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Radiation Enhances Regulatory T Cell Representation

Evelyn L. Kachikwu, MD¹, Keisuke S. Iwamoto, PhD¹, Yu-Pei Liao, PhD¹, John J. DeMarco, PhD¹, Nzhde Agazaryan, PhD¹, James S. Economou, MD PhD², William H. McBride, DSc¹, and Dörthe Schae, PhD¹

¹ Radiation Oncology, David Geffen School of Medicine at UCLA, Los Angeles, California, USA

² Surgical Oncology, David Geffen School of Medicine at UCLA, Los Angeles, California, USA

Abstract

PURPOSE—Immunotherapy (IT) could be a useful adjunct to standard cytotoxic therapies such as radiation in patients with micrometastatic disease although successful integration of IT into treatment protocols will require further understanding of how standard therapies affect the generation of anti-tumor immune responses. This study was undertaken to evaluate the impact of radiation therapy on immunosuppressive T regulatory (Treg) cells.

MATERIALS and METHODS—Tregs were identified as a CD4⁺CD25^{hi}Foxp3⁺ lymphocyte subset and their fate followed in a murine TRAMP-C1 model of prostate cancer in mice with and without radiation therapy.

RESULTS—CD4⁺CD25^{hi}Foxp3⁺ Treg cells increased in immune organs following local leg or whole body radiation. A large part, but not all, of this increase following leg-only irradiation could be ascribed to radiation scatter and Tregs being intrinsically more radiation resistant than other lymphocyte subpopulations resulting in their selection. Their functional activity on a per cell basis was not affected by radiation exposure. Similar findings were made with mice receiving local RT to murine prostate tumors growing in the leg. The importance of the Treg population in the response to RT was shown by systemic elimination of Tregs, which greatly enhanced radiation-induced tumor regression.

CONCLUSIONS—We conclude that Tregs are more resistant to radiation than other lymphocytes resulting in their preferential increase. Treg cells may form an important homeostatic mechanism for tissues injured by radiation, and in a tumor context may assist in immune evasion during therapy. Targeting this population may allow enhancement of radiotherapeutic benefit through immune modulation.

Keywords

T regulatory cells; radiation therapy; transgenic adenocarcinoma of the mouse prostate; anti-CD25 therapy

AUTHOR OF CORRESPONDENCE: Dr. Dörthe Schae, Department of Radiation Oncology, David Geffen School of Medicine at UCLA, B3-109 CHS, 10833 Le Conte Avenue, Los Angeles, CA 90095-1714. Telephone: (310) 825-5069, FAX: (310) 206-1260, dschae@mednet.ucla.edu.

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INTRODUCTION

Radiation Therapy (RT) is a first line treatment option to control many solid cancers. However, locally invasive or metastatic disease demands additional systemic approaches. Immunotherapy (IT) could be an attractive adjunctive treatment, in particular for patients with no definitive evidence of distant spread.

For RT and IT to be integrated into a combined protocol, it is imperative to understand how radiation affects immune function. There is increasing evidence that radiation has a spectrum of immune-modulating effects ranging from enhancing immunity to favoring immune tolerance. For example, we have shown that radiation inhibits endogenous processing of certain specific tumor-associated antigens by dendritic cells but enhances processing and presentation of the same antigen by the exogenous pathway (1). Our experience in animal tumor models and in patients is that RT increases anti-tumor immune responses (2,3) in some cases, but only to an extent that is unlikely to be clinically important without further IT interventions.

Of the many immune escape mechanisms that have been proposed for cancer, T regulatory cells (Treg) have long been suspected as being a major force regulating anti-tumor immunity (4) both outside and within the tumor microenvironment (5–7). The critical physiological role of these cells is normally the maintenance of peripheral immune tolerance, which they execute primarily through cell-to-cell contact mechanisms, though aided by release of cytokines such as IL-10, IL-4 and TGF- β (8–11) which in turn may be linked to the expression of extracellular ectonucleotidases catabolizing nucleotides such as ATP in sites of damage to produce adenosine (12,13). In fact, adenosine has long been known to play a critical, non-redundant role in the protection of normal tissues from collateral damage during inflammation (14–16), including radiation-induced tissue damage (17).

