune effector cells to produce more IFN $\gamma$ , which then leads to the growth arrest of Treg cells. The inhibition of Treg cells may be critical in the early phase of an adaptive immune response, when accessory cells start to present tumor antigens, and also produce cytokines like IL-12 and IFN $\gamma$ . These cytokines may work in a positive feedback loop to ensure a potent adaptive immune response, which could end prematurely should Treg cells also become activated.

A previous report suggested that IL-12 treatment induced the apoptotic cell death of intratumoral CD4<sup>+</sup>CD25<sup>+</sup> T cells *in vivo* (33). In that study, Annexin V was used to measure apoptosis; local IL-12 treatment increased the percentage of CD4<sup>+</sup>CD25<sup>+</sup> T cells that stained positive for Annexin V. However, CD25 is probably not a reliable Treg marker for *in vivo* mechanistic studies (40), especially when IL-12 is used to stimulate effector T cells during an anti-tumor response. It is possible that the observed death in CD4<sup>+</sup>CD25<sup>+</sup> T cells was in fact the activation-induced death of effector T cells, rather than Treg cells. To clarify this issue, we used splenocytes from Foxp3-GFP knock-in mice in co-culture assays, and used 7-AAD to measure cell death. No significant cell death was observed in the CD4<sup>+</sup>GFP<sup>+</sup> Treg cell compartment (Figure S7); however, IL-12 treatment did increase cell death in the CD4<sup>+</sup>GFP<sup>-</sup> effector T cell compartment. Because cells can lose GFP when membrane integrity is lost, this data alone cannot exclude Treg cell death that occurs in this setting. However, our cell cycle analyses suggest that the anti-proliferative mechanism is important.

Our data show that IL-12 indirectly inhibits Treg cells, and that IFN $\gamma$  signaling accounts for at least part of this phenotype. While an additional, probably IL-2 dependent mechanism may also account for part of IL-12 mediated inhibition of Treg proliferation, it is also possible that cellular components are involved. Purified Treg cells do not expand when co-cultured with tumor cells; Non-T accessory cells in the co-cultures are required to promote Treg expansion, presumably by presenting tumor antigens and/or by producing growth factors. Therefore, it is



certainly possible that IL-12 can prevent Treg expansion by altering the function of an as yet unidentified population of accessory cells.

In summary, we show here that Treg inhibition by IL-12 may underlie some of its anti-tumor effects. Although the initial trials were suspended a decade ago because of dose- or regimen-related toxicities (41,42), this cytokine has recently been revitalized for clinical studies in cancer patients (43-45). Since Treg cell proliferation (induced by tumors or even vaccines) can considerably dampen tumor immunity (9-11), this study provides mechanistic evidence to support the notion that IL-12, by inhibiting Treg cell proliferation, may serve as an adjuvant for tumor vaccine-based immunotherapy.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

