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Decreased survival after combining thoracic irradiation and an anti-PD-1 antibody is correlated with increased T cell infiltration into cardiac and lung tissues

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Abstract

Purpose—Lung cancer is the leading cause of cancer-related mortality in the United States. Radiation, a common component of treatment, can cause acute damage to critical organs including the lungs and the heart, but the serious toxicities from radiotherapy alone is relatively rare. A recent addition to the treatment regimen is immunotherapy, such as anti-PD-1 antibody, which blocks the inhibition of activated T cells. Combining anti-PD-1 treatment and thoracic radiation has potential for improving the outcomes of locally advanced lung cancer over traditional chemoradiation regimens, but the effect of combining these therapies on non-malignant lung tissue has not yet been investigated in preclinical models.

Materials and Methods—6–8 week old C57Bl/6 mice were treated with either anti-PD-1 antibody or control IgG with or without thoracic radiation (20Gy), and survival was monitored as an end point to determine any potential increase of toxicity from the combination therapy. Immune cell infiltration into the irradiated cardiac and lung tissues was analyzed via flow cytometry and histologically.

Results—At 21 days post-radiation, 70% of animals in the IgG + radiation group survived, significantly more than the anti-PD-1 + radiation group (36%; p=0.0169). T cell counts were significantly elevated in both cardiac and pulmonary tissues after combination therapy as compared to anti-PD-1 antibody alone (heart: 6.1 vs 22.4, p<0.001; lung 3.4 vs 20.8, p<0.001) or control IgG plus radiation (heart 11.3 vs 22.4, p<0.05; lung 12.2 vs 20.8, p<0.05) in flow cytometric studies. Histologic analysis confirmed this increase in the comparison of anti-PD-1 antibody alone versus antibody plus irradiation (heart: 464 vs 679 cells per field, p<0.001; lung:

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Combining immunotherapy and irradiation has the potential to greatly increase survival in cancer patients, but the effects of this combination on surrounding tissues has not been studied. We treated mice with either antibody or anti-PD-1 antibody, with or without thoracic irradiation, monitored survival, then analyzed immune cell infiltration into cardiac and lung tissues. Animals treated with both anti-PD-1 and thoracic irradiation had significantly decreased survival, and significantly increased infiltration of thoracic organs by CD3+ cells.

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780 vs 1109, p<0.001) and control IgG plus radiation or combination therapy (heart: 526 vs 679 cells per field, p<0.001; lung: 848 vs 1109, p<0.05).

Conclusions—Combining anti-PD-1 antibody and thoracic irradiation results in T cell infiltration into lung and heart tissue and increases mortality in a preclinical model. We conclude that healthy tissue damaged by irradiation is more susceptible to further damage by activated T cells.

Keywords

PD-1; thoracic irradiation; lung cancer

Introduction

Lung cancer is the leading cause of cancer-related mortality in the United States, with the American Cancer Society estimating that ~155,870 Americans will die of the disease in 2017(1). 85% of these are non-small cell lung cancers (NSCLC)(2,3), which has a 49% 5 year survival overall rate; this number drops to 1% for metastatic (stage IV) disease (1). Traditional treatments have centered around chemotherapy and radiation treatment, providing both systemic and local treatment for inoperable locally advanced lung cancer patients.

Although it can be an extremely effective therapy, thoracic radiation can have both acute and long-term detrimental effects to the highly radiation-sensitive lung tissue, in the form of pneumonitis and fibrosis, respectively, which can greatly affect post-treatment quality of life. Up to 15% of patients develop pneumonitis within 2–3 months of chest radiation (4,5). Evidence for an immunological component in radiation pneumonitis is strong: several groups have found that patients meeting radiological (evidence of pulmonary infiltrates on chest x ray) and clinical criteria respond positively to steroid treatment, with disease relapse on discontinuation of treatment (6). T cells constitute an important part of the immune cells infiltrating the lung tissue (7–11), and patients have elevated CD4/CD8 ratios in bronchoalveolar lavage fluid (6, 9–11). Importantly, this increase is also seen in animal models of radiation pneumonitis (12–14). However, it is still controversial whether cells from the innate and adaptive immune system directly contribute to radiation-induced tissue damage or only modulate disease progression. In this regard there is evidence from preclinical and clinical investigations that T cells constitute an important part of the immune cells infiltrating the lung tissue upon irradiation of the thoracic region (7–11).