Of the different subtypes of Tregs, naturally occurring CD4⁺CD25^{hi}Foxp3⁺ cells (nTregs) that exit the thymus as a functionally mature T cell population already primed for suppressive function are currently thought to be most relevant for tumor escape (18). The forkhead box transcription factor (Foxp3) acts as a master switch in the regulation and development of CD4⁺ Tregs (19) with both Foxp3-transduced T cells and CD4⁺CD25^{hi} Tregs having marked suppressor activity (20,21).

There are many unanswered questions concerning the effect of cancer therapy on Tregs. The development of more specific markers for Tregs, namely Foxp3 and CD25 allowed us to revisit the concept of radiation-induced alterations in this T cell subpopulation within the context of local RT of solid tumors.

METHODS and MATERIALS

Mice and cell lines

Female 6–8 week old C57Bl/6, and C57Bl/6 mice engineered with a bicistronic Foxp3 allele linking the expression of Foxp3 with that of the green fluorescent reporter protein EGFP (Foxp3^{EGFP} mice; a kind gift from Dr. Chatila, UCLA) were bred and maintained in a defined-flora environment in the American Association of Laboratory Animal Care (AALAC)-accredited Animal Facility of the Department of Radiation Oncology, UCLA. Experiments adhered to all local and national animal care guidelines. Transgenic Adenocarcinoma of the Mouse Prostate C1 cells (TRAMP C1) were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in DMEM (Mediatech, Herndon, VA) with 10% FBS (Omega Scientific, Tarzana, CA), 10,000 IU penicillin, 10,000 µg/ml streptomycin, 25 µg/ml amphotericin (Mediatech), 5 µg/ml insulin

and 10^{-8} M dihydrotestosterone (Sigma-Aldrich, St. Louis, MO). TRAMP C1 cells were routinely maintained in vivo as subcutaneous tumors to prevent antigenic drift. To prepare single cell suspensions, TRAMP C1 tumors were harvested and digested with collagenase D (Roche) and DNase 1 (Type IV; Sigma), and small clumps removed by filtration. Mice were inoculated s.c. on the outer right thigh with 1×10^5 freshly harvested TRAMP C1 tumor cells. Tumors reached 4–7mm average diameter within 2–3 weeks and were used for experiments at this stage.

Radiation treatment

Mice were anaesthetized with an i.p. injection of 2.4mg ketamine/0.12mg xylazine (Ketaject, Xyla-Ject, Phoenix Pharmaceutical, Inc., St. Joseph, MO) and positioned in a Lucite jig with 5cm lead shielding the body, except for the right leg, which was irradiated using a Gamma cell 40 irradiator (Cs-137 source; Atomic Energy of Canada Ltd., Ottawa, Canada) at a dose rate of approximately 67cGy/min. Radiation ranging from 0–20Gy was given to the right thigh with 4 mice per group. Multiple experiments using Harshaw TLD-100H (LiF:Mg, Cu, P) and film (GAFCHROMIC EBT2, International Specialty Products, Wayne, NJ) dosimetry were performed and calibrated against a clinical cobalt-60 irradiator (Theratron-1000, MDS Nordion, Ontario) indicating that leakage and scatter of gamma rays to the shielded areas reached about 10.9–14.1% of the total dose (not shown).

Treg enumeration

Lymphocytes were harvested from spleens, depleted of red blood cells by ACK treatment and re-suspended as a single cell suspension. Where indicated, lymphocytes were also isolated from inguinal and axillary lymph nodes, blood and lung. Lymph nodes were homogenized by mortar and pestle grinding, while heparin-treated blood samples from the posterior vena cava were separated over a Fico/Lite-LM (mouse) gradient (Atlanta biologicals). Lung tissue was minced and enzymatically digested with 1U/ml Dispase II and 1mg/ml collagenase D (Roche Applied Science, Mannheim, Germany) for 1 hour at 37°C. For Treg staining, 2×10^6 cells were blocked with 1% (w/v) BSA in PBS prior to staining with PE-CD25 (clone PC61, BD Pharmingen), and PE-Cy5-CD4 (clone L3T4, BD Biosciences Pharmingen). For intracellular FoxP3, cells were then fixed in Fix/Perm working solution and permeabilization buffer added prior to staining with FITC-Foxp3 (clone FJK-16s, eBioscience). When indicated PE-CD25 was substituted with anti-mouse PE-CD39 (clone 24DMS1, eBioscience). Flow cytometric analysis was performed on 100,000 events using a FACSCalibur system (BD Biosciences, Mountain View, CA). Cells from Foxp3^{EGFP} mice were stained for surface markers only.