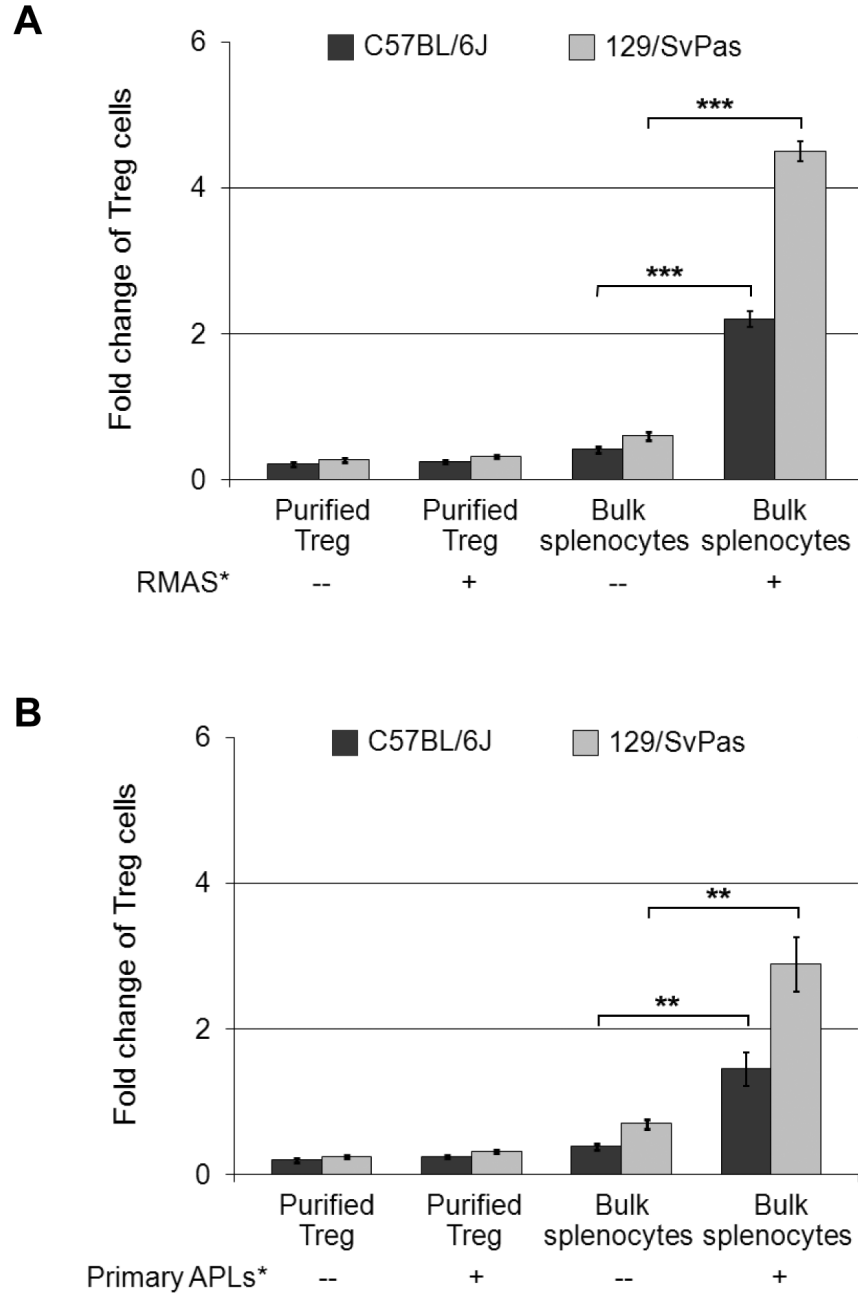
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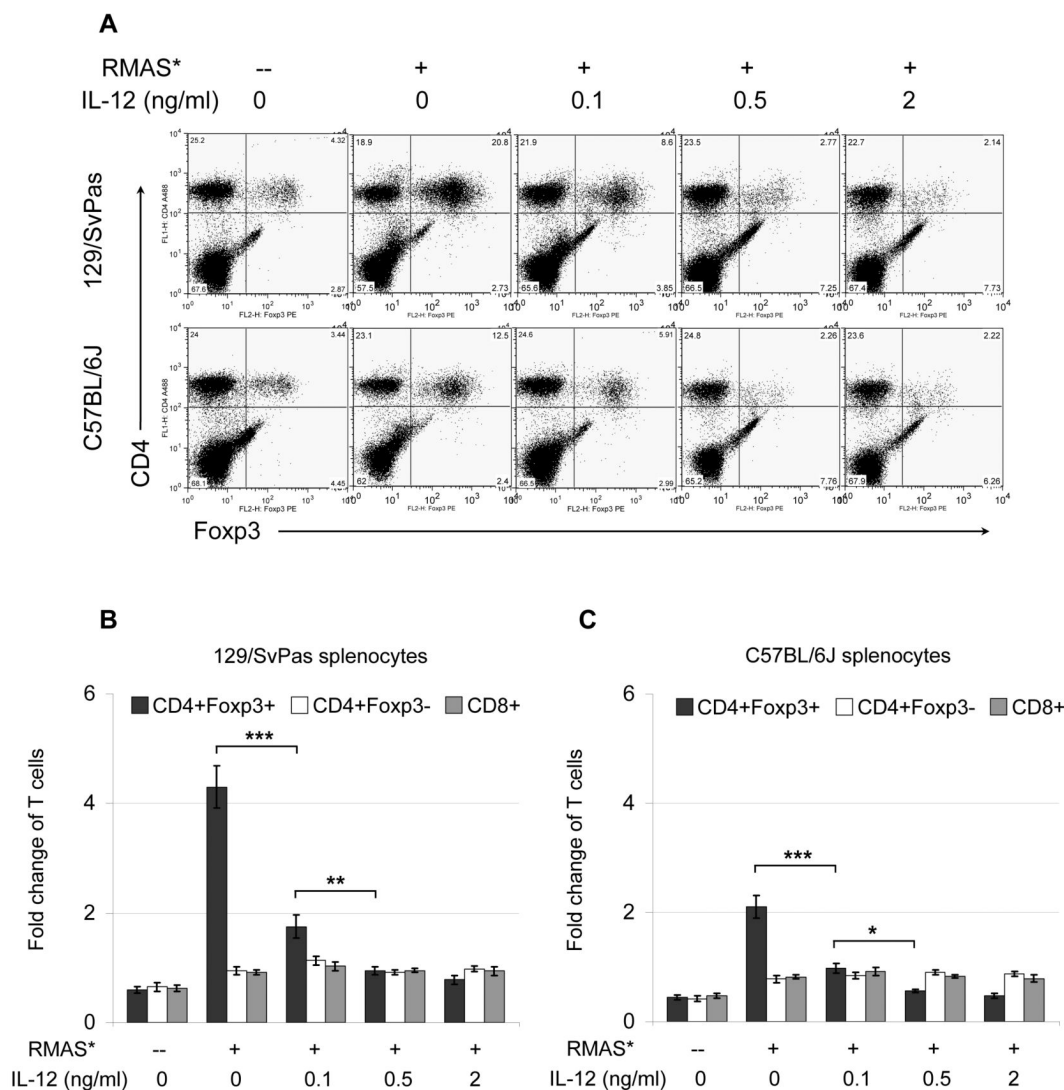
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**Figure 1. Irradiated RMAS and primary APL tumor cells induce Treg expansion when co-cultured with unfractionated splenocytes, but not purified Treg cells**

