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**ORIGINAL ARTICLE**

# Experimental investigation of skin toxicity after immune checkpoint inhibition in combination with radiation therapy

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## Abstract

**Immune checkpoint inhibitors (ICIs) have revolutionized cancer therapy. However, structured knowledge to mitigate a patient's specific risk of developing adverse events are limited. Nevertheless, there is an exponential growth of clinical studies combining conventional therapies such as radiation therapy (RT) with ICIs. Cutaneous reactions are among the most common adverse events after monotherapy with either ICIs or RT. So far, little is known about interindividual differences for the risk of developing severe tissue toxicity after the combination of RT with ICIs, and the underlying biological mechanisms are ill defined. We used experimental models of RT-induced skin injury to analyze skin toxicity after simultaneous application of ICIs. We compared different RT regimens such as fractionated or stereotactic RT with varying dose intensity. Strikingly, we found that simultaneous application of RT and ICIs did not significantly aggravate acute skin injury in two different mouse strains. Detailed examination of long-term tissue damage of the skin revealed similar signs of epidermal hyperplasia, dermal fibrosis, and adnexal atrophy. In summary, we here present the first experimental study demonstrating the excellent safety profiles of concurrent treatment with RT and ICIs. These findings will help to interpret the development of adverse events of the skin after radioimmunotherapy and guide the design of new clinical trials and clinical decision-making in individual cases.**


**Keywords:** immune checkpoint inhibition; radiation therapy; immune-related adverse events; skin toxicity; radiodermatitis; epidermal hyperplasia; dermal fibrosis; adnexal atrophy

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## Introduction

Approximately two-thirds of all cancer patients undergo radiation therapy (RT) at some point during their course of illness. While RT is highly effective in treating local tumors, it is primarily not intended or effective in curing metastatic disease [1]. However, rare observations describe regression of distant metastasis outside the RT area of a primary tumor site. Termed the abscopal effect (AE), this immune-mediated response can be boosted by combining RT with immune checkpoint inhibitors (ICIs) [2,3]. ICIs target different immune-inhibitory receptor

systems (e.g. PD-1 or CTLA-4), thereby increasing expansion and cytolytic function of tumor-specific T cells. Since their first approval in 2011, the number of different combination therapy regimens with ICIs has increased rapidly [4]. The combination of RT with ICIs in particular promise high synergistic potential, ultimately leading to priming of tumor-specific T cells, and development of potent antitumor immune responses. These expectations resulted in an exponential increase of clinical studies combining RT with ICIs [1–3,5]. First and foremost, patients suffering from oligometastatic disease—defined by a limited number of metastases—have benefitted from this combination therapy, and such patients now even stand a chance of cancer cure [6,7].

While RT and ICIs can work hand-in-hand, the greatest limitation to their combination is the potential risk of enhanced or even unknown toxicity [5]. Considering both treatment components individually, many studies have addressed the various side effects of RT. Irradiated tumor cells undergo programmed cell death caused by DNA double-strand breaks and formation of reactive oxygen species, a process resulting in local tissue inflammation in the tumor microenvironment [5,8,9]. In the tumor-adjacent healthy tissue, changes in chemokine expression after RT plays an important role in the recruitment of different immune cells (e.g. T cells) and the release of inflammatory cytokines (e.g. interleukin-6 (IL-6), tumour necrosis factor (TNF), or transforming growth factor (TGF)-β), which contribute to local tissue inflammation. This process is the basis for acute side effects after RT as well as long-term tissue damage due to chronic inflammation or tissue remodeling [5]. Importantly, both the beneficial immune-stimulating effects of RT and the severity of toxic effects are highly dependent on the total radiation dose, the dose per fraction, and the volume of irradiated tissue. There are several strategies to reduce healthy tissue toxicity. Splitting the overall dose of RT into many small fractions (fractionated RT) is a historically applied method to reduce radiogenic side effects. Modern techniques of image-guided and stereotactic RT enable high-precision irradiation of target volumes with a high dosage per single fraction or even without fractionation (radiosurgery). Both techniques, fractionated RT and stereotactic RT, can significantly prevent side effects and are therefore commonly used in patient care [10,11]. In contrast, much less is known about the biological processes leading to ICI-induced toxicity, and ongoing investigations address the mechanisms resulting in immune-related adverse events (irAEs) [2,12]. ICIs act systemically and can induce undesired effects in all organs, but most commonly affect the lungs, intestines, glands, and skin [12,13]. Many frequent adverse events after RT or ICIs are not life-threatening but often reduce a patient's quality of life, therapy compliance, and severe cases can necessitate termination of treatment [14,15].

Up to 95% of all patients experience some grade of cutaneous reaction during RT; therefore, skin toxicity might be the most important limitation to this type of therapy [10,14,16]. Irradiation harms the highly proliferative stem/progenitor cells in the basal layer of the skin, leading to a dysfunction of the epithelial protective barrier [17]. Acute damage symptoms of RT-induced skin injury (radiodermatitis) range from hair loss, erythema, dry or moist desquamation to ulceration, and necrosis. Chronic damage driven by the remodeling of the extracellular matrix via TGFβ signaling can lead to development of skin fibrosis as well as epidermal hyperplasia and adnexal atrophy, even months after treatment, impairing patients' quality of life [5,10,14,18,19]. Dermatologic toxicity is also one of the most common irAEs after ICI application [12]. Skin irAEs are observed in 47–68% of all anti-CTLA-4-treated cancer patients and in 20–40% of all anti-PD-1/PD-L1-treated patients [20,21]. Importantly, the combination of anti-PD-1 and anti-CTLA-4 generally results in increased rates of irAEs [20,21]. It is unclear whether and how the biological mechanisms leading to both individual toxicities interact and possibly aggravate during a combination of RT and ICIs [22]. Of note, previous studies found that combination therapy of RT with targeted therapies (e.g. BRAF inhibitors) significantly increased skin toxicity [15,23]. In general, clinicians worry about enhanced toxicity after a combination of RT with additional systemic therapies (e.g. ICIs), and ongoing studies aim to create a better understanding to evaluate the risk for such side effects [22,24,25]. Importantly, therapy interruption and delay due to combinatorial toxicity impose the risk of systemic tumor progression during the time of localized RT, and reduce the chance to induce a systemic antitumor immune response.

