Expression of Arginase I in Myeloid Cells Limits Control of Residual Disease after Radiation Therapy of Tumors in Mice

Marka R. Crittenden, Taticia Savage, Benjamin Cottam, Jason Baird, Paulo C. Rodriguez, Pippa Newell, Kristina Young, Andrew M. Jackson and Michael J. Gough

* Earle A. Chiles Research Institute, Robert W. Franz Cancer Center, Providence Portland Medical Center, Portland, Oregon; † The Oregon Clinic, Portland, Oregon; ‡ Stanley S. Scott Cancer Center, Louisiana State University Health Sciences Center, New Orleans, Louisiana; § Providence Hepatobiliary and Pancreatic Cancer Program, Providence Portland Medical Center, Portland, Oregon; ‡ Department of Radiation Medicine, Oregon Health and Sciences University, Portland, Oregon; and ‡ Host: Tumour Interactions Group, Academic Unit of Clinical Oncology, University of Nottingham, United Kingdom


An accumulating body of evidence demonstrates that radiation therapy can generate adaptive immune responses that contribute to tumor control. However, in the absence of additional immune therapy, the adaptive immune response is insufficient to prevent tumor recurrence or affect distant disease. It has been shown in multiple models that tumor-infiltrating myeloid cells exhibit alternative activation phenotypes and are able to suppress adaptive immune responses, and recent data suggests that the myeloid response in tumors treated with cytotoxic therapy limits tumor control. We hypothesized that tumor myeloid cells inhibit the adaptive immune response after radiation therapy through expression of the enzyme arginase I. Using a myeloid cell-specific deletion of arginase I in mice, we demonstrate an improved tumor control after radiation therapy. However, tumors still recurred despite the conditional knockdown of arginase I. Since multiple alternative factors may combine to inhibit adaptive immunity, we propose that targeting macrophage differentiation may be a more effective strategy than targeting individual suppressive pathways. © 2015 by Radiation Research Society

INTRODUCTION

Macrophages that infiltrate tumors exhibit an alternative activation phenotype that has been termed M2 differentiation to distinguish them from classically activated or M1 macrophages that exhibit a very different response to inflammatory stimuli (1, 2). These alternatively activated macrophages and other myeloid derived suppressor populations are able to suppress T-cell responses (3, 4) and limit the effect of T-cell immunotherapies (5). The alternative differentiation pathways in macrophages have been associated with wound healing and may play a role in resolving adaptive immune responses to permit tissue repair (6, 7). These observations are highly relevant to cytotoxic therapies. Recent studies have demonstrated that tumors treated with radiation therapy or different chemotherapy agents generate myeloid responses that limit tumor control (8–10). We recently demonstrated that macrophages in tumors after irradiation exhibit an alternative activation phenotype (11), and that preventing tumor macrophages from developing a suppressive phenotype improved tumor control by radiation therapy and resulted in long-term tumor-specific immunity (11).

The enzyme arginase I is a classical marker of alternative activation in murine macrophages (12, 13). Hydrolysis of arginine by arginase I lead to the production of ornithine and urea. Ornithine accumulation provides substrate sources of free proline for collagen synthesis in healing wounds (7). In addition, it enables the formation of polyamines, which promote tumor cell proliferation. Arginase induction can be detected in myeloid cells after trauma and surgery (14, 15). By contrast, classically activated macrophages do not express arginase I and instead express iNOS and utilize L-arginine to generate nitric oxide with its attendant vascular inflammatory effects (13). Arginase I expression limits T-cell responses in infectious models (16), and expression of arginase I by tumor-associated macrophages and myeloid-derived suppressor cells have been demonstrated to suppress antitumor effector T-cell responses (3, 5).

We recently observed upregulation of arginase I in tumor macrophages after radiation therapy as part of an alternative activation phenotype that prevented tumor control (11). Therefore, we hypothesized that tumor macrophages inhibit the adaptive immune response after radiation therapy.
through expression of the enzyme arginase I. To test this hypothesis, we treated tumors in mice lacking arginase expression in myeloid cells using LysM-Cre mediated excision of a floxed arginase I gene (17, 18). We demonstrate that specific loss of arginase I improves tumor control by radiation therapy, but that the effect is restricted to a transient window after treatment. In addition, we demonstrate that this window after radiation therapy correlates with peak T-cell involvement in tumor control, but that tumors recur despite this immune activity. These data are consistent with a window of adaptive immune involvement after radiation therapy that is limited by macrophage expression of arginase I, although tumors recur even if arginase I is not expressed despite adaptive immune responses in the host.