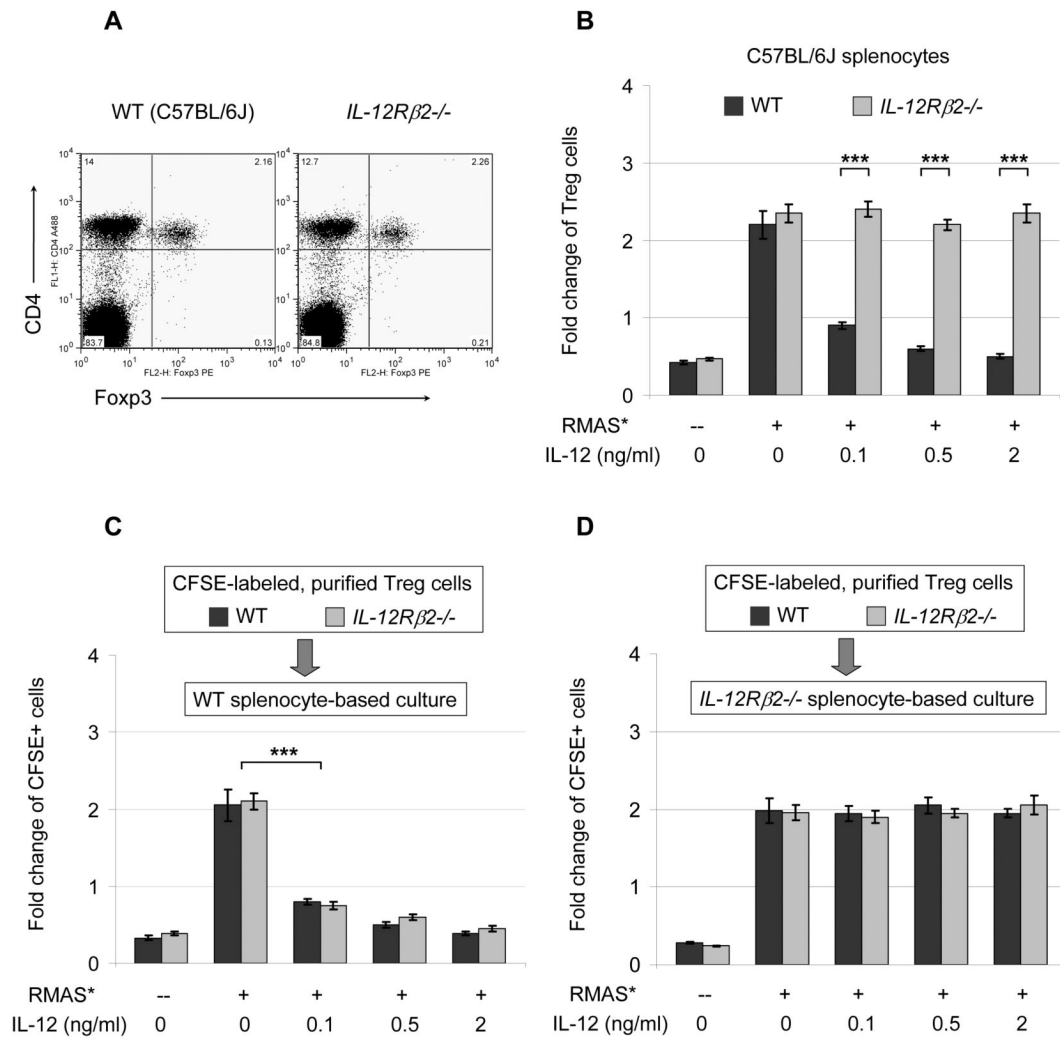
**A.**  $1 \times 10^6$  CD4<sup>+</sup>CD25<sup>+</sup> T cells were isolated from the resting spleens of WT 129/SvPas or C57BL/6J mice, and cultured in 1 ml regular K10 medium only or with  $1 \times 10^6$  irradiated (3,000 Rads) RMAS tumor cells. In addition,  $4 \times 10^6$  unfractionated splenocytes were cultured in 2 ml regular K10 medium only or with  $1.2 \times 10^5$  irradiated RMAS tumor cells. **B.** Instead of RMAS cells, cryopreserved primary APL tumor cells harvested from the spleens of 3 independent leukemic mCG-PML-RAR $\alpha$  transgenic mice were irradiated (3,000 Rads) and used at the same doses in the co-cultures. At the beginning and the end of co-cultures, absolute numbers of CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells were obtained by multiplying the percentages (determined