Utilizing different RT regimens with or without concurrent ICI treatment, we studied the development of acute injury and long-term tissue damage of the skin in detail, taking advantage of an experimental and controlled preclinical system without patient-related confounders.

## Materials and methods

### Mice and animal studies

All animal experiments were approved by the local governmental authorities (Regierung von Oberbayern, Munich, Germany) and conducted according to the guidelines of the study protocol (55.2-2532.Vet_02-19-22) to ensure animal welfare.

Five-week-old, female C57BL/6J and Balb/c mice were purchased from Charles River Laboratories (Research Models and Services, Germany GmbH, Sulzfeld, Baden-Württemberg, Germany) and acclimatized for 1 week before the start of the experiments. Mice were kept in individually ventilated cages and had access to food and water *ad libitum*.

### Radiation therapy

Mice were anesthetized with an intraperitoneal (i.p.) injection of medetomidin (0.5 mg/kg), midazolam (5 mg/kg), and fentanyl (0.05 mg/kg) and were fixed

on their back on a plastic disc before irradiation. Afterwards, the central part of the right thorax (area of 1 cm × 1 cm, Figure 1B) of the mice were treated with a single dose (15 or 30 Gy) of RT or fractionated RT on 5 consecutive days (9 Gy per day, cumulative dose of 45 Gy). Lead plates (3 mm) were used to shield the rest of the body from radiation. RT was performed using the Gulmay RS225A (Gulmay Medical, Camberley, Surrey, UK) at a dose rate of 0.95 Gy/min (15 mA, 200 kV). Biological effective doses (BEDs) of different RT regimens were calculated using an $\alpha/\beta$ ratio of 10 [26]. Experiments were performed with RT regimens with three different BEDs to address the question of the dose-specific effects. 1 × 15 Gy equals a low BED of 37.5 Gy, 5 × 9 Gy equals a medium BED of 85.5 Gy and 1 × 30 Gy equals a high BED of 120 Gy.

### Immunotherapy with immune checkpoint inhibitors

Immunotherapy with ICIs was performed according to treatment protocols adapted from previous reports [27]. The indicated experimental groups received i.p. injections with anti-PD-1 (250 µg, clone RMP1-14, Bio X Cell, West Lebanon, NH, USA) and anti-CTLA-4 (250 µg, clone 9H10, Bio X Cell) in a total volume of 300 µl phosphate-buffered saline (PBS) on days 0, 7, 14, and 21 after the start of RT (Figure 1A). Control mice were injected with 300 µl PBS i.p. without ICIs.

### Scoring of acute skin injury

Scoring of acute skin injury was performed in line with previous studies [28]. In brief, acute skin injury was evaluated using an ordinal scale-ranked score from 0 (no injury) to 9 (skin necrosis and ulceration) points. Details of the clinical score are presented in Figure 1C and representative pictures to each score are depicted in Figure 1D. Mice were scored at least once per week for 4 months until the end of the experiments. The experiments presented in Figure 1H–J were performed fully blinded.

### Skin tissue sample preparation

Mice were euthanized and the chest area was shaved. Afterwards, shaved skin samples of the right thorax (area of RT) and the left thorax (control area that did not receive RT) were obtained from each mouse.

### Histopathological analysis and long-term skin damage

Skin samples were fixed in 4% formaldehyde for at least 24 h and embedded in paraffin wax. Standard hematoxylin and eosin (H&E) staining protocols were used for histopathological analysis of tissue sections. Long-term damage of the epidermis (grading of epidermal thickness investigating signs of hyperplasia), dermal connective tissue (grading of pathological compaction of connective tissue indicating signs of fibrosis) and skin adnexa (grading of loss of density indicating adnexal atrophy) were evaluated individually with a semiqualitative score (0 = no damage; 1 = moderate damage; 2 = severe damage). The size of the affected area was analyzed with a semiquantitative score (0 = 0% damaged tissue; 0.5 = less than 10% damaged tissue; 1.0 = 10–50% damaged tissue; 1.5 = more than 50–90% damaged tissue; 2.0 = more than 90% damaged tissue). Scores of the grading of the damage and the size of the affected area were summed to a pooled damage score with a range from 0 to 4 points. Additionally, we calculated an overall damage score (from 0 to 12 points) of each skin sample, which included the cumulative scores of the epidermis, dermal connective tissue, and skin adnexa. The assessment of long-term skin damage was blinded.