MATERIALS AND METHODS

Animals and Cell Lines

The Panc02 murine pancreatic adenocarcinoma cell line (19) (C57BL/6) was kindly provided by Dr. Woo (Mount Sinai School of Medicine, New York, NY). The Lewis lung carcinoma (3LL) cell line (20) (C57BL/6) was obtained from the ATCC (Manassas, VA). Six- to eight-week-old C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA) for use in these experiments. Mice with floxed arginase I crossed with LysM-Cre mice were provided by P. Rodriguez and previously generated (18). All animal protocols were approved by the Earle A. Chiles Research Institute IACUC (Animal Welfare Assurance No. A3913-01).

Antibodies and Reagents

Fluorescently conjugated antibodies CD11b-AF700, Gr1-PE-Cy7, IA (MHC class II)-e780, CD4-e450, FoxP3-APC, CD4-PerCP Cy5.5 and CD8-FITC were purchased from Abcam (Cambridge, MA). Western blotting antibodies used include arginase I (BD Biosciences, San Jose, CA), iNOS (Cayman Chemical Corp., Ann Arbor, MI), GAPDH, anti-mouse-HRP and anti-rabbit-HRP (all from Cell Signaling Technology, Danvers, MA).

Preparation of Bone Marrow Macrophages

Bone marrow cells isolated from long bones of mice were cultured for a total of 7 days in complete media containing 40 ng/ml macrophage colony-stimulating factor (M-CSF) (eBioscience), with additional growth media provided after 3 days of culture. Adherent cells were harvested and macrophage differentiation confirmed by flow cytometry for CD11b, F4/80, Gr1 and IA. Macrophages were differentiated into M1 or M2 phenotypes by culture for 24 h in the presence of IFN-γ (10 ng/ml eBioscience) plus 1 µg/ml Ultrapure lipopolysaccharide (LPS, Invivogen, San Diego, CA) or IL-4 (10 ng/ml, eBioscience), respectively.

Western Blotting

Cells were lysed in RIPA buffer and denatured in SDS loading buffer containing β2-mercaptoethanol, electrophoresed on 10% SDS-PAGE gels and transferred to nitrocellulose. Blocked blots were probed overnight at 4°C with primary antibodies followed by HRP-conjugated secondary antibodies. Binding was detected using a SuperSignal West Pico Chemiluminescent Substrate (Pierce/Thermo Fisher Scientific, Rockford, IL) and exposure to film.

Flow Cytometry of Tumor and Blood

For analysis of tumor-infiltrating cells, the tumor was dissected into approximately 2 mm fragments followed by agitation in 1 mg/mL collagenase (Invitrogen, Carlsbad, CA), 100 µg/mL hyaluronidase (Sigma, St. Louis, MO) and 20 mg/mL DNase (Sigma) in PBS for 1 h at room temperature. The digest was filtered through 100 µm nylon mesh to remove macroscopic debris. Splenocytes were isolated by first crushing and then red blood cell lysis using ACK buffer. For flow cytometry analysis, cell suspensions were washed and stained with antibodies specific for surface antigens, then cells were washed and fixed using a T regulatory cell staining kit (eBiosciences) and intracellularly stained for FoxP3, Ki67 and CD3-zeta, as appropriate. The proportion of each infiltrating cell type was analyzed on a BD LSRII (Beckman Dickinson, Franklin Lakes, NJ). Flow sorting of tumor macrophages was performed as previously described (11, 21) using a BD FACSaria cell sorter to greater than 98% purity.

Radiation Therapy of Tumors

Tumors were injected subcutaneously in the right leg below the knee at a dose of 2 × 105 Panc02 or 5 × 105 3LL and allowed to establish for 14 days (Panc02) or 10 days (3LL) before initiation of treatment. Dosing was based on dose escalation studies in mice and recent clinical studies (22), with three daily 20 Gy treatment fractions given using an Elekta Synergy linear accelerator (Atlanta, GA) with 6 MV photons incorporating a half beam block to minimize dose to the torso and 1 cm bolus.

