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Metastatic potential of B16-F10 melanoma cells is enhanced by extracellular S100A4 derived from RAW264.7 macrophages

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ABSTRACT

S100A4, synthesized and secreted from both tumor and stroma cells, modulates an aggressive tumor phenotype in various cancers by intracellular and extracellular interactions which are not completely understood. Because of the high content of tumor-associated macrophages in melanoma, here, a syngeneic model (coculture of mouse B16-F10 melanoma cells (Mel) and RAW264.7 macrophages (Mϕ); administration (i.v.) of Mel and Mϕ/Mel in NMRI *nu*/*nu* mice) was used to investigate synthesis and secretion of (a) S100A4, (b) S100A4-mediated signaling and activation of NFκB, and (c) S100A4-mediated modulation of Mel invasiveness *in vitro* (transwell assay, transwell matrigel assay) and *in vivo* (metastatic lung colonization), respectively. In this model substantial S100A4 synthesis and secretion is demonstrated in Mϕ. Macrophage-derived S100A4 promotes Mel invasiveness in a paracrine manner *in vitro*, which is further substantiated in control experiments using recombinant human S100A4 and Mel stably transfected with mouse S100A4. Moreover, the participation of S100A4-mediated signaling, e.g., via the receptor for advanced glycation endproducts (RAGE), resulting in activation of NFκB was demonstrated in all experimental settings. Finally, we demonstrated that interaction of macrophage-derived S100A4 with Mel results in increased metastatic lung colonization *in vivo*.

1. Introduction

Tumor-associated macrophages (TAM) are involved in several carcinogenic processes including tumor growth, angiogenesis and modulation of extracellular matrix, and thereby also affect the metastatic cascade [1,2]. The important role of TAM is further supported by clinical studies showing a correlation between the high number of macrophages in tumor tissue and poor prognosis [3]. The identification of molecular targets implicated in TAM driven metastasis essentially thus will result in improved therapeutic approaches to treat advanced stage cancer. Among promising candidates is S100A4, a member of the S100 protein family, which has been implicated in tumor progression and metastasis and, moreover, is suggested to be a potential prognostic marker in several tumor entities including skin cancers [4–6]. Inhibition of S100A4 expression *in vitro* and *in vivo* results in impaired stroma organization and suppresses the metastatic potential of tumor cells [7,8]. Similar to other S100 proteins, S100A4 possesses both intracellular and extracellular functions. Extracellular S100A4 protein is able to stimulate angiogenesis, decreases cell adhesion as well as induces expression and secretion of extracellular matrix degrading enzymes leading to increased cell migration and invasion [9]. Next to its expression in tumor cells, S100A4 has also been found to be substantially synthesized in macrophages, but also in fibroblasts, lymphocytes, and bone marrow-derived cells [10–12]. Deletion of S100A4 provides the genetic evidence that S100A4 contributes to the normal physiological motility of macrophages, and demonstrated a role of this protein in regulating macrophage recruitment and chemotaxis *in vivo* [11]. Colocalization of S100A4 with a macrophage-specific antigen showed that S100A4 is expressed in fetal macrophages. Interestingly, these macrophages share some of the properties of metastatic tumor cells, such as invasiveness, high mobility, and degradation of extracellular matrix components [13]. However, the exact mechanism how extracellular S100A4 interferes melanoma metastasis, and, in this context, the involvement of macrophage-tumor cell interactions is still unknown.

In this regard, the receptor for advanced glycation endproducts (RAGE) expressed both by melanoma cells and macrophages is assumed to be a putative receptor for extracellular S100A4 [6]. We report that S100A4 expression and secretion is enhanced and

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further modulated by a prooxidative microenvironment in a mouse macrophage model. S100A4 derived from macrophages promotes an aggressive phenotype in a mouse melanoma model *in vitro* and *in vivo*. Furthermore, the participation of S100A4-RAGE interaction in these processes is demonstrated.

2. Materials and methods

2.1. Cell culture

Mouse RAW264.7 macrophages (Mϕ) and the metastatic melanotic B16-F10 mouse melanoma cell line (Mel) were obtained from American Type Culture Collection (LGC Standards, Wesel, Germany). Cells were cultured and cultivated as described previously [14].