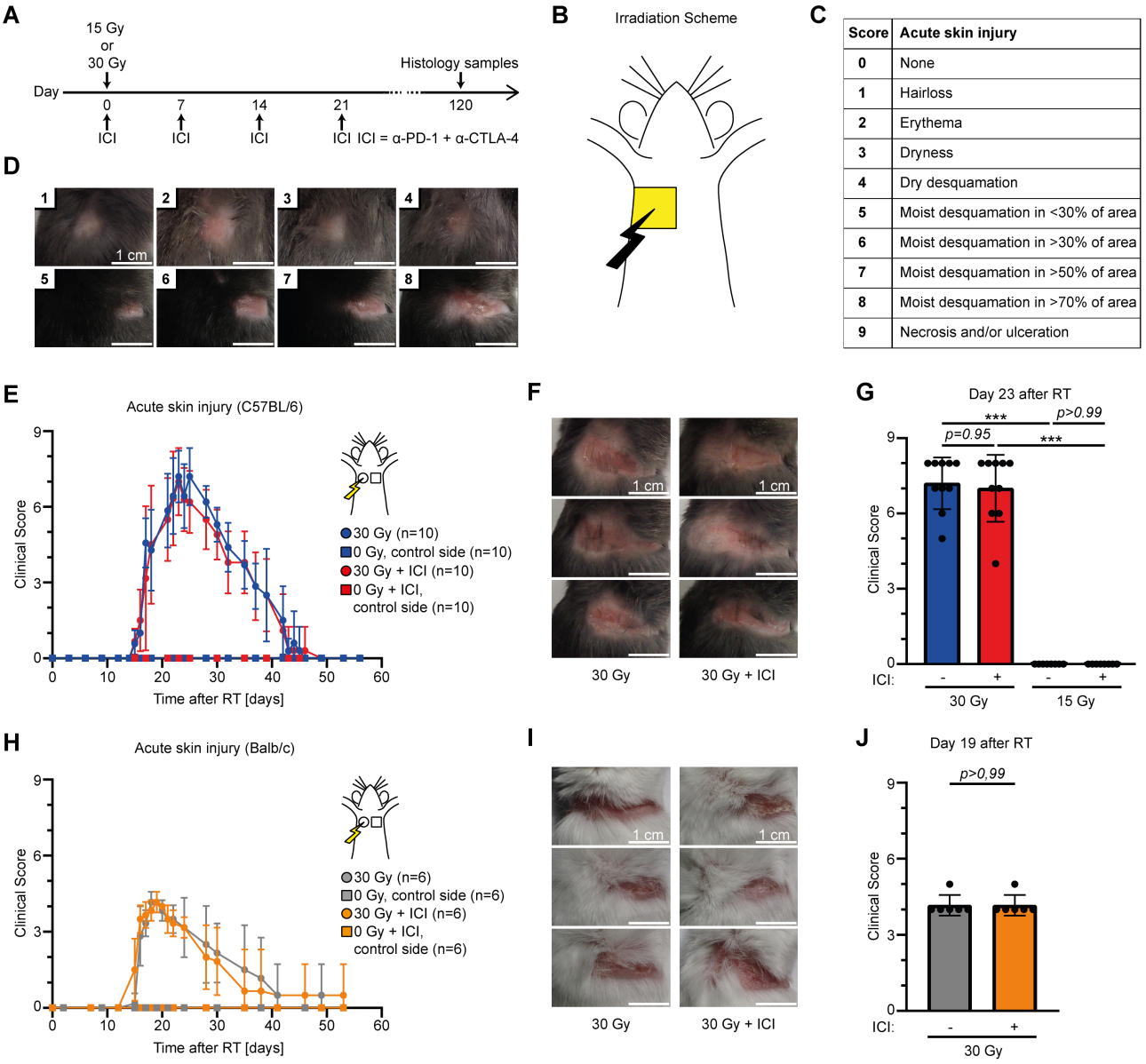
### Statistics

All data are presented as the mean ± standard deviation (SD) from two or three independent experiments, as indicated in the figure legends, which also depict the animal numbers per group ($n$). Statistical analyses were performed using GraphPad Prism v. 9.3.0 (GraphPad Software, San Diego, CA, USA). Differences between means of experimental groups were analyzed using ordinary one-way analysis of variance (ANOVA) for multiple comparisons or unpaired $t$-test if only two groups were compared. Significance was set at $p < 0.05$, $p < 0.01$, and $p < 0.001$ (respectively *, **, and ***). All $p$ values >0.05 are shown in the figures.

## Results

### Dual immune checkpoint inhibition does not exacerbate acute skin injury after unfractionated RT

By simultaneously applying RT in combination with an intensive regimen of dual immune checkpoint inhibition, we hypothesized provoking enhanced skin toxicity (Figure 1A). In our experimental model, C57BL/6 mice were treated with RT to a defined area of the right thorax, while the left side of the chest served as the nonirradiated control (Figure 1B). Acute skin reactions were evaluated using a scoring system of clinical signs of RT-induced skin injury (Figure 1C,D). Mice treated with a high dose of RT (30 Gy) showed first the signs of visible skin reactions 15 days after RT (Figure 1E). Starting with a small area of hair loss, clinical signs of skin injury with erythema, dryness, and desquamation progressed rapidly and peaked on day 23 after RT with severe signs of toxicity (Figure 1F,G). Strikingly, mice that received additional treatment with anti-PD-1 and anti-CTLA-4 did not show significantly increased skin toxicity at day 23 after RT or at any timepoint during the acute phase of disease (Figure 1E,G). All mice from all experimental groups underwent a complete healing process, and the nonirradiated side of the chest never showed any clinical signs of acute skin injury. We concluded that the simultaneous combination of ICIs with a high dose of




| Score | Acute skin injury |
|---|---|
| 0 | None |
| 1 | Hairloss |
| 2 | Erythema |
| 3 | Dryness |
| 4 | Dry desquamation |
| 5 | Moist desquamation in <30% of area |
| 6 | Moist desquamation in >30% of area |
| 7 | Moist desquamation in >50% of area |
| 8 | Moist desquamation in >70% of area |
| 9 | Necrosis and/or ulceration |