Proliferation assay

Magnetic bead sorting was used to separate CD4⁺CD25⁺ Tregs from CD4⁺CD25⁻ cells (Dynabeads, Invitrogen Dynal AS, Oslo, Norway). Aliquots of the two fractions were stained and analyzed for CD4 and CD25 as above to assess purity. Tregs were isolated from control and experimental spleens 48h post treatment and co-cultured with 1×10^5 CD4⁺CD25⁻ T effector cells from control mice at different ratios in 96-well round-bottom plates in triplicate for three days in RPMI 1640 medium (Mediatech), with 10% FBS, 1% antibiotics-antimycotic solution and 50µM 2-Mercaptoethanol in the presence of 0.25ug/ml CD3e (clone 145-2C11, BD Biosciences Pharmingen) and 1ug/ml aCD28 (clone 37.51, BD Biosciences Pharmingen). Control groups included CD4⁺CD25⁺ cells and CD4⁺CD25⁻ cell populations alone. Wells were pulsed with 1µCi of ³H-thymidine for the last 18 hours of culture. Cells were harvested onto glass microfibre filters and beta-emissions counted in a Wallace 1450 Microbeta Trilux Counter.

Cell viability assay

Apoptotic, necrotic, and viable cells were distinguished by Annexin V binding and 7-AAD exclusion according to the manufacturer's instruction (BD Pharmingen). Briefly, 1×10^6 splenocytes were washed with ice-cold PBS and stained with PE-Annexin V and 7-AAD in 100 μ l Annexin V binding buffer for 15–20 mins at RT followed by the addition of 400 μ l of binding buffer and FACScan of 100,000 cells as above. Percent viable cells in the splenocyte gate were defined by double negative for Annexin V and 7-AAD.

In vivo tumor studies with CD25⁺ cell depletion

Mice with Tramp C1 tumors, were randomized to receive 200 μ l of anti-CD25 mAb i.p. (PC61, BioExpress, NH) or PBS on days -4 , -1 , $+7$, $+17$, and $+21$, and to receive 0 or 10Gy irradiation to the tumor site on day 0. All groups contained 2–6 mice. Tumors were measured prior to starting treatment, and every three to four days thereafter, using Vernier calipers.

Statistical analysis

Data were analyzed for statistical significance using the two-tailed Student's t-test at the 5% level.

RESULTS

Treg profile after irradiation

Foxp3 expression is an established marker of Treg cells and Foxp3^{EGFP} C57Bl/6 mice have a functionally activated Foxp3 promoter driving EGFP expression that allows their convenient monitoring (22). Flow cytometric analysis of splenocytes from untreated Foxp3^{EGFP} C57Bl/6 mice typically identified 2% of splenocytes expressing EGFP (Figure 1A) that belonged almost exclusively (97%) to the CD4 lineage (not shown). This compares well to the results of intracellular antibody staining for Foxp3 of splenocytes in C57Bl/6 mice (eBioscience). Although not all-inclusive, most Foxp3⁺ cells expressed the IL-2 receptor CD25 and they did that with high intensity, which is associated with the Treg phenotype (Figure 1B). Clearly, Foxp3 can be expressed by CD4⁺CD25[−] cells, as has been reported in the literature (18), but overall there is a good correlation between CD4⁺/CD25^{hi} and CD4⁺/CD25^{hi}/Foxp3⁺ populations in spleen, lymph nodes, blood and lung (Figure 1C and D), and both these phenotypes can be considered as reliable indices of Treg cells. All Foxp3⁺ T cells also expressed CD39 (Figure 1F), an extracellular ATPase- which was recently suggested to be preferentially expressed on Tregs more than on other CD4 T cells (13). Yet, the bulk of cells expressing CD39 were not of the CD4 lineage (Figure 1E). CD4⁺/CD25^{hi}/Foxp3⁺ Tregs were most abundant in spleen and lymph nodes, representing 6% of CD4⁺ cells, whereas lung and blood had only 4% or less (not shown).

As a prelude to tumor studies, mice were irradiated with 10Gy gamma to the right leg only. Remarkably, Tregs significantly increased in spleen, lymph nodes, blood, and lung within 2 days, returning to normal by 10 days after exposure (Figure 1G), indicating that local irradiation of normal tissue could generate a systemic immunoregulatory response.