2.2. In vitro cell models

Besides experiments using either Mϕ or Mel single cell monolayer cultures also a macrophage and melanoma cell coculture system (Mϕ/Mel) was investigated. For this purpose Mϕ and Mel were mixed at a rate of 4:1 according to Zhang and colleagues [15]. In order to stress cells regarding their S100A4 synthesis/secretion and RAGE expression, respectively, all cell models were exposed to various regulative situations. First, pro-inflammatory activation was induced by moderate dose X-ray irradiation following a protocol described in detail elsewhere [16]. Therefore, Mϕ, Mel and Mϕ/Mel were plated, irradiated (5 Gy; 20 Gy; single dose) and analyzed after 1 and 6 days and compared to non-irradiated (control cells). Second, the impact of conditioned medium (CM) both from Mel and Mϕ/Mel was explored. For this purpose, conditioned medium of (i) irradiated Mel (20 Gy, 6 days, further termed as CM1) and (ii) Mϕ/Mel (20 Gy, 6 days, further termed as CM2) was collected; and the medium of Mel and Mϕ/Mel was replaced with CM1 or CM2 for 2 days. Moreover, for invasion experiments cells were further incubated for 24 h with 5 μM recombinant human S100A4 protein, which was obtained following a protocol described previously [14].

2.3. RNA extraction and quantitative real-time PCR

The procedure was performed as described previously [17]. The following mouse intron spanning primers were purchased from Metabion (Planegg-Martinsried, Germany): β-actin-forward 5′-ACC TTC AAC ACC CCA GCC ATG-3′, β-actin-reverse 5′-GCT CGG TCA GGA TCT TCA TGA GG-3′, S100A4-forward 5′-TCA GCA CTT CCT CTC TCT TGG-3′, S100A4-reverse 5′- AGC TCC CTG GTC AGT AGC TC-3′, RAGE-forward 5′-TCC ACT GGA TAA AGG ATG GTG-3′, RAGE-reverse 5′-GAC CCT GAT GCT GAC AGG AG-3′.

2.4. Preparation of cell lysates

The procedure of cell extraction was done as already described in detail [14].

2.5. SDS–PAGE and Western blotting

The procedure of SDS–PAGE and Western blotting was done as already described in detail [14].

2.6. Cell migration and cell invasion assay

Cell migration was investigated as already described in detail [16]. For cell invasion assay, all procedures were carried out as in the migration assay except that Matrigel (20 mg/ml, BD Biosciences, Heidelberg, Germany) was coated on the upper side of a Boyden chamber PET membrane.

2.7. Preparation of nuclear extracts and measurement of NFκB p65 subunit activity

The procedure was done as already described in detail [18]. For this purpose, Mel were further stably transfected with mouse S100A4 (^{mS100A4}Mel) using the pIRES2-AcGFP1 cloning vector (Clontech, Saint-Germain-en-Laye, France) according to the protocol published elsewhere but using mouse cDNA (NM_011311) [19].

2.8. Metastatic lung colonization in NMRI-nu/nu mice

The procedure was done as already described in detail with some modifications [16,20]. Three independent experiments were performed with four animals in each group. In brief, female NMRI-*nu*/*nu* mice were injected into the tail vein either with (i) Mel (5×10^5) or (ii) Mϕ/Mel (2.0×10^6 Mϕ and 5×10^5 Mel) in 300 μl isotonic solution for infusion.

2.9. Data analysis

Data are given as means ± standard deviation of at least three independent experiments. To test statistical significance, one-way analysis of variance ANOVA with a Bonferroni *post hoc* test was used for multiple comparisons. Data of the corresponding untreated control cells are set as 100%, $^*p < 0.05$.

3. Results

3.1. Increased S100A4 and RAGE mRNA expression after irradiation

In order to induce a prooxidative regulative situation cells were exposed to single dose X-ray irradiation. Fig. 1A shows the relative expression level of S100A4 (left) and RAGE (right) compared with non-irradiated corresponding control cells. A significantly increased S100A4 and RAGE expression was found in all cell lines after irradiation as model stressor (Fig. 1A). In all experiments a dose- and time-dependent increase of S100A4 and RAGE was visible (data not shown in detail), with the most pronounced effects at 20 Gy and 6 days.

3.2. Modulation of intra- and extracellular S100A4 in Mϕ

Highly metastatic B16-F10 melanoma cells revealed no detectable intracellular monomeric S100A4 protein (Fig. 1B). In contrast, mouse macrophages showed a high content of intracellular S100A4 protein even increasing after moderate dose X-ray irradiation in a dose- and time-dependent manner. Also the Mϕ/Mel coculture showed a higher extent of intracellular S100A4 protein levels, which is suggested to be completely contributed by Mϕ. Interestingly, the intracellular S100A4 in Mϕ/Mel decreased after irradiation, and it has to be highlighted that after 20 Gy and 6 days intracellular S100A4 could not be detected any longer (Fig. 1B). Control bands of β-actin confirmed similar protein content. Secretion analysis showed no extracellular S100A4 monomers/dimers in cell culture supernatants of Mel. However, positive protein signals at ~37 kDa and ~55 kDa could be detected in Mel supernatants, supposed to be keratins and keratin degradation products detected by cross-reactivity of the antibody used as published by Cabezon and colleagues [21]. Of note, these bands only were visible after using an enhancing procedure to detect blotted proteins in the femtomolar range. In contrast, Mϕ showed high secretion of S100A4 which was further elevated after irradiation (Fig. 1B).