**Figure 1.** Dual immune checkpoint inhibition does not exacerbate acute skin injury after unfractionated RT. (A,B) The schemes show the experimental set-up of the following experiments. The right thorax of C57BL/6 or Balb/c mice was irradiated with a single dose of either 15 or 30 Gy. Selected experimental groups received an additional treatment with anti-PD-1 and anti-CTLA-4 at the indicated timepoints. (C) The table presents the scoring system for evaluating clinical signs of acute skin injury. (D) Representative images of skin injuries corresponding to each score in C57BL/6 mice. A score of 9 was never observed in this experimental setting. Scale bar, 1 cm. (E) Development of acute skin injury after 30 Gy RT and additional treatment with ICIs. C57BL/6 mice were scored for acute skin injury on the irradiated chest as well as on the unirradiated control side. Pooled data from three independent experiments with 10 mice per group. (F) Images of acute skin injury on day 23 after 30 Gy RT ± ICIs of one representative experiment. Scale bar, 1 cm. All images were processed with ImageJ 1.53k (NIH, Bethesda, MD, USA) to enhance the brightness. (G) Acute skin injury after 30 Gy RT or 15 Gy RT ± ICIs on day 23 after RT (day of peak acute skin injury). Pooled data of four independent experiments including three experiments evaluating RT with 30 Gy (10 mice per group) and one experiment with 15 Gy (8 mice per group). (H) Development of acute skin injury of the irradiated chest and the unirradiated control side after 30 Gy RT ± ICIs in Balb/c mice. Pooled data from two independent experiments with six mice per group. (I) Representative images of acute skin injury on day 19 after the start of RT with 30 Gy ± ICIs of one representative experiment in Balb/c. Scale bar = 1 cm. (J) Acute skin injury after 30 RT ± ICIs on day 19 in Balb/c mice (day of peak acute skin injury). All data are shown as mean ± SD. For statistical analysis, one-way ANOVA with multiple comparisons or unpaired *t*-test was performed. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

unfractionated RT does not significantly exacerbate severe signs of RT-induced skin injury. It is known that experimental models of inflammatory tissue toxicities (e.g. colitis) can be exacerbated by simultaneous immune checkpoint inhibition, although ICIs alone do not induce such irAEs in these models. [29]. Therefore, we performed RT with a lower dose and questioned whether ICIs would induce enhanced development of RT-induced skin injuries. Mice undergoing RT with only 15 Gy did not develop any clinical signs of acute skin injury (supplementary material, Figure S1A). Importantly, combining ICIs and 15 Gy RT also did

not induce skin injury (Figure 1G and supplementary material, Figure S1A). Finally, we conducted additional experiments in Balb/c mice to address the possibility of mouse strain-specific differences. We observed the first visible reactions on day 15 after the start of RT with 30 Gy (Figure 1H). Skin reactions peaked on day 19, and subsequently underwent a smooth healing process (Figure 1H). Again, the unirradiated control side did not show any signs of acute skin injury. We did not find significantly enhanced signs of acute skin toxicity after combining RT with ICIs (Figure 1H–J). Based on these findings in two different mouse strains, we conclude that concurrent combination of ICIs with a single fraction of RT does not promote development of increased skin injury.

## Dual immune checkpoint inhibition does not aggravate long-term skin damage after unfractionated RT

Healthy skin in mice is characterized by a physiological architecture of the epidermis, dermal connective tissue, and the adnexa. With respect to the rodent skin of the wall of the thorax, the latter is equivalent with the pilosebaceous unit, which comprises the hair follicle, its associated sebaceous glands, and the arrector pili muscle. In the skin of untreated animals, the adnexal structures appear with high density (Figure 2A). Using histopathological analysis, we evaluated signs of long-term skin damage 4 months after exposure to RT and ICIs in C57BL/6 mice (Figure 1A). Increased epidermal thickness (due to epidermal hyperplasia), a more compact appearance of the dermal connective tissue (signs of fibrosis), and reduced density of skin adnexa was scored considering the degree of tissue damage and the extent of the affected area. As expected, we observed signs of severe skin damage after RT with 30 Gy, whereas nonirradiated skin from the same mice was unaffected (Figure 2B). Importantly, concomitant application of ICIs did not aggravate overall skin toxicity (Figure 2B). In detail, RT and its combination with ICIs increased thickness of the epidermis to a similar degree (Figure 2C,D). In this regard, we did not observe significant differences in either grading of tissue damage or the size of the affected epidermal area