In addition to pulmonary damage, cardiovascular effects such as pericardial effusion/carditis can be seen acutely after thoracic radiation (15–25). Although cancer survivorship has been improving over the last several decades, this cohort has to contend with a new source of potential health complications, including cardiovascular disease, which is now the second leading cause of death in this group (8). In particular, cardiac fibrosis and remodeling can cause extensive pathology that can severely limit cardiac function (26–27). Multiple kinds of cancer may be treated with thoracic radiation, and in many of these cohorts, increased post-radiation cardiovascular disease has been studied, including breast cancer (28–33), Hodgkins

lymphoma (34–35). As serious these complications are, their incidence is low in patients treated with radiation alone (36–37).

The relatively recent addition of immunotherapeutic treatment has proven immensely beneficial for cancer patients, including some with lung cancer. One target of immunotherapy is PD-1, a member of the same family as CTLA-4, which functions to regulate T cell activity by inhibiting activated T cells upon engaging its ligand, PD-L1. PD-L1 expression is upregulated on tumor cells, including breast (38–39), pancreatic (41), colorectal (39–40), ovarian (39, 42), brain (43), and lung cancers (39, 44–46). Isolation of tumor infiltrating lymphocytes has shown that these cells express increased levels of PD-1 (47), indicating that they are active, yet susceptible to the protective PD-L1 upregulation seen almost ubiquitously across tumor types. Clinical trials have shown the efficacy of both anti-PD-1 and anti-PD-L1 blocking antibodies in enhancing the anti-tumor activity of chemo- and radiation therapy, and several drugs have been FDA approved for lung cancer treatment, including atezolizumab (TECENTRIQ, Genentech Oncology), erlotinib (TARCEVA, Astellas Pharm), and nivolumab (Opdivo, Bristol-Myers Squibb). Pembrolizumab (Keytruda, Merck) has been approved as a first-line treatment for stage IV NSCLC with a high level of PDL1. PD1 inhibitors are being combined with thoracic RT to treat stage III NSCLC, in some studies with concurrent initiation of both therapies.

Although the efficacy of anti-PD-1 treatment is being heavily investigated, and the deleterious effects of radiation on thoracic organs has been well-established, the effect of combining these therapies on non-malignant lung tissue has not yet been investigated. Here, we provide evidence that the combination of anti-PD-1 antibody and thoracic irradiation results in T cell infiltration into lung and heart tissue that increases mortality in an animal model ubiquitous in the study of cancer.

Materials and methods

Animals

C57Bl/6 mice were and bred in the pathogen-free animal facility. All protocols were approved by the Institutional Animal Care And Use Committee (IACUC) and complied with the Guide for the Care and Use of Laboratory Animals. Commercially prepared food and water were provided without restriction.

Survival analysis

6–8 week old C57Bl/6 mice were pretreated with 200ug of either control IgG or anti-PD-1 antibody in 100uL PBS 4 days before irradiation, 2 days before irradiation, and just before irradiation. Full thoracic x ray irradiation was applied, with the head, neck, abdomen, and lower body shielded with a custom-designed lead cylinder. Five mice were irradiated in parallel. One irradiation provided 20Gy at 170cGy/min through a 1/4mm copper 1mm aluminum filter. Control animals were exposed to 0Gy. After radiation, mice were returned to a quarantine facility and monitored daily with weighing and behavior observation. They received booster injections of 100ug of antibody 3, 7, 10, 14, and 17 days post-irradiation to maintain circulating levels. Mice were followed until death or weight loss of >20%, at which

point they were sacrificed using carbon dioxide and death was confirmed with cervical dislocation, as per IACUC-approved protocols. Mice surviving until 21 days post-irradiation were sacrificed using carbon dioxide and death was confirmed with cervical dislocation.

Lung tissue analysis

Lung tissue was collected from sacrificed animals and ground through a 70um filter. The resulting suspension was rinsed with DMEM with 10% fetal bovine serum and placed on ice to await staining and flow cytometric analysis.