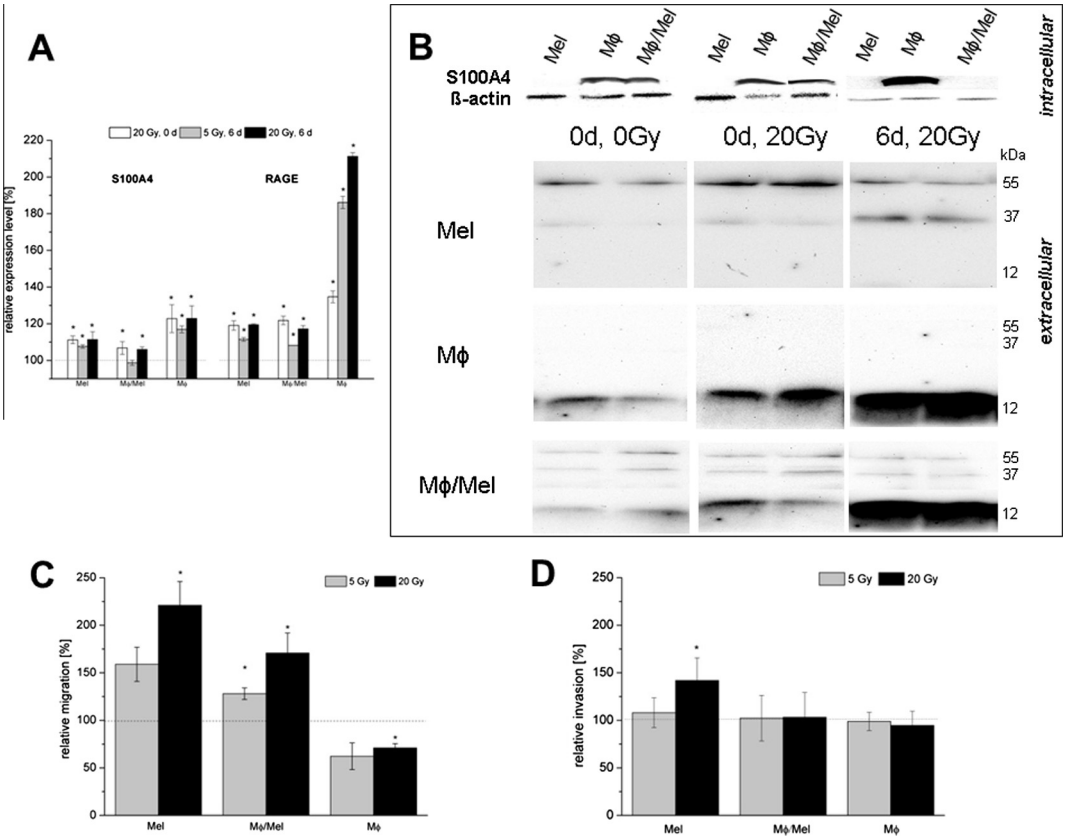

Fig. 1. Increased expression and secretion of S100A4, and regulation of migration and invasion after X-ray irradiation. (A) Relative mRNA synthesis of S100A4 and RAGE in B16-F10 cells (Mel), RAW264.7 macrophages (Mϕ), and Mϕ/Mel after single dose irradiation. Arithmetic chart of the threshold cycle (C_t) for S100A4 and RAGE was normalized to house-keeping-gene β-actin. (B) Intracellular and extracellular protein expression and synthesis of S100A4 in Mel, Mϕ, and Mϕ/Mel after single dose irradiation. Relative migration (C) and invasion (D) of Mel, Mϕ, and Mϕ/Mel after irradiation.

Secretion analysis in Mϕ/Mel demonstrated also the positive signals at ~37 kDa and ~55 kDa supposed to be keratins from Mel as discussed above, and, furthermore, S100A4 (~12 kDa; referring to S100A4 monomer) with increasing intensity after irradiation supposed to be secreted from Mϕ alone (Fig. 1B). In summary, this experimental approach results in a prooxidative microenvironment that is characterized by baseline and elevated extracellular S100A4 levels exclusively secreted by Mϕ. We then hypothesized that macrophage derived S100A4 affects metastatic properties of melanoma cells.