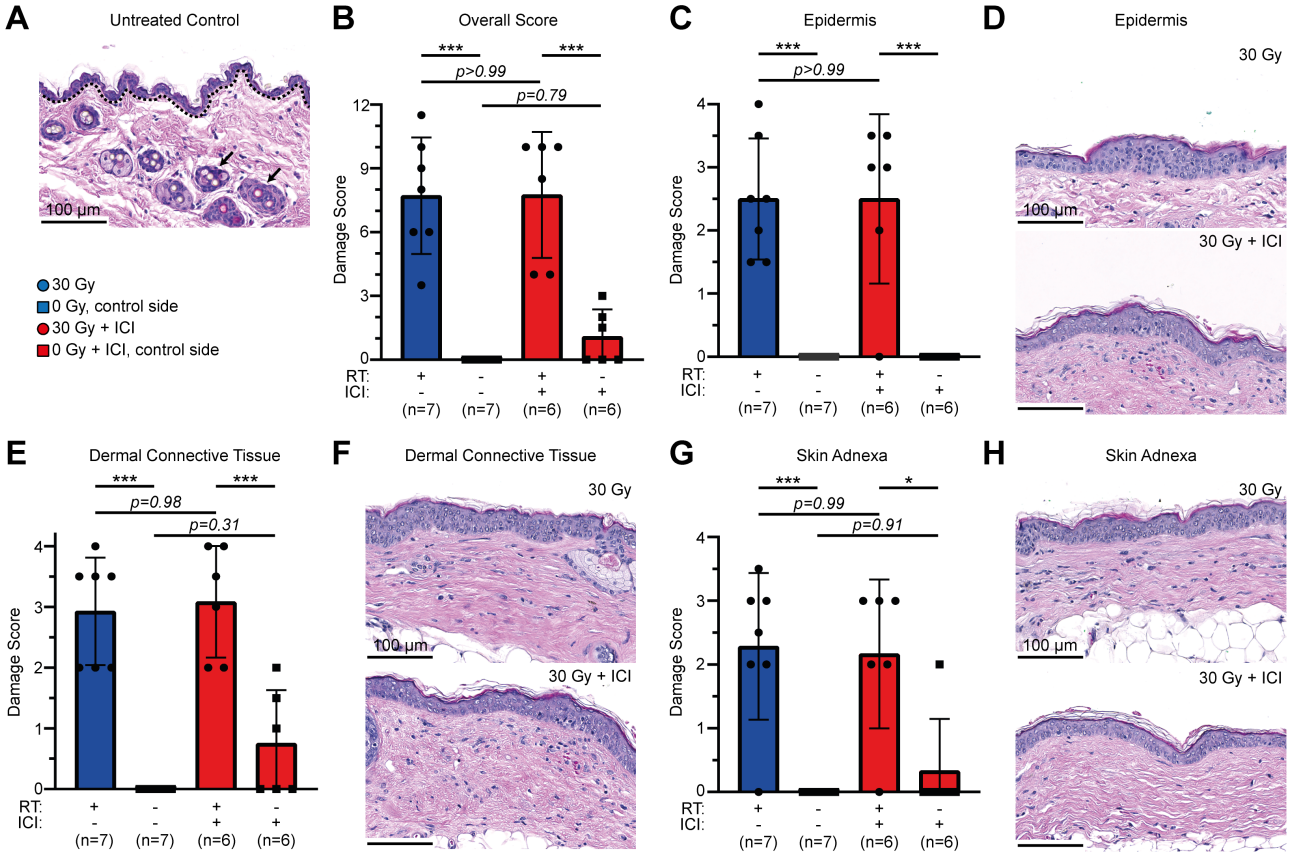



**Figure 2.** Dual immune checkpoint inhibition does not aggravate long-term skin damage after unfractionated RT. Skin damage of C57BL/6 mice was evaluated via histopathological analysis of H&E staining 4 months after 30 Gy RT ± treatment with ICIs. Skin samples of the irradiated area as well as the control side were obtained on day 120 after RT. (A) H&E staining of skin tissue derived from an untreated control mouse. The dashed line differentiates epidermal cell layer and underlying dermis consisting of connective tissue and adnexa (arrows). (B) Overall damage score (whole skin) comprising cumulative scores for the epidermis, dermal connective tissue, and skin adnexa. (C,D) Individual scores and representative images of epidermal hyperplasia, (E and F) dermal fibrosis and (G,H) adnexal atrophy. Scale bar, 100 µm. Data show mean ± SD and are pooled from two independent experiments with six or seven mice per group. For statistical analysis, one-way ANOVA with multiple comparisons was performed. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

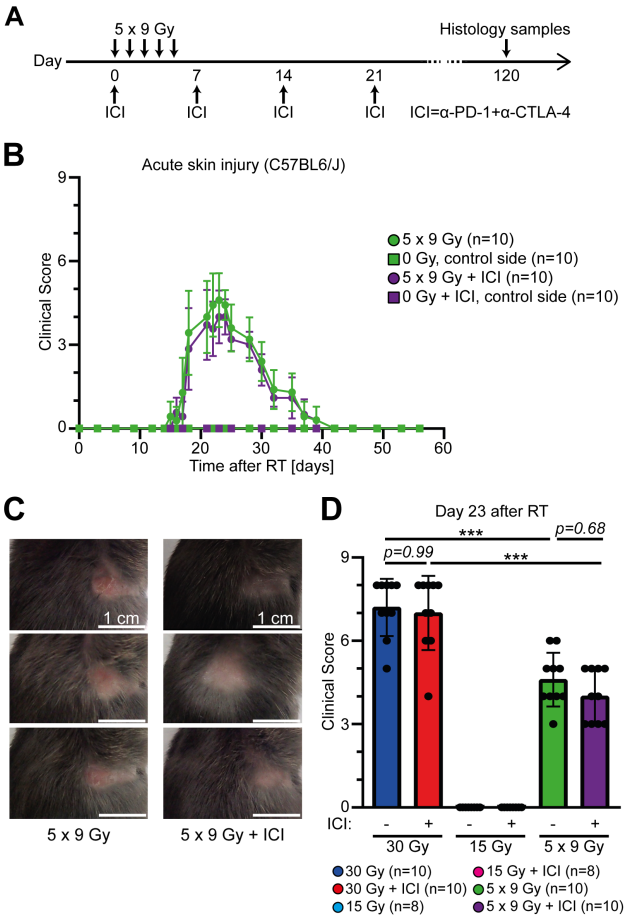



**Figure 3.** Fractionated radiation therapy significantly lowers development of acute skin injury independent of simultaneous immune checkpoint inhibitor treatment. (A) Experimental set-up of fractionated RT with 5 × 9 Gy and treatment with ICIs in C57BL/6 mice. (B) Clinical scores of acute skin injury after fractionated RT ± ICIs. Mice were scored for acute skin injury on the irradiated chest as well as on the unirradiated control side. Data show mean ± SD. (C) Images of acute skin injury on day 23 after fractionated RT ± ICIs of one representative experiment. Scale bar, 1 cm. All images were processed with ImageJ 1.53k to enhance the brightness. (D) Acute skin injury after 30 Gy RT or 15 Gy or 5 × 9 Gy RT in combination with ICIs on day 23 after RT (day of peak acute skin injury). Data are shown as mean ± SD and pooled from four independent experiments including three experiments evaluating RT with 30 Gy and 5 × 9 Gy, and one experiment with 15 Gy. For statistical analysis, one-way ANOVA with multiple comparisons was performed. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