by flow cytometry as described in the Methods, and represented in Figure S1) by the total numbers of live cells harvested from the cultures. Treg expansion is presented as fold change by the equation:  $\text{Fold change} = (\text{Absolute numbers of Treg cells at the end of co-culture}) / (\text{Absolute numbers of Treg cells at the beginning of co-cultures})$ . Data points are shown as mean  $\pm$  SD; Two-tailed t-tests were used to determine statistical significance (\*\*P<0.01, \*\*\*P<0.001).



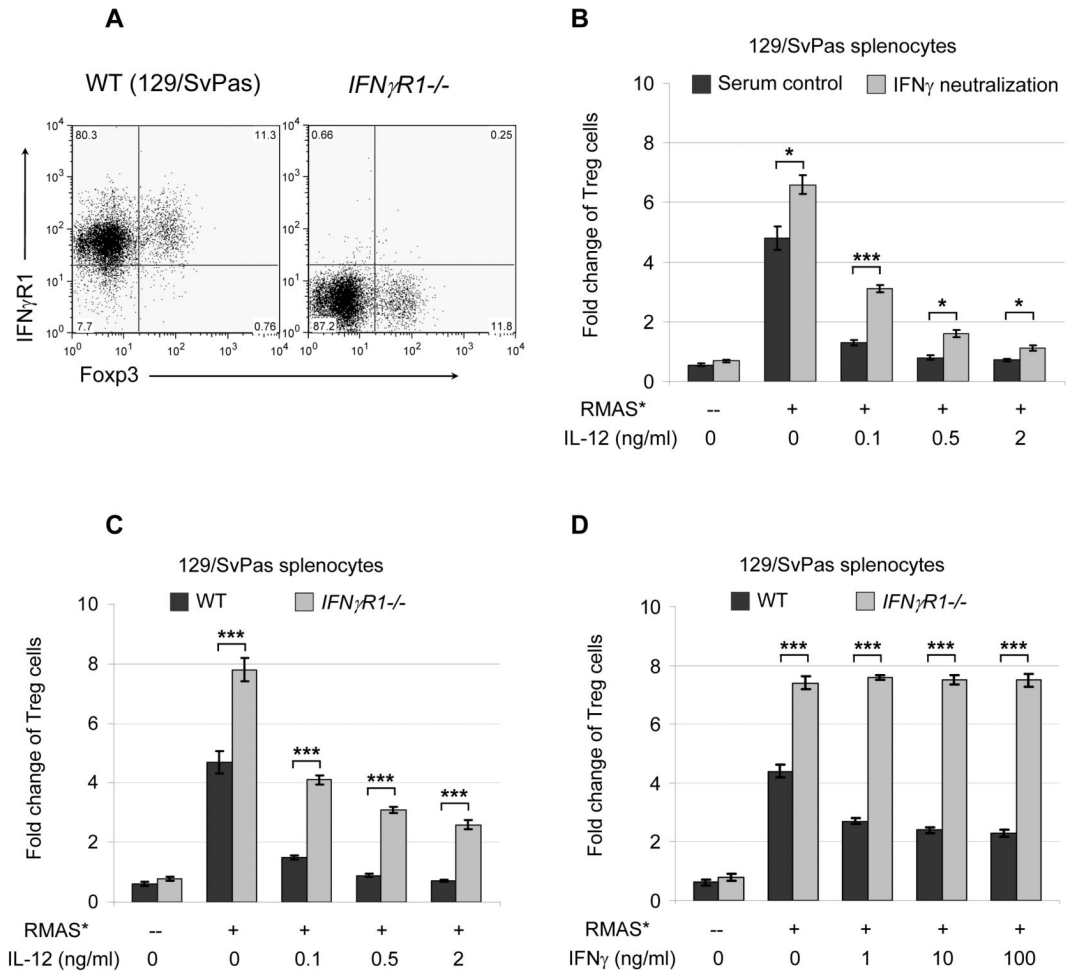
**Figure 2. IL-12 treatment inhibits tumor-induced Treg expansion**