3.3. Prooxidative microenvironment regulates Mel migration and invasion

As shown in Fig. 1C irradiation significantly enhanced migration properties in Mel by 121% after 20 Gy, and in Mϕ/Mel by 28% after 5 Gy, and by 70% after 20 Gy. The effect was more pronounced in Mel. Interestingly, in Mϕ migration was significantly suppressed after irradiation (Fig. 1C). Also invasive properties were significantly increased in Mel by 41% after 20 Gy (Fig. 1D). In contrast, Mϕ and Mϕ/Mel showed no shift on the invasive behavior (Fig. 1D).

3.4. Modulation of S100A4 and RAGE mRNA expression after exposition to conditioned media

As shown in Fig. 2A, a significant decrease of S100A4 (left) and RAGE (right) mRNA expression was found in both Mel and Mϕ/Mel after treatment with either CM1 or CM2 compared with untreated corresponding control cells (Fig. 2A).

3.5. Regulation of Mel migration and invasion after exposition to conditioned media

Migration was significantly increased in Mel by 30% and in Mϕ/Mel by 27% after incubation with CM1 (Fig. 2B), whereas CM2 only provoked a significant increase in Mel by 76% compared to untreated control cells. An enhanced migration was also visible in Mϕ/Mel, but was not statistically significant. No changes in the migratory behavior were detectable in Mϕ (Fig. 2B). For invasion studies a significant higher effect was found after incubation of Mϕ/Mel with CM2 by 75% (Fig. 3C). CM1 and CM2 did not change invasion behavior of both Mel and Mϕ (Fig. 2C).

3.6. Increased invasion by incubation with recombinant S100A4 protein

As shown in Fig. 3A incubation with 5 μM recombinant human S100A4 protein significantly up-regulated invasion in non-irradiated Mel by 52%, in Mϕ/Mel by 90%, and in Mϕ by 45%. A significantly up-regulated invasion was observed after single dose irradiation and further incubation with 5 μM recombinant S100A4 in Mel by 93%, in Mϕ/Mel by 95%, and in Mϕ by 71% (Fig. 3A). In summary, the data demonstrate macrophage derived and, as control, human S100A4 to be involved in regulation of metastatic properties of melanoma cells.

3.7. Regulation of NFκB activity

Former investigations demonstrated S100A4 to be a modulator of a prooxidative and proinflammatory tumor microenvironment

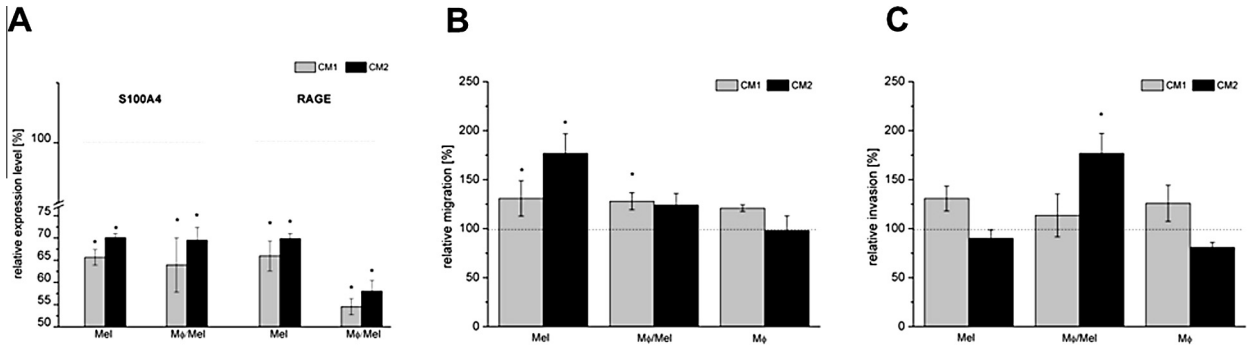

Fig. 2. Effects of conditioned medium referring to expression, migration and invasion. Mel and Mϕ/Mel were incubated with CM1 and CM2 for 2 days. Decreased mRNA synthesis of S100A4 and RAGE (A), the corresponding migration (B) and invasion (C) activity is demonstrated.