(supplementary material, Figure S2A). Consistent with these observations, grading and size of the dermal areas showing signs of fibrosis were comparable in mice treated with RT or its combination with ICIs (Figure 2E,F and supplementary material, Figure S2B). Furthermore, concerning therapy-induced loss of skin adnexa (adnexal atrophy), we found similar grades of density reduction and affected area in the skin of animals within both experimental groups (Figure 2G,H and supplementary material, Figure S2C). In summary, these data demonstrate that a combination of RT and ICIs does not result in increased long-term skin damage.

## Fractionated radiation therapy significantly lowers development of acute skin injury independent of simultaneous immune checkpoint inhibitor treatment

Fractionation of the overall RT dosage is a highly relevant clinical strategy to reduce tissue toxicity such as radiation-induced skin injury. Therefore, we assessed possible risks of increased skin damage after application of ICIs combined with fractionated RT with a total dose of 45 Gy, divided into five consecutive daily fractions (Figure 3A). Acute skin reactions after fractionated RT showed similar spatiotemporal progression as that after unfractionated 30 Gy RT, starting at day 15 and peaking on day 23 after RT (Figure 3B). Again, the nonirradiated left side of the chest did not show any signs of toxicity (Figure 3B). As expected, fractionated RT with 45 Gy resulted in significantly less severe acute skin injury compared to unfractionated RT with 30 Gy (Figure 3D). Importantly, and in agreement with our results on unfractionated RT, the combination of ICIs with fractionated RT did not exacerbate acute skin injury (Figure 3B–D).

## Concomitant dual immune checkpoint inhibition does not aggravate long-term skin damage after fractionated RT

Again, we observed that a combination of ICIs with RT, in this case fractionated RT, did not aggravate the overall histopathological scores of skin toxicity (Figure 4A). In particular, there were no significant differences regarding epidermal hyperplasia, signs of dermal fibrosis, and adnexal atrophy in mice treated with RT or RT in combination with ICIs (Figure 4B–G and supplementary material, Figure S3A–C), supporting the results from previous experiments with unfractionated RT. In summary, we did not observe significantly increased long-term skin damage after fractionated RT combined with ICIs. Furthermore, we found that fractionated RT resulted in less severe long-term toxicity with a numerically, but not significantly lower overall scores compared to unfractionated RT (supplementary material, Figure S3D). Notably, histopathological assessment of the unirradiated skin revealed low-grade signs of tissue damage in individual mice. We observed this phenomenon in mice treated with ICIs, but also in mice that had not been treated with ICIs (Figure 4A,D,F, and supplementary material, Figure S3B–D). Of note, this finding was also apparent after unfractionated RT (Figure 2B,E,G, and supplementary material, Figure S2B,C). However, we did not find significantly enhanced skin injury after treatment with ICIs (Figures 2, 4, and supplementary material, Figure S3D). To enhance the statistical power of the comparison, we performed a pooled analysis including all mice treated with ICIs and all mice that had not been treated with ICIs (supplementary material, Figure S3E). In total, we observed signs of skin damage in 2/14 mice that had not received ICIs and in 4/12 mice that had received ICIs. In sum, we did not observe significantly increased damage to