Tregs respond to radiation dose

Our experience with Foxp3^{EGFP} C57Bl/6 mice indicated that CD4⁺CD25^{hi} cells were largely Foxp3^{EGFP} positive and were representative of the Treg population, allowing us to use these markers along with intracellular staining for Foxp3 to monitor radiation responses of Tregs in standard C57Bl/6 mice. Foxp3⁺CD25⁺ cells in splenocytes were 5–6% of CD4⁺,

as in the FoxP3 transgenics, letting us conclude that our intracellular Foxp3 staining was accurately reflecting Foxp3 expression in vivo.

Using this approach, we confirmed that local irradiation of the right leg increased Treg in the spleen 2 days later in a radiation dose-dependent fashion, even after clinically relevant single or fractionated doses as low as 2Gy (Figure 2A). Mice receiving 20Gy leg irradiation had double the fraction of splenic CD4⁺ Tregs as controls ($5.4 \pm 1.6\%$ to $11.7 \pm 2.2\%$). Fractionating a larger dose into smaller, daily fractions lessened the final magnitude of the effect without statistical significance but presumably prolonged it. Interestingly, the proportion of CD4⁺ splenocytes fell following local leg irradiation, although a clear dose response was not seen despite repeated experiments (Figure 2B). The total number of splenocytes dropped noticeably in mice following exposure to the higher doses with some dose sparing after fractionation (Figure 2C).

Radiation spares Tregs

Since radiation delivered locally to the leg may have leaked to the shielded body by 10–14%, we used whole body irradiation to C57Bl/6 mice to pinpoint the minimum dose necessary for Tregs to increase. The rise in Tregs became measurable within 48h of radiation with 0.5Gy (Figure 3C) and was accompanied by a drop in spleen weight and cellularity (Figure 3A and B).

Whether or not this increase in Tregs relative to other lymphocytes was the result of induction, recruitment or simply a matter of differences in inherent radiation sensitivities was addressed by in vitro experiments using Foxp3^{EGFP} C57Bl/6 splenocytes. The extent of radiation-induced death of Treg and non-Treg cells was determined by Annexin V and 7-AAD staining and monitored over a time frame of up to 48h following in vitro irradiation. Again, GFP expression served as a marker to track Tregs without the need of further intervention. Sample FACS dot-blots of 0h and 24h are shown in Figure 4A. The fraction of viable cells as indicated by Annexin V/7-AAD double negative cells fell with dose from 12h onwards and was more marked in all (Figure 4B) than in Foxp3⁺ splenocytes (Figure 4C). As a result the proportion of splenocytes expressing Foxp3⁺ increased (Figure 4D) suggesting that these naturally occurring suppressor T cells had a relatively higher radiation resistancy. This was also reflected in the absolute increase in viable Tregs compared to an absolute decrease in viable non-Tregs in each well (supplementary figure).

Local irradiation does not affect the functional integrity of Tregs

To test Treg function, CD4⁺CD25⁺ and CD4⁺CD25[−] splenocyte populations were isolated (Figure 5A right panel). Initial selection of CD4⁺ cells essentially eliminated CD4[−] cells from the sample (Figure 3A left panel) and further positive selection for CD25 yielded a population that were 84% CD4⁺CD25⁺. The number of CD25⁺ cells in the CD4⁺CD25[−] fraction was negligible (middle panel).

T effector CD4⁺CD25[−] cells from spleens of untreated mice exposed to CD3 plus CD28 stimulation strongly responded with uptake of tritiated thymidine and served as our internal baseline. These stimulated T effector cells were equally inhibited in their proliferative capacity by about 40% when mixed with Tregs, regardless of whether the Tregs came from control mice or from locally-irradiated mice (Figure 5B).

CD4⁺CD25⁺ Treg cells mediate tumor evasion in vivo

Clearly, radiation-induced fluctuations in Tregs may be most pertinent in a cancer therapy setting and one must therefore ask the question as to whether Tregs also rise following tumor irradiation and whether intervention - systemic or otherwise – would influence the

outcome or RT. To address this, syngeneic TRAMP C1 tumors were grown s.c. in the leg in C57Bl/6 mice up to 6.5mm in size and treated with 10Gy local irradiation. Indeed, 48h later Tregs clearly increased in the spleen and, importantly, also in the tumor (Figure 6A).