Statistics

Data were analyzed and graphed using Prism (GraphPad Software, La Jolla, CA). Blood myeloid numbers over time were fitted to second order polynomial curves using Prism. Individual data sets were compared using Student’s t test and analysis across multiple groups was performed using ANOVA with individual groups assessed using Tukey’s comparison.
RESULTS

To test the effect of high-dose fractionated radiation therapy on adaptive immune cells in the tumor, we used a previously described model of in vivo radiation therapy in immunocompetent mice (11). C57BL/6 mice with 14 days injected subcutaneously Panc02 pancreatic adenocarcinoma tumors were treated with 20 Gy × 3 focal irradiation to the leg over a period of 3 days. As expected, radiation therapy resulted in a loss of both CD8 (P < 0.01) and CD4 (P < 0.001) T cells in the treated tumor 1 day after the final treatment dose [Fig. 1A(i–ii)]. Importantly, 7 days after the final treatment dose CD8 T cells returned to the tumor, while CD4 T cells rebounded to higher levels than untreated tumors (P < 0.005). Among the CD4 T cells was a large population of regulatory T cells, identified by expression of FoxP3 [Fig. 1A(iii)], and these cells were decreased 1 day after irradiation (P < 0.0001) before rebounding to higher proportions 7 days after irradiation (P < 0.05). To determine the T-cell response to tumor irradiation in secondary lymphoid organs outside of the treatment field,
we examined the spleen for expression of proliferative marker Ki67 in T cells. While there was no change in T-cell proliferation over days 1 and 4 after radiation therapy, by day 7 after irradiation there was a spike in proliferation of CD8, nonregulatory CD4 and regulatory T cells, which declined over time (Fig. 1B). The timing of this T-cell proliferative response is consistent with the timing of antigen-specific T-cell responses in secondary lymphoid organs following antigen challenge (23, 24), suggesting that endogenous T-cell responses may be triggered after radiation therapy. Nevertheless, despite systemic T-cell activation and the recruitment of T cells back to the treated tumor, the tumors recurred locally [Fig. 1C(i–iii)]. These data suggest that while radiation induces adaptive T-cell responses, they are unable to control the tumor. These data are consistent with prior studies showing that while T cells contribute to control of tumors treated with radiation therapy in the absence of additional immunotherapy the T-cell response is insufficient to eliminate tumors (24–27).

To investigate why the residual cells in irradiated tumors resist adaptive immune control, we further examined the tumor immune environment. We previously demonstrated that irradiation of tumors resulted in increased infiltration of tumors with macrophages exhibiting an alternative activation (M2) phenotype (11). These tumor macrophages increase expression of arginase I, as measured by microarray and Western blot analysis (11). We hypothesized that arginase I expression by tumor macrophages and other tumor-infiltrating myeloid cells after irradiation suppressed the antitumor T-cell response, permitting outgrowth of residual disease. For this reason, we performed experiments using mice with a myeloid-specific deletion of arginase, i.e., mice with a floxed arginase gene crossed with mice expressing Cre recombinase under the LysM promoter (18). Once Cre excises the arginase I gene in myeloid cells, the cells are permanently unable to express arginase, although these myeloid cells can otherwise undergo normal differentiation. To confirm the conditional deletion of arginase, we prepared bone marrow macrophages from Argfl/fl mice with matched littermates that either were heterozygous for Cre (LysM-Cre–/–-arginase-deficient) or did not express Cre (LysM-Cre–/–-normal-arginase expression). To generate M1 or M2 differentiation, bone marrow macrophages were treated with IFN-γ and LPS, or IL-4, respectively. As expected, in LysM-Cre–/– bone marrow macrophages IFN-γ treatment generated M1 macrophages expressing iNOS but not arginase, while IL-4 treatment generated M2 macrophages expressing arginase but not iNOS (Fig. 2A). LysM-Cre+ bone marrow macrophages were unable to produce arginase, but iNOS remained unaffected, showing induction by IFN-γ but not IL-4.