Cardiac tissue analysis

Cardiac tissue was collected from sacrificed animals, cut into 2–3mm slices, and ground through a 70um filter. Red blood cells were lysed. The resulting suspension was rinsed with DMEM with 10% fetal bovine serum and placed on ice to await staining and flow cytometric analysis.

Flow cytometry

All samples were pretreated with CD16/CD32 FcR blocker before staining. Cells were labeled with anti-mouse CD3. Staining was performed as per the manufacturer's protocols. Data was collected on a BD LSRII Flow Cytometer, and analyzed using FlowJo software.

Histological analysis and quantification

At the time of death, the lungs of each animal were collected for histological analysis, as previously described (48). At the time of death, the thorax and neck were dissected to expose the trachea and thoracic organs. The trachea was cannulated using a 22 gauge needle attached to rubber tubing filled with PBS, and the lungs allowed to fill via gravity. The lungs were then removed and placed in formalin. After removal of the lungs, the cardiac vasculature was flushed with PBS and the heart removed and placed in formalin. Whole organs were embedded in paraffin, and 5μm thick slices were mounted on slides. Slides were stained with anti-CD3 antibody, appropriate for paraffin-preserved samples, as per the manufacturer's protocols. Slides were photographed at 10× (heart) or 20× (lung) magnification and the number of positively staining cells calculated by blinded analysis.

Statistical analysis

Kaplan-Meier analysis was used to determine significant differences in survival. ANOVA with pairwise comparison was performed with Prism 5.0 software to accommodate multiple groups. Statistical significance was set at the level of $p < 0.05$.

Results

Combining thoracic radiation and T cell stimulation decreases survival

We first examined whether the combination of thoracic radiation and anti-PD-1 antibody would decrease survival as compared to irradiation and a control IgG antibody or treatment with anti-PD-1 antibody alone. Mice were pretreated with control or anti-PD-1 antibody and irradiated as described above. The first death was seen on day 4, in the IgG plus radiation

group; the first deaths in the anti-PD-1 plus radiation group were seen on day 7 (Figure 1). The last deaths were seen on day 12 and 14, respectively. At 21 days post-radiation, 100% of animals in the antibody-only groups were still alive. 70% of animals in the IgG + radiation group survived, significantly more than the anti-PD-1 + radiation group (36%; p=0.0169).

Combination immunotherapy and radiation significantly increases T cell influx into thoracic organs

Flow cytometric analysis of T cells isolated from cardiac tissue or lung tissue showed a significant increase in the number of immune cells present after treating animals with both radiation and anti-PD-1 antibody as compared to anti-PD-1 antibody alone (p=0.0003; p=0.0006) or control IgG and radiation (p=0.02; p=0.03). Anti-PD-1 alone did not significantly alter the T cell count in either organ over treatment with control IgG (Figure 2A). In the heart, the difference in infiltration was not different after control IgG and radiation as compared to control IgG alone, although a significant difference was seen in the lungs (p=0.02).

Histological analysis supports these results. The differences in the cardiac tissues (Figure 2B) were significant when comparing control IgG against anti-PD-1 (p=0.000003) or control IgG and radiation ($p=8.8\times10^{-11}$), or comparing anti-PD-1 antibody to anti-PD-1 plus radiation ($p=7.1\times10^{-10}$). Comparing both groups receiving radiation also revealed a significant difference (p=0.0000002). The differences in the lung tissues (Figure 2C) also revealed significant differences when comparing control IgG against anti-PD-1 (p=0.008) or control IgG and radiation (p=0.001), or comparing anti-PD-1 antibody to anti-PD-1 plus radiation (p=0.0009). Comparing both groups receiving radiation also revealed a significant difference (p=0.008).

Discussion

Cancer immunotherapy officially began in the early 1980s when the Rosenberg group used adoptive therapy to treat several different types of cancers, infusing lymphocytes and attempting to induce tumor regression (49). In the 30 years since, therapy has become much more specific and much more aggressive, in some cases resulting in impressive decreases in tumor growth. With the growing popularity of immunotherapy, many models have been developed to study how alteration of immune cell function can affect tumors. Combination immuno- and chemotherapy has been studied in a variety of cancers, and several studies combining immuno- and radiotherapy are underway, with promising preliminary results. This paper reflects the preliminary yet critical findings of increased mortality after immune cell infiltration in animals treated with a combination of radiation and anti-PD-1 blocking antibody, an immunostimulatory molecule.