An anti-CD25 monoclonal antibody was used to deplete Tregs in vivo to determine if they contributed to suppressing anti-tumor immunity and possibly lessen the efficacy of radiation treatment of TRAMP C1 tumors. TRAMP C1 were grown s.c. in the leg in C57Bl/6 mice and treated with 10Gy local irradiation as above (Figure 6B). Antibody treatment alone caused, at best, transient tumor growth delay and RT alone was also largely ineffective (Figure 6C). However, mice receiving antibody injections in combination with RT had the longest tumor growth delay and showed signs of transient regression ($p=0.003$; day7; Figure 6C). By the time all animals in other groups had to be euthanized because of large tumors, 60% of those in the combined therapy group had acceptable tumor volumes ($<1.5\text{cms}$ diameter) (Figure 6D). This suggests to us that targeting Tregs systemically might benefit local tumor RT and improve overall tumor control.

DISCUSSION

The frequency of $\text{CD4}^+/\text{CD25}^{\text{hi}}/\text{Foxp3}^+$ Treg cells that we detected in spleens of C57Bl/6 mice was about 6% of the CD4^+ population, which is well within the previously published range (18). Our observation that local leg irradiation increased Tregs in the spleen and other organs is strikingly similar to our experience with colorectal cancer patients undergoing local RT, although patients with prostate cancer did not respond in a similar manner (3). It may also be relevant to abscopal radiation effects reported by others (23,24) and it raises many questions.

Firstly, what is the anatomical target of the local radiation? It seems that these effects partly, though not completely, stem from radiation scattered outside the field. This is supported by findings that splenocyte counts fell with increasing dose and that whole-body exposure of 0.25 – 0.5Gy is sufficient to increase splenic Tregs. The fact that WBI increases Tregs has recently been reported (25) although the dose response was not examined. In the clinic, doses in excess of these levels may be delivered to large areas of the body even though the margins treated to high dose are tight. Volume and dose levels outside the planned volume will vary with the system, and will be higher for some, such as IMRT, than others (26). Close examination of the data however suggest that this is not the only mechanism involved and in-field effects such as lymphocytes trafficking through the radiation field, or amount of the bone marrow irradiated, or the location may contribute.

The second question is, what is the physiological mechanism behind the increase in Tregs? Are they generated in response to radiation-induced damage or selected for. Traditionally, modulation of immune cell function by ionizing radiation has been somewhat overshadowed by the focus on its cytotoxic action. In general, B cells and naïve T cells are relatively more radiosensitive than memory T and NK cells (27). Our in vitro data support the view that Tregs are a radioresistant subset but this does not necessarily exclude additional scenarios. For example, radiation is able to induce and activate the powerful immune-suppressive cytokine $\text{TGF-}\beta$ (28), which is known to drive Tregs (29) and the concept that Tregs may be activated in response to radiation damage must be considered plausible. Furthermore, we were able to detect the ectonucleotidase CD39 on the Treg population, as well as other non-lymphocytic cells. This has also been observed in cancer patients (30). Adenosine production through nucleotide catabolism is probably the most primitive immunosuppressive mechanism triggered by cell death. Our finding that the suppressive capacity of Tregs from these animals on a per cell basis remained unaltered by the radiation while their proportion increased, is important, as it suggests that these animals end up in an

overall heighten state of systemic immune suppression, at least temporarily, as a result of local or whole body RT. Ultimately, any of these scenarios, be it differences in intrinsic radiation sensitivities favoring Tregs over other T cell subsets or Treg cell activation, the outcome is the same, namely radiation appears to tailor the immune system towards tolerance. It is important to distinguish our studies from those done by North and colleagues more than 30 years ago showing that T suppressor cells were eliminated by sublethal, whole-body gamma-irradiation leading to partial or complete tumor regression in immunocompetent, but not in immunoincompetent, mice (31). The difference is that the suppressor T cells population targeted then belonged to a tumor-induced and tumor-specific CD8 positive lineage, as supposed to naturally occurring CD4 Tregs in our study. This is illustrated by the fact that the timing of the radiation exposure with respect to tumor implantation was crucial for North and colleagues to be able to observe the radiation effect.