Cre-mediated excision of arginase in myeloid cells did not affect tumor infiltration by macrophages (Fig. 2B). To investigate the effect of irradiation on tumor macrophage expression of arginase in this model, Argfl/fl LysM-Cre–/– and Argfl/fl LysM-Cre+ were challenged with Panc02 tumors and left untreated or treated with 3 × 20 Gy focal irradiation matching the prior model (11). Seven days after irradiated tumors were harvested and sorted tumor-infiltrating CD11b+ cells were analyzed by Western blotting for iNOS and arginase. Radiation therapy caused increased expression of arginase in tumor macrophages and this did not occur in mice with macrophage-specific Cre-regulated excision of arginase (Fig. 2C). These data demonstrate that the Argfl/fl LysM-Cre model provides a technique to
specifically prevent the radiation-mediated upregulation of arginase in tumor macrophages.

To determine whether tumor myeloid expression of arginase limited tumor control by radiation therapy, Panc02 tumors in Argf/fl LysM-Cre\(^{+/−}\) and Argf/fl LysM-Cre\(^{+/−}\) littersmates were left untreated or were treated with \(3 \times 20\) Gy focal irradiation. In untreated mice, tumor growth was unaffected by myeloid arginase expression (Fig. 3A). However, after radiation therapy, Argf/fl LysM-Cre\(^{+/−}\) mice that lacked arginase I in myeloid cells had smaller tumors than the Argf/fl LysM-Cre\(^{+/−}\) littersmates (Fig. 4A). Similarly, T-cell responses and independent of arginase expression did not affect the level of CD3-zeta on CD8 T cells in the presence or absence of myeloid arginase expression (Fig. 4A(i)). One of the major effects of L-arginine depletion by overexpression of arginase appears to be loss of CD3-zeta in tumor-infiltrating T cells, leading to loss of T-cell function (28). To determine whether CD3-zeta expression was altered in the treated tumors in the presence or absence of arginase, we analyzed the infiltration of CD8 T cells expressing the effector molecule granzyme B and activated CD4 nonregulatory (FoxP3\(^{−}\)) T cells expressing the effector molecule the presence or absence of arginase, we analyzed the infiltration of CD8 T cells expressing the effector molecule.

These data demonstrate that tumor control is enhanced after radiation therapy by knockout of arginase in myeloid cells, consistent with arginase-mediated suppression of T-cell responses in the tumor. However, unexpectedly, tumors recur despite T-cell responses and independent of arginase expression. These data are consistent with current studies showing limited capability of adaptive immune cells to control tumors in the absence of additional immune stimulation.

To examine the function of T cells in the treated tumors in the presence or absence of arginase, we analyzed the infiltration of CD8 T cells expressing the effector molecule macrophage arginase in radiation responses. Macrophages directed towards M2 phenotypes express arginase \(in vitro\), and after radiation therapy tumor macrophage expression of arginase is increased \(in vivo\) (Fig. 2). Eliminating arginase expression using Cre-regulated excision of a floxed arginase gene significantly improved tumor control through a transient window after radiation therapy, but did not prevent disease recurrence (Fig. 3). Eliminating arginase expression did not change the proportion of effector or activated T cells in the tumor after radiation therapy, and did not change CD3-zeta expression on T cells in the treated tumor (Fig. 4). These data support a model where radiation therapy engenders a CD8 T-cell response that participates in tumor control in a transient window after treatment. Arginase expression by M2 differentiated tumor macrophages limits tumor control through this window, but in the absence of additional intervention, adaptive immune cells remain unable to control residual disease.