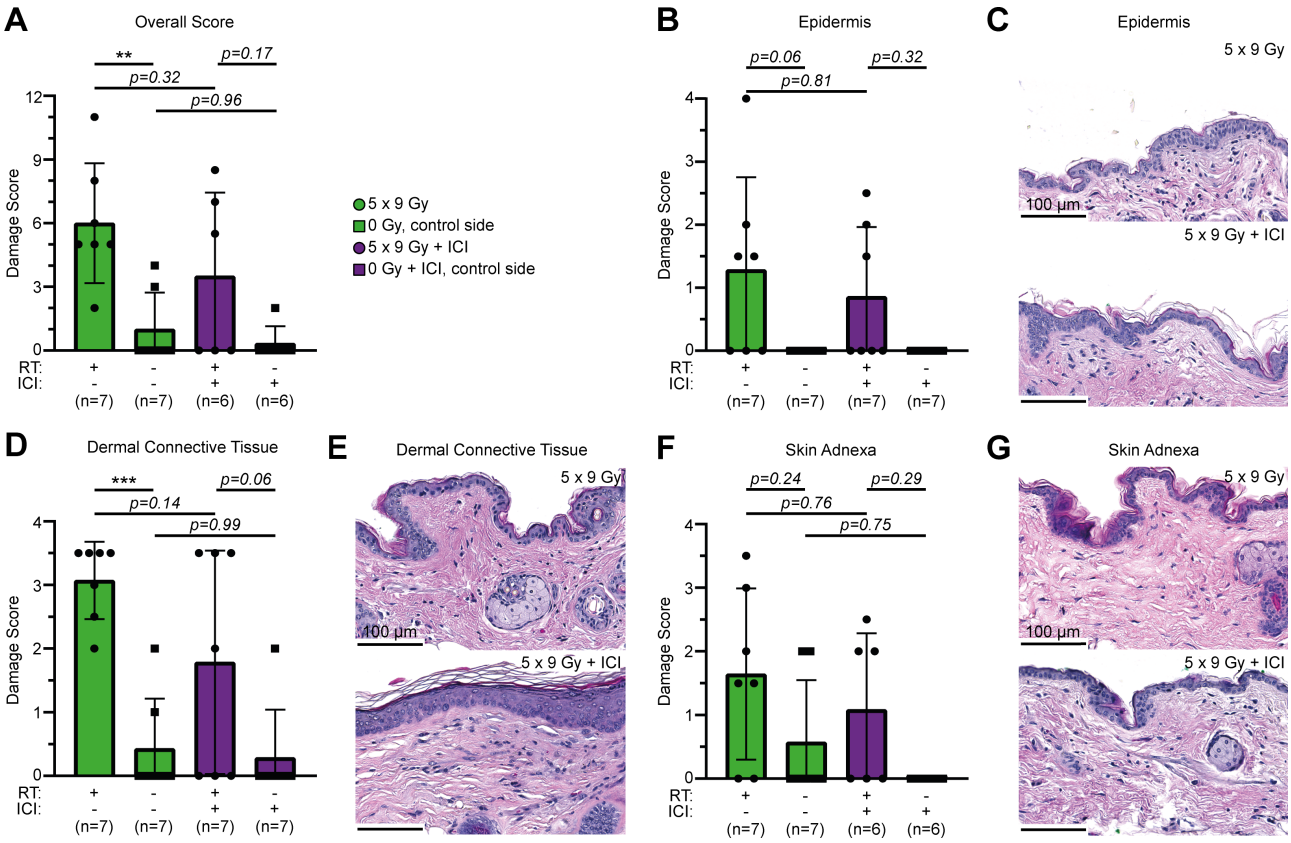



**Figure 4.** Concomitant dual immune checkpoint inhibition does not aggravate long-term skin damage after fractionated RT. Skin damage of C57BL/6 mice was evaluated via histopathological analysis of H&E staining 4 months after fractionated RT with 5 × 9 Gy ± treatment with ICIs. Skin samples of the irradiated area as well as control side were obtained on day 120 after RT. (A) Overall damage score (whole skin) comprising cumulative scores of the epidermis, dermal connective tissue, and skin adnexa. (B,C) Individual scores and representative images of epidermal hyperplasia, (D,E) dermal fibrosis and (F,G) adnexal atrophy. Scale bar, 100 µm. Data show mean ± SD and are pooled from two independent experiments with six or seven mice per group. For statistical analysis one-way ANOVA with multiple comparisons was performed. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

nonirradiated skin after treatment with ICIs (supplementary material, Figure S3E).

## Discussion

Recently, Jagodinsky *et al* observed an exponential growth in clinical trials combining RT with ICIs, peaking in more than 500 trials at the time of publication in 2020 [3]. Current studies estimate that there are now thousands of clinical trials ongoing or in development combining RT with immunotherapy [5]. In comparison to the analysis of enhanced tumor response rates, little attention was paid to the postulated risk of aggravated immune-mediated adverse effects and their pathogenesis [2,3,5]. However, the recent premature discontinuation of a clinical study on the combination of hypofractionated RT with anti-PD-1 in bladder cancer due to unexpected grave adverse effects highlights the crucial importance of safety considerations [30]. Thus, the great expectations for the potential of new therapeutic regimens combining ICIs with RT contrasts with the lack of detailed knowledge about the risk of aggravated irAEs and guidelines for their clinical management [5,31–33].

Regarding aggravated skin toxicity during combinatorial RT and systemic therapies, most of our experience stems from cytotoxic or other targeted agents. The randomized controlled trial RTOG 1016 showed that fractionated RT combined with the monoclonal anti-epidermal growth factor receptor (EGFR) antibody cetuximab significantly increased the risk of skin injury as compared to RT combined with chemotherapy [34]. Moreover, in addition to classical cytotoxic reagents [15], several more targeted therapies have been implicated in increasing skin toxicity after RT including BRAF inhibitors [15,35], the multi-kinase inhibitor sorafenib [36,37], and the small molecule EGFR tyrosine kinase inhibitor erlotinib [38,39]. All of these agents can induce skin toxicity independent of concomitant RT, presumably by direct cytotoxicity to keratinocytes and dermal stem cells. So far, there are only fragmentary data on underlying biological mechanisms and risk factors driving combinatorial tissue toxicities with RT and possible involvement of the immune system [2,5,40]. Accordingly, clinically translatable mouse models are essential to gain detailed insights into the pathogenesis

of tissue toxicity during such new regimens. To the best of our knowledge, we here report the first experimental study specifically investigating toxicity of the skin after RT combined with immunotherapy blocking immune checkpoints.

In line with accepted radiobiological concepts, our experiments demonstrate that RT regimens with different biological effective doses (BEDs) result in different severity of skin injury [26]. Consistent with published experimental studies, unfractionated RT with a low dose ($1 \times 15$ Gy, which equals a BED of 37.5 Gy) did not induce clinical signs of acute skin damage, whereas unfractionated RT with a high dose ($1 \times 30$ Gy, BED of 120 Gy) resulted in severe skin injury [41,42]. As expected, fractionated RT with an intermediate dose ($5 \times 9$ Gy $= 45$ Gy, BED of 85.5 Gy) induced only moderate disease.