Combining immunotherapy with traditional chemotherapy has been studied in many cancers, including bladder (50), esophageal (51), urothelial, hepatocellular (52), colorectal (53), and non-small cell lung cancer (54), with promising results reporting synergisitic effects but an accompanying increase in side effects. This may be due to the fact that both treatments are systemic, leading to nonspecific activity that can affect more than the target tissues. Radiation is a commonly used tool in the treatment of many cancers, and it can be

applied selectively to limit damage to non-cancerous tissue. Exploring the combination of radiation and immunotherapy is an exciting new area being studied in many tumor types, including melanomas (55), and breast (56–57), colorectal (58), pancreatic (59), prostate (60), and lung (61) cancers.

In addition to analyzing the efficacy of this treatment in tumors being directly irradiated, the combination of anti-PD-1 and radiation has been analyzed for abscopal anti-tumor effects, with a small number of patients showing decreased metastatic disease. Radiation is able to affect change at abscopal sites via its effects on the immune system. At the site of treatment, dead cells release damage-associated molecular patterns (DAMPs), such as ATP, which in turn activate local dendritic cells, increasing antigen presentation (62–63). Radiation can also increase the diversity of presented antigens (64) and localize macrophages to the tumor (65). However, these effects are primarily seen in immunostimulatory tumors such as renal cell carcinoma, melanoma, and hepatocellular cancer (66), and are dose- and method-dependent. For less immunogenic cancers, combining radiation with immunomodulatory molecules may provide the boost needed to see anticancer effects at distant sites. Preclinical data regarding the combination of radiation with IL-2, Flt3-L, TLR ligands (63), and CTLA-4 (67–68) have been published, with promising results. A limited number of studies have looked at treating lung cancer patients with radiation and GM-CSF (69) or ipilimumbab (66), but never before have the combination of anti-PD-1 and irradiation been examined (although Deng et al recently published on the combination of anti-PD-L1 antibody and radiation in the TUBO breast cancer model, 70).

This indicates that the effects of combining these treatments is not restricted to the target area (71–72). Although this has potentially beneficial effects for treating metastatic disease, it also puts non-targeted tissue at risk. Typical tumor regression models involve inoculation with tumor cells on a limb, providing easy access to the site for radiation, but lacking an assessment of how surrounding tissues are affected by irradiation. Ectopic models provide more accurate information regarding the role of the tumor microenvironment, as it is located in the tissue from which the tumor would normally develop, but these models are notoriously difficult to replicate in a uniform manner. Thus, most preliminary studies are conducted in orthotopic models, but further exploration is necessary before parallels can be drawn between the model system and it clinical applications. Our lab’s primary interest is lung cancer; therefore, we explored the consequences of combined thoracic radiation and anti-PD-1 antibody to understand how non-malignant tissue may be inadvertently affected during the treatment of lung cancer.

Analysis of damage to non-target tissue has not been reported in these studies. With the growing popularity of immunotherapy, many models have been developed to study how the alteration of immune cell function can affect tumors. We explored the consequences of combined thoracic radiation and anti-PD-1 antibody to understand how healthy tissue may be inadvertently affected during the treatment of lung cancer.

Both radiation (73) and anti-PD-1 antibody (74) given alone have been shown to cause acute pneumonitis in lung cancer patients. Thankfully, most patients with lung cancer will not experience this complication, but for the 10–15% of patients that do, the consequences can

be devastating, negatively impacting quality and length of life. In addition, irradiation is known to cause both long- and short-term cardiac damage (75); the effect of anti-PD-1 on cardiac function has not been studied. Analysis of damage to cardiac and pulmonary non-target tissue has not been reported in the abovementioned combination treatment studies.