The alternative macrophage differentiation pathway is characteristically seen in Th2-type responses to parasites (29) and in infectious models and in tumors it is closely associated with the effects of IL-4 (30, 31). While multiple differentiation phenotypes can be detected in tumor macrophages, the preponderance of macrophage differentiation in tumors includes many features of alternative differentiation (1, 2), which in mice includes arginase expression (12). This arginase expression limits T-cell function in the tumor (3, 31) and limits the efficacy of T-cell targeted immunotherapy (5). While the metabolites of L-arginine may play a role in T-cell suppression, T-cell function can be restored through exogenous provision of L-arginine (16, 32). Thus, T-cell dysfunction may relate more to the low bioavailability of L-arginine to proliferating T cells (32). These data may explain why blocking other L-arginine metabolizing enzymes is also able to improve T-cell activation (31, 33) and means that despite loss of arginase I in our model, other arginase-depleting enzymes may persist. In addition, arginase I is only one of the features of alternatively activated macrophages that may suppress adaptive immunity at the tumor. Furthermore, the presence of the arginase II isoform could compensate for the absence of arginase I in myeloid cells. Macrophages exposed to irradiated cancer cells produce IL-10 on stimulation (11) and IL-10 has a broad spectrum of immune modulatory functions (34) and can suppress T-cell activity in the tumor (35). Similarly, TGFβ is
FIG. 3. Response to radiation therapy in LysM-Cre Argfl/fl mice. Cre–/– and Cre+/– Argfl/fl littermates bearing d14 Panc02 tumors were left untreated (NT) or treated with 3 × 20 Gy focal irradiation over 3 days (RT). Panel A: Graphs show, (i) mean and standard error of tumor size over time and (ii) survival. Panel B: Tumor sizes in individual mice in the window after radiation therapy, (i) day 24 after challenge (day 8 after final RT dose), (ii) day 27 after challenge (day 11 after final RT dose), (iii) day 34 after challenge (day 18 after final RT dose). Panel C: Survival studies in Cre–/– and Cre+/– Argfl/fl littermates bearing day 10 3LL tumors left untreated (NT) or treated with 3 × 20 Gy focal irradiation over 3 days (RT). *P < 0.05, NS = not significant.
induced in macrophages by dying cells (36, 37), is a well-described feature of the postirradiation treatment tumor environment (38, 39) and suppresses adaptive immune function in tumors (40, 41). These cytokines can combine to generate extremely poor environments for functional adaptive immunity (42) and together with arginase I direct inflammatory resolution to establish a macrophage-directed wound repair environment in the treated tumor (43).

For these reasons, it may be more effective to broadly target macrophages rather than individual features of M2 differentiation. Preventing macrophage recruitment to the postirradiation environment by targeting Mac1 has been shown to improve the efficacy of radiation therapy (9), and macrophage depletion using small molecule inhibitors of the CSF1R improved tumor control with radiation therapy (10). However, these approaches also block potential positive effects of macrophages, since M1-differentiated macrophages may be needed to sustain auto-destructive T-cell-mediated immune responses (44) and can produce effector cytokines such as TNFα that can assist in tumor destruction after irradiation (45, 46). These data suggest that the immune environment after irradiation limits adaptive immune responses, but in keeping with other studies, in the absence of powerful immune therapies residual cancer cells can escape immune control. Further studies that more extensively track the onset and resolution of T-cell responses in treated tumors from a range of pathologies will be valuable to identify consistent patterns, markers and targets for immune modulation. Similarly, more extensive studies identifying patterns of macrophage differentiation in the tumor during inflammatory and resolution phases after radiation therapy could similarly provide new targets and determine the optimal timing for intervention. Strong adaptive immune responses result in influxes of IFN-γ-producing CD8+ T cells to the tumor (21). These data suggest that the immune balance in the tumor after treatment may influence the outcome of radiation therapy, and approaches that skew macrophage differentiation to prevent M2 responses (11) or prevent the macrophage influx after irradiation (9, 10) have the potential to improve adaptive immunity in the tumor and may synergize well with immunotherapies that target T cells.

Received: July 30, 2012; accepted: December 2, 2013; published online: 00 00, 00

**FIG. 4.** T cell activation in tumors in LysM-Cre Argfl/fl mice. Cre−/− and Cre+/− Argfl/fl littermates bearing d14 Panc02 tumors were left untreated or treated with 3 × 20 Gy focal irradiation over 3 days (RT). Panel A: Tumors were harvested 7 days after the last dose of radiation and analyzed for the proportion of tumor infiltrating, (i) CD8+ GranzymeB+ cells, (ii) CD4+CD25+FoxP3− cells and (iii) CD4+FoxP3+ cells. Panel B: Tumors treated and harvested, as in panel A, were analyzed by flow cytometry for CD3-zeta mean fluorescent intensity on tumor infiltrating CD8+ T cells. NS = not significant.
REFERENCES