Finally, our study raises the question whether fluctuations in Tregs need any intervention in the context of cancer care. If the radiation-induced rise in Tregs that we observed turns out to be a genuinely temporal phenomenon, then a combination of RT with IT may not necessitate anything other than appropriate timing. On the other hand, our data on mice receiving combined RT and anti-CD25 antibody, showed substantial tumor regression that exceeded either treatment alone, would argue in favor of an intervention. This has been demonstrated in other tumor models (32) and it opens exiting new avenues for future cancer therapies that will be enhanced if they are used to unmask the potential of the RT to boost systemic immunity to help achieve local and distant tumor control.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Liao YP, Wang CC, Butterfield LH, et al. Ionizing radiation affects human MART-1 melanoma antigen processing and presentation by dendritic cells. *J Immunol*. 2004; 173:2462–2469. [PubMed: 15294960]
2. Tsai CH, Hong JH, Hsieh KF, et al. Tetracycline-regulated intratumoral expression of interleukin-3 enhances the efficacy of radiation therapy for murine prostate cancer. *Cancer Gene Ther*. 2006; 13:1082–1092. [PubMed: 16841082]
3. Schaeue D, Comin-Anduix B, Ribas A, et al. T-cell responses to survivin in cancer patients undergoing radiation therapy. *Clin Cancer Res*. 2008; 14:4883–4890. [PubMed: 18676762]
4. Dannull J, Su Z, Rizzieri D, et al. Enhancement of vaccine-mediated antitumor immunity in cancer patients after depletion of regulatory T cells. *J Clin Invest*. 2005; 115:3623–3633. [PubMed: 16308572]
5. Liyanage UK, Moore TT, Joo HG, et al. Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. *J Immunol*. 2002; 169:2756–2761. [PubMed: 12193750]
6. Curiel TJ, Coukos G, Zou L, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med*. 2004; 10:942–949. [PubMed: 15322536]

7. Miller AM, Lundberg K, Ozenci V, et al. CD4+CD25high T cells are enriched in the tumor and peripheral blood of prostate cancer patients. *J Immunol.* 2006; 177:7398–7405. [PubMed: 17082659]
8. Shevach EM. CD4+ CD25+ suppressor T cells: more questions than answers. *Nat Rev Immunol.* 2002; 2:389–400. [PubMed: 12093005]
9. Nelson BH. IL-2, regulatory T cells, and tolerance. *J Immunol.* 2004; 172:3983–3988. [PubMed: 15034008]
10. Bluestone JA, Abbas AK. Natural versus adaptive regulatory T cells. *Nat Rev Immunol.* 2003; 3:253–257. [PubMed: 12658273]
11. Chen ML, Pittet MJ, Gorelik L, et al. Regulatory T cells suppress tumor-specific CD8 T cell cytotoxicity through TGF-beta signals in vivo. *Proc Natl Acad Sci U S A.* 2005; 102:419–424. [PubMed: 15623559]
12. Louis NA, Robinson AM, MacManus CF, et al. Control of IFN-alphaA by CD73: implications for mucosal inflammation. *J Immunol.* 2008; 180:4246–4255. [PubMed: 18322237]
13. Mandapathil M, Hildorfer B, Szczepanski MJ, et al. Generation and accumulation of immunosuppressive adenosine by human CD4+CD25highFOXP3+ regulatory T cells. *J Biol Chem.* 2010; 285:7176–7186. [PubMed: 19858205]
14. Ohta A, Madasu M, Kini R, et al. A2A adenosine receptor may allow expansion of T cells lacking effector functions in extracellular adenosine-rich microenvironments. *J Immunol.* 2009; 183:5487–5493. [PubMed: 19843934]
15. Sitkovsky M, Lukashev D, Deaglio S, et al. Adenosine A2A receptor antagonists: blockade of adenosinergic effects and T regulatory cells. *Br J Pharmacol.* 2008; 153 (Suppl 1):S457–464. [PubMed: 18311159]
16. Sitkovsky MV, Kjaergaard J, Lukashev D, et al. Hypoxia-adenosinergic immunosuppression: tumor protection by T regulatory cells and cancerous tissue hypoxia. *Clin Cancer Res.* 2008; 14:5947–5952. [PubMed: 18829471]
17. Sheth S, Bleibel W, Thukral C, et al. Heightened NTPDase-1/CD39 expression and angiogenesis in radiation proctitis. *Purinergic Signal.* 2009; 5:321–326. [PubMed: 19337853]
18. Sakaguchi S. Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol.* 2004; 22:531–562. [PubMed: 15032588]
19. Fontenot JD, Rudensky AY. A well adapted regulatory contrivance: regulatory T cell development and the forkhead family transcription factor Foxp3. *Nat Immunol.* 2005; 6:331–337. [PubMed: 15785758]
20. Jaekel E, von Boehmer H, Manns MP. Antigen-specific FoxP3-transduced T-cells can control established type 1 diabetes. *Diabetes.* 2005; 54:306–310. [PubMed: 15591438]
21. Mottet C, Uhlig HH, Powrie F. Cutting edge: cure of colitis by CD4+CD25+ regulatory T cells. *J Immunol.* 2003; 170:3939–3943. [PubMed: 12682220]
22. Haribhai D, Lin W, Relland LM, et al. Regulatory T cells dynamically control the primary immune response to foreign antigen. *J Immunol.* 2007; 178:2961–2972. [PubMed: 17312141]
23. Van der Meeren A, Monti P, Vandamme M, et al. Abdominal radiation exposure elicits inflammatory responses and abscopal effects in the lungs of mice. *Radiat Res.* 2005; 163:144–152. [PubMed: 15658889]
24. Dewan MZ, Galloway AE, Kawashima N, et al. Fractionated but not single-dose radiotherapy induces an immune-mediated abscopal effect when combined with anti-CTLA-4 antibody. *Clin Cancer Res.* 2009; 15:5379–5388. [PubMed: 19706802]
25. Qu Y, Jin S, Zhang A, et al. Gamma-ray resistance of regulatory CD4+CD25+Foxp3+ T cells in mice. *Radiat Res.* 2010; 173:148–157. [PubMed: 20095846]
26. Shueng PW, Lin SC, Chang HT, et al. Toxicity risk of non-target organs at risk receiving low-dose radiation: case report. *Radiat Oncol.* 2009; 4:71. [PubMed: 20043839]
27. Belka C, Ottinger H, Kreuzfelder E, et al. Impact of localized radiotherapy on blood immune cells counts and function in humans. *Radiother Oncol.* 1999; 50:199–204. [PubMed: 10368044]
28. Martin M, Lefaix J, Delanian S. TGF-beta1 and radiation fibrosis: a master switch and a specific therapeutic target? *Int J Radiat Oncol Biol Phys.* 2000; 47:277–290. [PubMed: 10802350]