In our study, animals with no tumor burden showed increased mortality when given a combination of anti-PD-1 and total thoracic irradiation (a single dose of 20Gy, an easily reproducible dose over that typically described as causing mortality in mice). Dosing and strength were optimized for survival to be used as an end point in order to demonstrate a difference in toxicities. The choice of C57Bl/6 mice, typically known for being fibrosis-prone, for these experiments was a result of much research demonstrating physiological (76), cytologic (77–81), and pathological (76, 78, 82–83) evidence of acute pneumonitis in this model in the setting of thoracic irradiation. These animals are prone to developing Th1-weighted responses, and, as the T cell population is weighted towards this response in acute radiation pneumonitis (84), they are indeed an appropriate model for this study. In addition, modeling this effect in animals that require more intervention to develop this response lends weight to the importance and universality of this finding. At 21 days post-radiation, 70% of animals in the IgG + radiation group survived (Figure 1), significantly more than the anti-PD-1 + radiation group (36%; p=0.0169).

Limited analysis of breath rate and ejection fraction differences between these groups provide preliminary evidence that, with the accumulation of activated immune cells, organ function is compromised (data not shown). In addition, we found that cardiac-targeted irradiation does not decrease survival as dramatically as total thoracic irradiation (data not shown), indicating that the significantly decreased survival is due to damage to multiple organs. The consequences of this finding are critical, as patients undergoing combination therapy are at higher risk from treatment-induced pathology. Understanding the origin of this phenomenon is key to specifically targeting cancer cells while inducing as little unnecessary damage as possible.

T cell counts were significantly elevated in both cardiac and pulmonary tissues after combination therapy as compared to treatment with radiation alone, indicating that, while prolonging the action of immune cells may enhance their anti-tumor activity, non-malignant tissue damaged by irradiation is susceptible to accumulation of and further damage by activated T cells. Flow cytometric and histological analysis of lung and cardiac tissue showed a significant increase in the number of immune cells present after treating animals with both radiation and anti-PD-1 antibody (Figure 2). Simply the presence of these cells undoubtedly affected organ efficiency, as infiltrates of any type are known to interfere with normal functioning. Immune cell infiltrates can be particularly damaging, as activated cells can cause damage beyond disruption of the normal structure and connections, including destruction of tissue. Early studies of PD-1 function, using knockout mice to assess its function, show that animals develop immune infiltrates that cause premature mortality. This is the first report of such a finding in an experimental treatment model, indicating that collateral damage induced when normal tissue is exposed to anti-tumor treatments has the potential to negatively affect patient. Preliminary data (not shown) from these infiltrates indicated a higher level of IFNγ expression in T cells isolated from animals in the

combination treatment group. Further analysis of these cells' functionality will be critical to future studies.

The next step is to understand which cell types are responsible for this damage, including a more in-depth analysis of differences between the T cell population in animals that survived radiation alone or combination therapy. Understanding differences in cytokine production and proportional representation of cell subtypes will allow us to explore the influence of combination therapy in particular on these cells. Once a determination has been made as to what T cell subtype(s) is/are responsible for this damage, and whether they are the same types active in the anti-tumor response, alterations in the therapeutic regime designed to protect non-targeted tissue can be more thoroughly explored.

Our study has a few limitations, namely the use of only one strain of mice. We have limited preliminary findings in Balb/c mice showing the same effect, but further exploration of this nature in a variety of genetic backgrounds will shed more light on this phenomenon. In addition, the radiation dose chosen for these experiments is not directly comparable to that which would be used in a clinical setting. 20Gy allowed us to use mortality as the endpoint, whereas translation of these experiments to the clinic would assess morbidity.

There are currently over 60 studies combining radiation and anti-PD-1 therapy in various stages of development, including 12 currently recruiting patients with lung cancer (no lung cancer studies have begun treatments or data collection). None have yet published data on efficacy or complications. Our experiments are attempting to foresee complications that might arise from applying these treatments at the same time, particularly in light of their known complications individually. We are of course limited by variables that exist in the clinic and may have an effect on the development of cardiopulmonary complications, such as genetic variations, COPD, underlying heart disease, or overall functional status, but seeing these effects in animals that have overall healthy tissues makes it even more likely that they will be seen in patients with significant comorbidities. It is our hope that our findings will provide some insight as to potential complications of treatment and, after further study, may help to guide the development of treatment protocols to minimize complications.

In conclusion, although the combination of radiation and immunotherapeutic treatments has the potential to greatly decrease tumor burden and increase survival in lung cancer patients, healthy tissues may also be affected to the extent that the treatment may be as bad as the disease. A more extensive understanding of the mechanisms underlying these findings may shed light on how to best decrease tumor burden with a minimum amount of collateral damage.