Many radiobiologists currently think that a simultaneous combination of stereotactic RT with ICIs could aggravate healthy tissue toxicity, whereas sequential application of ICI after RT may circumvent such enhanced risks of adverse events [24]. To challenge these nonevidence-based assumptions, we aimed to provoke enhanced side effects of the skin by applying an intensive and simultaneous immunotherapy regimen, composed of a high dosage of anti-CTLA-4 and anti-PD-1 in a preclinical model. Strikingly, dual immune checkpoint inhibition did not sensitize the skin to RT-induced acute skin injury after a low dose of unfractionated RT, nor did it exacerbate acute skin injury after a high dose of unfractionated RT. Of note, this observation was reproducible in two different and genetically diverse mouse strains. Furthermore, the combination therapy did not worsen long-term damage to the epidermis, dermal connective tissue, or skin adnexa. From a clinician's point of view, this aspect is of crucial importance because late and often irreversible side effects such as fibrosis can lead to physiological dysfunctions that can severely impair the patients' quality of life [10,19]. Importantly, contrary to our observations investigating RT-induced skin injury, recent studies have shown that a genetic deficiency of *Pdcd1* (the gene encoding PD-1) or use of PD-1 inhibitors have the pathogenic potential of exacerbating skin diseases, as demonstrated in mouse models of contact hypersensitivity and psoriasis-like dermatitis [43,44].

However, our findings seem to be heavily context- and tissue-dependent. Using similar mouse models, ICIs have recently been shown to increase cardiac toxicity after RT [45,46]. Furthermore, there are controversial experimental data about RT-induced lung toxicity after additional treatment with ICIs [47,48]. As stated above, there are no experimental studies addressing skin injury in this context.

In contrast to stereotactic RT, which is usually restricted to local tumor ablation of small metastases, fractionated RT is a commonly used clinical strategy to reduce toxicity in adjacent healthy tissue after RT of large tumors next to the organs at risk [5,11]. We found that application of ICIs in combination with fractional RT did not significantly exacerbate acute or late skin toxicity. Our data can be interpreted in line with a large clinical study that observed no increased signs of radiation skin injury in head and neck cancer patients after chemoradiotherapy (CRT) combined with anti-PD-L1 (radiation skin injury in 38% of all patients) compared to CRT alone (40% of all patients) [49]. However, Antonia *et al* analyzed more than 700 patients, and found that there might be enhanced risks of adverse events of the skin after fractionated CRT and sequential application of anti-PD-L1 in patients with advanced stage lung cancer [50]. They found that 12% of patients treated with the combination therapy developed rash or pruritus, but only 5–8% after CRT without additional anti-PD-L1 therapy. In addition, 1.5% of patients developed signs of dermatitis after combination therapy compared to 0.5% of patients treated with CRT without anti-PD-L1. Almost all observed events were of mild or moderate severity [40,50]. However, the authors state that their study is limited in its ability to distinguish or assign causality for the observed adverse events [40], particularly with regard to the effect of simultaneously applied chemotherapy. Both studies have this limitation, which is partly due to the fact that the study design did not include patients treated with anti-PD-L1 without CRT [49,50]. Importantly, pruritus and rash are among the most common adverse events in patients treated with ICI monotherapy. In this context, a recently published meta-analysis did not find significantly increased rates of rash or pruritus after ICIs in combination with RT; unfortunately, the authors did not analyze RT-induced dermatitis [51]. Interestingly, a systematic review of published case reports demonstrated that a combination of ICIs with RT might enhance the risk of skin injury. Nevertheless, the authors state that it is difficult to conclude whether the patients developed adverse events of the skin because of the combination of RT and ICI or if the side effects were caused by either ICI or RT alone [52]. Addressing limitations in our own study, we found nonsevere signs of late tissue damage in the nonirradiated skin in 23% of all analyzed mice ($n = 6/26$) (supplementary material, Figure S3E). However, the aim of our study design was not the investigation of nonirradiated skin, and our data do not allow us to address the question of whether monotherapy with ICIs leads to significantly enhanced signs of late skin injury in our mouse models. In line with previous studies, we did not observe signs of acute skin injury after monotherapy with ICIs [43].

In sum, reliable clinical data concerning skin toxicity after RT combined with ICIs are lacking, even though skin injury is one of the most common side effects of ICIs or RT. Our study provides the first experimental evidence that the simultaneous application of an intensive regimen of dual immune checkpoint inhibition combined with different RT regimens does not enhance acute or late skin injury. Such studies are important to bridge

experimental and clinical data in the rapidly developing field of radioimmunotherapy for evidence-based clinical decision-making [5,53].

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## Author contributions statement

LLR and HF established, performed, and analyzed experiments. LLR (equal first author) began conducting the project's experiments and contributed to all analyses. HF (equal first author) started to contribute to the experiments early on in the project and later began writing the first draft of the article. SMN, CNW and JG helped to perform experiments. AF performed histopathological analysis. KS contributed to the histopathological processing of skin tissue. TES, SH and SEC provided intellectual input and helped in writing the article. LLR, HF and JCF wrote the article. All authors received and approved the article. JCF designed and guided the study. This work is part of the medical doctoral thesis of LLR at TUM.

## Data availability statement

All data relevant to the study are included in the article, including the supplementary information. Any additional information is available from the corresponding author (JCF) upon request. This study did not generate new unique reagents.

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## SUPPLEMENTARY MATERIAL ONLINE

**Figure S1.** Irradiation with 15 Gy and simultaneous ICI application does not induce acute skin injury

**Figure S2.** Detailed histopathological analysis of long-term skin damage after RT with 30 Gy and concomitant ICI treatment

**Figure S3.** Detailed histopathological analysis of long-term skin damage after RT with 5 × 9 Gy and simultaneous ICI application