29. Chen W, Jin W, Hardegen N, et al. Conversion of peripheral CD4+CD25– naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med.* 2003; 198:1875–1886. [PubMed: 14676299]
30. Mandapathil M, Szczepanski MJ, Szajnik M, et al. Increased ectonucleotidase expression and activity in regulatory T cells of patients with head and neck cancer. *Clin Cancer Res.* 2009; 15:6348–6357. [PubMed: 19825957]
31. North RJ. Radiation-induced, immunologically mediated regression of an established tumor as an example of successful therapeutic immunomanipulation. Preferential elimination of suppressor T cells allows sustained production of effector T cells. *J Exp Med.* 1986; 164:1652–1666. [PubMed: 2945892]
32. Kudo-Saito C, Schlom J, Camphausen K, et al. The requirement of multimodal therapy (vaccine, local tumor radiation, and reduction of suppressor cells) to eliminate established tumors. *Clin Cancer Res.* 2005; 11:4533–4544. [PubMed: 15958639]

1A

Foxp3 (FL1-H)

FSC-H

1B

CD25 (FL2-H)

Foxp3 (FL1-H)

1C **1D**

Figure 1.

Tregs increase temporarily in Foxp3^{EGFP} mice after local irradiation. Splenocytes, lymph nodes, blood and lung were stained for CD4 (FL3) and CD25 (FL2) and assessed by flow cytometry. **A)** Splenocyte dot blot of Foxp3 (FL-1) vs forward scatter. **B)** CD25 vs FoxP3 dot blot of CD4⁺ splenocytes. **C)** Box and whisker diagram summarizing the number of FoxP3⁺ cells and the number of CD4⁺/CD25^{hi}/Foxp3⁺ Tregs in the spleen. **D)** Correlation of CD4⁺ T cells that have high CD25 expression and FoxP3 in spleen, blood, lymph nodes and lung. **E)** Splenocytes dot blot of CD39 vs CD4. **F)** CD39 vs Foxp3 dot blot. **G)** Time course of Treg changes in spleen, lymph nodes, blood and lung after 10Gy gamma-irradiation of the right leg. Data are mean fold increases in all organs to control of $n=4 \pm \text{s.d.}$ * $p<0.05$.