$1.2 \times 10^5$  irradiated RMAS cells were co-cultured with  $4 \times 10^6$  unfractionated splenocytes for 5 days in the presence of the indicated dose of recombinant murine IL-12. **A**. Representative flow cytometric plots show the percentages of CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells and CD4<sup>+</sup>Foxp3<sup>-</sup> effector T cells in the end of the 5-day co-cultures. **B**. A summary of 3 independent co-culture experiments with 129/SvPas splenocytes is shown. **C**. A summary of 3 independent co-culture experiments with C57BL/6J splenocytes is shown. Treg expansion is presented as fold-change of the final yield over initial input as described in Figure 1. Data points are shown as mean  $\pm$  SD; Two-tailed t-tests were used to determine statistical significance (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).



### Figure 3. IL-12 signaling blocks Treg expansion indirectly

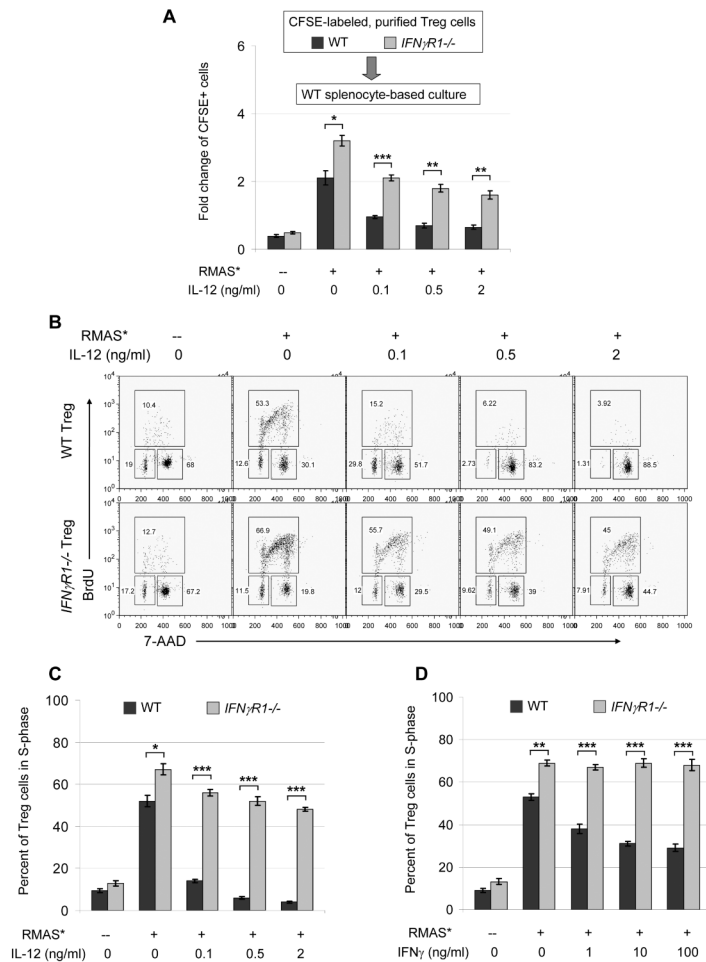
**A.** Representative flow cytometric plots show the percentages of CD4<sup>+</sup>Foxp3<sup>+</sup> and CD4<sup>+</sup>Foxp3<sup>-</sup> T cells in the spleens of naïve WT C57BL/6J (B6) mice and strain-matched IL-12 receptor β2-deficient (*IL12Rβ2-/-*) mice. 10 mice in each genotype were examined in 3 independent experiments, with similar results. **B.** RMAS tumor cells were cultured with WT B6 splenocytes or *IL12Rβ2-/-* splenocytes for 5 days. Summary data from 2 independent experiments are shown. **C** and **D.** Purified WT and *IL12Rβ2-/-* Treg cells were labeled with CFSE and mixed into co-cultures of tumor cells with either WT splenocytes (**C**) or *IL12Rβ2-/-* splenocytes (**D**) for 5 days, and expansion of the CFSE-labeled Treg cells are summarized for 2 independent experiments. Treg expansion is presented as fold-change of the final yield over initial input as described in Figure 1. Data points are shown as mean ± SD; Two-tailed t-tests were used to determine statistical significance (\*P<0.05, \*\*P<0.01, \*\*\*P<0.001).