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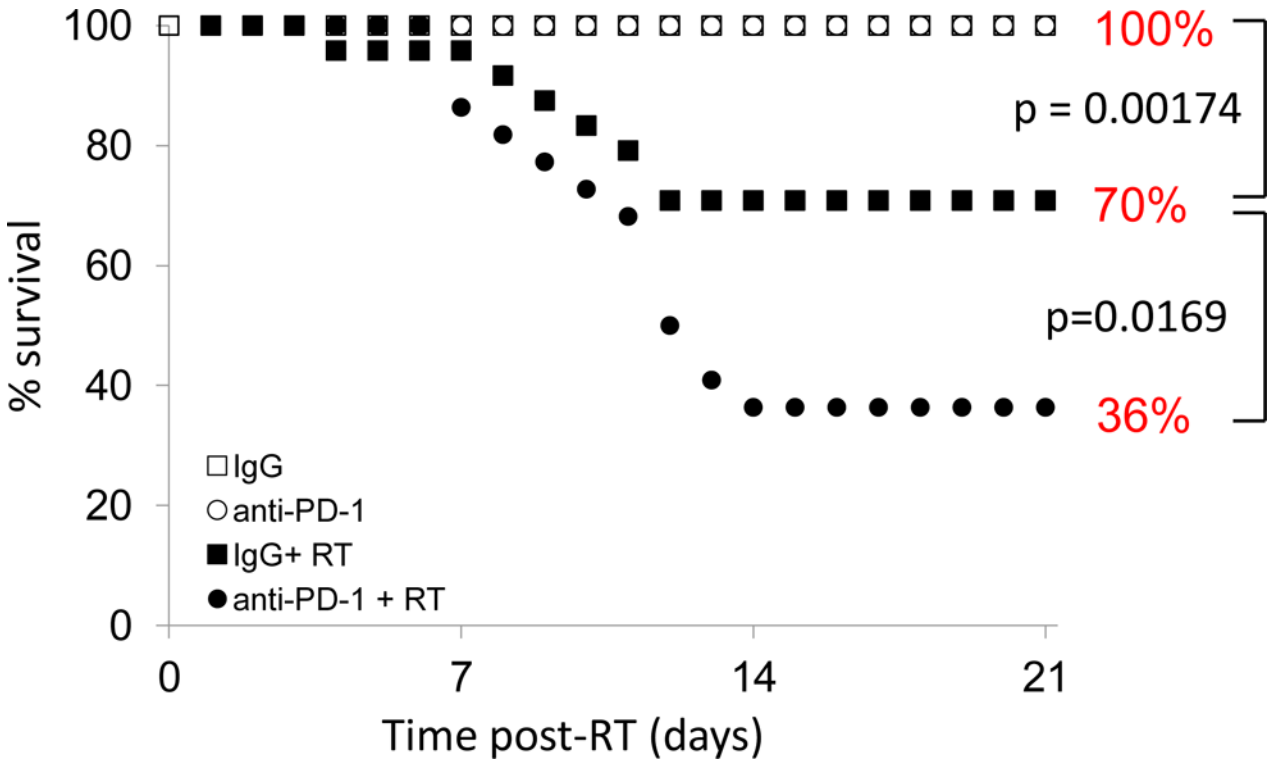

Figure 1.

Anti-PD-1 antibody decreases survival after thoracic radiation. Combining anti-PD-1 antibody and 20Gy irradiation significantly decreases survival. IgG + RT first death: day 4. Anti-PD-1 + RT first death: day 7. Day 5 survival: IgG + RT 96%, all other groups 100%. n=20–25 animals per group.