Figure 2.

The radiation-induced increase in Tregs in the spleen of C57Bl/6 mice is dose-dependent. The right leg was exposed to various doses of radiation 48h prior to analysis or left untreated. Splenocytes were stained for CD4, CD25 and Foxp3 and analyzed by FACScan. Dose-response of **A)** Treg changes, of **B)** CD4⁺ splenocyte levels and of **C)** total splenocyte numbers per spleen. Data are mean of $n=4 \pm$ s.d. of 2 independent experiments * $p<0.05$.

Figure 3.

Low radiation doses to the whole body reduced spleen size, weight and cell numbers while increasing the proportion of splenic Tregs. C57Bl/6 mice were exposed to X-irradiation ranging from 0.25 to 2Gy. 48h later spleens were weighed, splenocytes counted and analyzed as above. **A)** Dose-dependent fall in splenic weight and cell numbers. **B)** Photograph taken of spleens from 2Gy irradiated mice illustrating the change in size. **C)** CD4⁺/CD25^{hi}/Foxp3⁺ Treg increased with radiation dose. Data are mean of $n=3 \pm$ s.d. * $p<0.05$.

Figure 4.

Foxp3⁺ splenocytes are relatively more radioresistant than other spleen cells. Splenocytes were isolated from Foxp3^{EGFP} C57Bl/6 mice, irradiated in vitro with 0, 0.5, 1, 2, and 5Gy. Cell death was measured at 0, 6, 12, 24, and 48 hrs by 7-AAD/Annexin-V-staining **A**) Examples of 7-AAD vs Annexin-V gating for splenocytes 24hrs after 2 Gy showing

decreased viability in whole splenocyte populations (left). In contrast, Foxp3⁺-gated splenocytes showed little radiation-induced cell death. (the increase in viability is due to loss of dead cells during incubation). **B)** Dose- and time-dependent changes in the fraction of all viable splenocytes as Annexin-V⁻/7-AAD⁻ (lower left quadrants in 4A). **C)** time-dependent but dose-independent changes in the fraction of Foxp3⁺ viable splenocytes. **D)** Dose- and time dependent rise in Foxp3⁺ splenic cells.

Figure 5. $CD4^+/CD25^+$ Tregs suppressed cell proliferation of $CD4^+CD25^-$ T effector cells (T_{eff}) in vitro. Splenic Tregs were isolated from 10Gy leg-irradiated C57Bl/6 mice 48h post treatment. Tregs from healthy mice's spleen served as control. **A)** Data are FL3(CD4) vs FL2(CD25) dot plots of whole spleens (left panel), $CD4^+/CD25^-$ fraction (middle panel) and $CD4^+/CD25^+$ fraction (right panel). **B)** aCD3/aCD28 – stimulated T_{eff} from control mice were mixed with Tregs from control or from irradiated animals and proliferation was assessed according to 3H -Thymidine incorporation after 3 days. T_{eff} alone were incubated separately without CD3/CD28 stimulation. Data are mean counts per minute (cpm) of $n=4-6 \pm$ sem.

Figure 6.

Tumor control following local irradiation benefits from concurrent, systemic inhibition of Tregs. **A)** Local irradiation of TRAMP C1 tumors increased Tregs in the tumor and outside. Mice bearing tumors of 4.5–6.5mm in size were given radiation treatment with 10 and 20Gy and their spleen and tumors analyzed 48h later. CD4⁺/CD25^{hi}/Foxp3⁺ Tregs are shown as mean of $n=4 \pm \text{s.d.}$ **B)** Treatment of TRAMP C1 tumors with anti-CD25 antibody (aCD25 Ab) in combination with 10Gy local irradiation caused tumor growth delay. Tumor-bearing mice were given aCD25 Ab or PBS i.p. on day -4, -1, +7 and +14. Tumors were irradiated on day 0 with 10Gy or left untreated. Tumor size as mean mm diameter (2 dimension) of $n = 2-6 \pm \text{s.d.}$ **C)** Survival data with % of mice per treatment group alive. * $p<0.05$.