**Figure 4. IFN $\gamma$  signaling partially accounts for IL-12 mediated Treg inhibition**

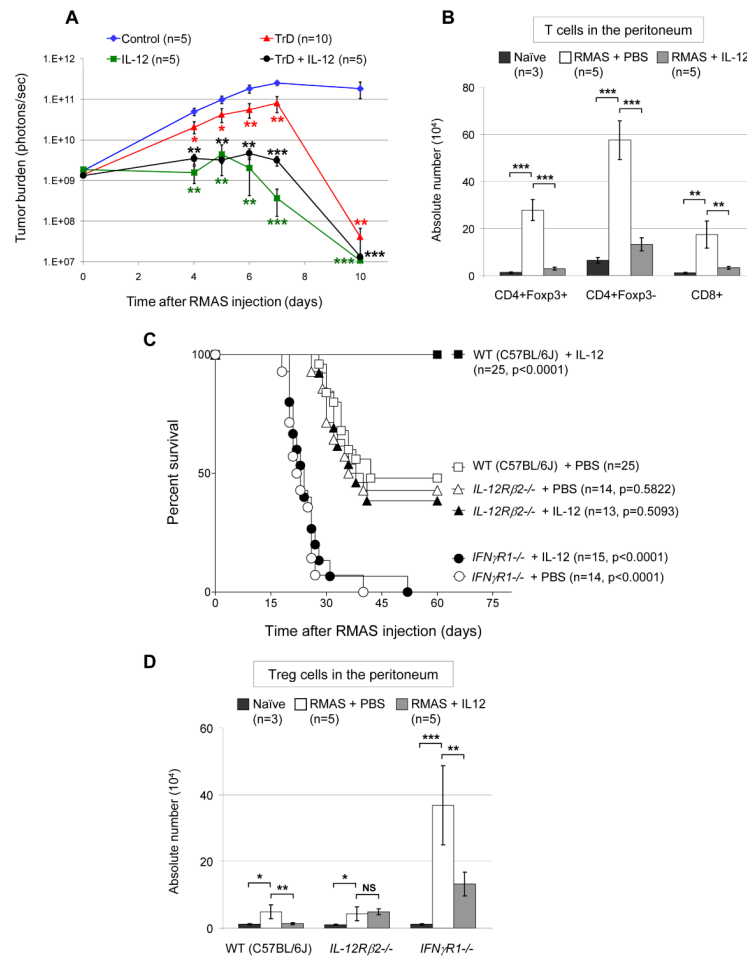
**A.** Representative flow cytometric plots for IFN $\gamma$  receptor 1 expression in naive WT 129/SvPas splenocytes and strain-matched IFN $\gamma$  receptor 1-deficient (*IFN $\gamma$ R1*<sup>-/-</sup>) splenocytes. Shown are cells in the CD4<sup>+</sup> gates. 6 mice in each genotype were examined in 2 independent experiments, with similar results. **B.** An IFN $\gamma$  neutralizing antibody, H22, or its isotype control, PIP, was added to a final concentration of 50  $\mu$ g/ml. A summary of 2 independent co-culture experiments is shown. **C.** RMAS tumor cells were cultured with WT 129/SvPas splenocytes or *IFN $\gamma$ R1*<sup>-/-</sup> splenocytes for 5 days. A summary of 3 independent experiments is shown. **D.** Indicated doses of IFN $\gamma$  were used to treat the co-cultures. A summary of 2 independent experiments is shown. Treg expansion is presented as fold-change of the final yield over initial input (as described in the Methods and Figures 1 and S1). Data points are shown as mean  $\pm$  SD; Two-tailed t-tests were used to determine statistical significance (\* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001).