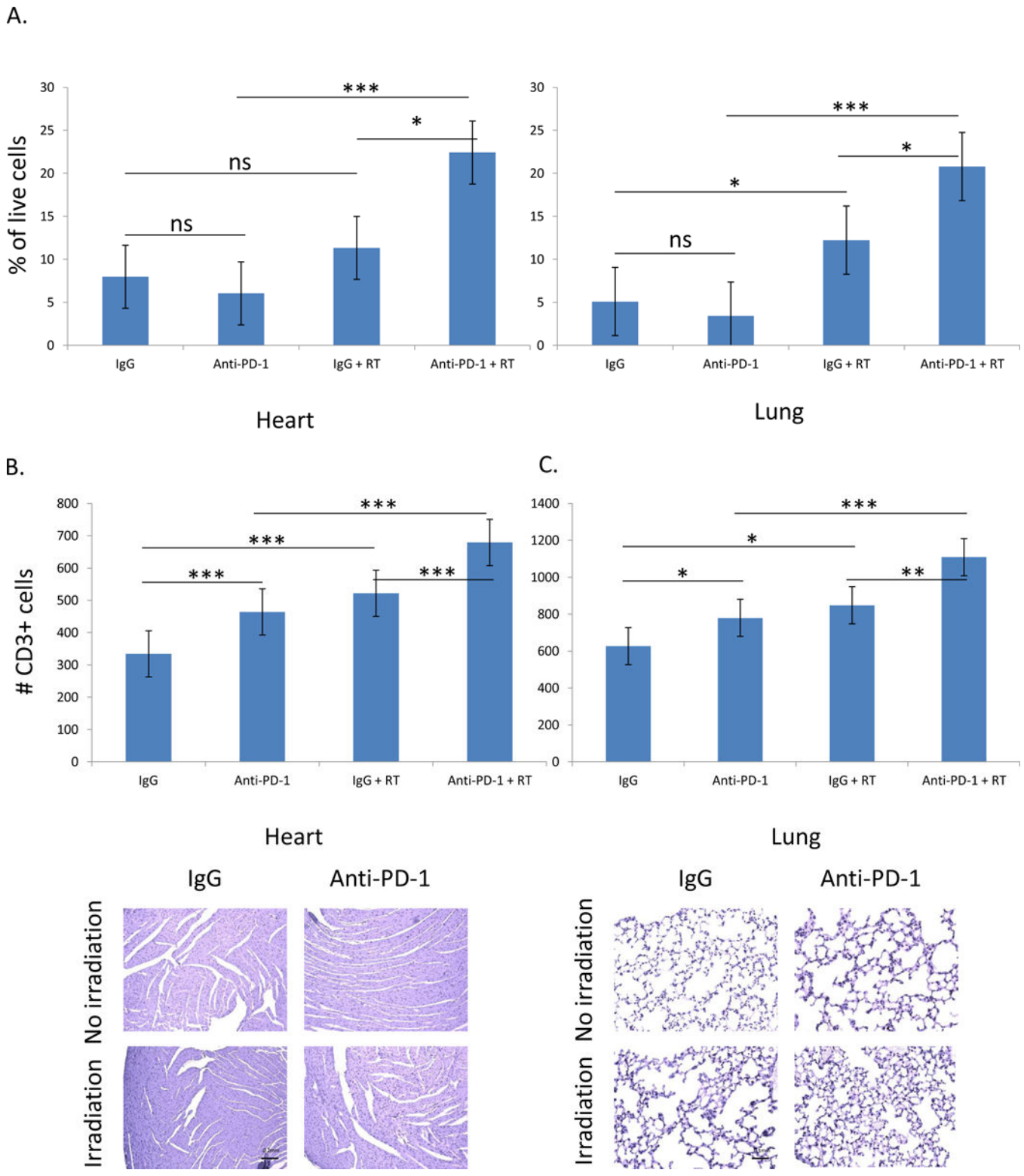

Figure 2.

CD3+ cells are significantly increased in the heart and lungs of animals receiving immunotherapy and thoracic irradiation. A. Combining irradiation and anti-PD-1

significantly increase the number of T cells isolated from heart and lung tissue. Irradiation alone significantly increased the number of T cells in the lung. Error bars represent standard error. B and C. Analysis of CD3+ stained samples showed a significant increase in T cells in both the heart and lungs after treatment with radiation, with anti-PD-1 antibody, and with combination treatment. Three views of each sample, 10 samples per group, were analyzed by a blinded observer. Analysis of heart (B) and lung (C) tissue involved calculation of the average number of cells per field and comparison between groups. Representative samples of tissues collected from each treatment group are shown below. *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$