### Figure 5. IFN $\gamma$ signaling directly inhibits Treg proliferation

**A.** Purified WT and IFN $\gamma$ RI<sup>-/-</sup> Treg cells were labeled with CFSE and mixed with WT splenocytes and irradiated RMAS cells for 4 days, and expansion of the CFSE-labeled Treg cells are shown as summary data from 2 independent experiments. Treg expansion is presented as fold-change of the final yield over initial input (as described in the Methods and Figures 1 and S1). **B.** Representative cell cycle analysis of CD4<sup>+</sup>FoxP3<sup>+</sup> Treg cells in cultures of irradiated RMAS cells and WT 129/SvPas splenocytes or IFN $\gamma$ RI<sup>-/-</sup> splenocytes. On day 5, BrdU was added to the cultures to a final concentration of 10  $\mu$ M and incubated for 4 hours before cells were harvested for analysis. **C.** Summary data of 2 independent experiments for cell cycle analyses, showing the percentage of Treg cells in S-phase. **D.** Instead of IL-12, indicated doses of IFN $\gamma$  were used to treat the co-cultures. Shown are summary data of 2 independent experiments for cell cycle analyses. Data points are shown as mean  $\pm$  SD; Two-tailed t-tests were used to determine statistical significance (\*P<0.05, \*\*P<0.01, \*\*\*P<0.001).



**Figure 6. IL-12 treatment inhibits Treg expansion and enhances tumor clearance *in vivo* in an IFN $\gamma$ -dependent fashion**

**A.** Four days prior to RMAS injection, 129/SvJ WT mice received a single IP injection of 400  $\mu$ g PC61 CD25 antibody to deplete Treg cells (TrD), or were injected with an isotype control antibody. From day 0 to day 7 after IP injection of  $2 \times 10^6$  luciferase-expressing RMAS cells, these mice were injected IP daily with equal volumes of PBS while a third set of mice received 100 ng of IL-12 IP daily. A fourth group of mice received both Treg depletion (TrD) and IL-12 treatment. Bioluminescence imaging was performed to monitor tumor burden. Shown are summary data of 2 independent experiments. **B.** Total cells were harvested by flushing the peritoneal cavity of naïve 129/SvJ WT mice or mice that were IP-injected with  $1 \times 10^6$  RMAS cells 5 days earlier, and treated with 100 ng IL-12 or equal volume of PBS control daily for 5 days. Cells were stained with primary conjugated antibodies directed against CD4, CD8, or Foxp3, and analyzed by flow cytometry. Absolute numbers of T cells were obtained by multiplying the percentage of the indicated T cell compartments by the total cell numbers harvested from the peritoneum. **C.** Kaplan-Meier survival curves of C57BL/6J WT mice versus strain-matched *IL12R $\beta$ 2*<sup>-/-</sup> mice and *IFN $\gamma$ R1*<sup>-/-</sup> mice, after intravenous injection with  $1 \times 10^4$  RMAS cells. From day 0 to day 40, these tumor-challenged mice received 50 ng of IL-12 IP or equal volume of PBS IP every other day. Shown are summary data of 3 independent experiments. **D.** C57BL/6J WT, strain-matched *IL12R $\beta$ 2*<sup>-/-</sup>, and *IFN $\gamma$ R1*<sup>-/-</sup> mice were IP-injected with  $1 \times 10^4$  RMAS cells. From day 0 to day 12, these tumor-challenged mice received 50 ng of IL-12 IP or equal volume of PBS IP every other day. On day 14, total cells were

harvested from the peritoneal cavity and analyzed by flow cytometry as described above in panel B to acquire the absolute numbers of CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells. Shown are summary data of 2 independent experiments. Data points are shown as mean  $\pm$  SD; Two-tailed t-tests were used to determine statistical significance (\*P<0.05, \*\*P<0.01, \*\*\*P<0.001).