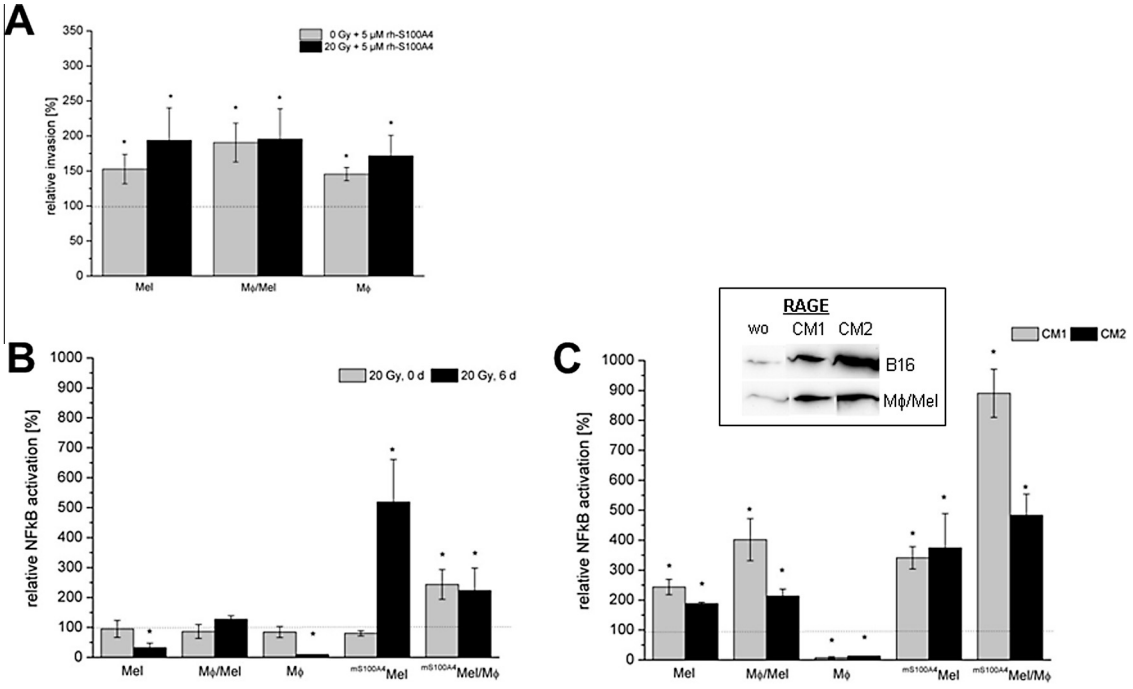

Fig. 3. Increased invasion by recombinant S100A4 protein and regulation of NFκB activity. (A) Invasion of Mel, Mϕ, Mϕ/Mel after incubation with 5 µM recombinant human S100A4. NFκB activation status after (B) X-ray irradiation and (C) incubation with CM1 and CM2 in Mel, Mϕ, and Mϕ/Mel as well as in ^{mS100A4}Mel and ^{mS100A4}Mel/Mϕ. An increased RAGE expression after incubation with CM1 and CM2 is shown (see the Western blotting insert).

via activation of the transcription factor NFκB [18]. Therefore, the aim of this experiment was to compare the activation status of NFκB p65 subunit between Mel and Mϕ/Mels. For this purpose, Mel were further stably transfected with mouse S100A4 (^{mS100A4}-Mel). As shown in Fig. 3B irradiation significantly inhibited NFκB in Mel by 70% and in Mϕ by 90% after 6 days whereas Mϕ/Mel had a tendency to up-regulate the NFκB status (Fig. 3B). In contrast, ^{mS100A4}Mel demonstrated a significantly increased activation of NFκB by 418% after irradiation and 6 days. Likewise ^{mS100A4}Mel/Mϕ-cocultures showed significantly increased NFκB activation by 143% (20 Gy, 0 days) and 123% (20 Gy, 6 days) (Fig. 3B). Otherwise, after incubation with CM1 the activation status of NFκB was significantly up-regulated in Mel by 143% and in Mϕ/Mel by 300%, respectively, and in ^{mS100A4}Mel by 241% and in ^{mS100A4}Mel/Mϕ-cocultures by 790% (Fig. 3C). Incubation with CM2 demonstrated a significant up-regulation in Mel by 87% and in Mϕ/Mel by 113%, and respectively in ^{mS100A4}Mel by 274% and in ^{mS100A4}Mel/Mϕ-cocultures by 383% (Fig. 3C). Interestingly, no NFκB translocation was found in Mϕ cells. The increased NFκB activity after incubation with CM correlates with an increased RAGE expression (see the Western blotting insert).

3.8. Enhanced metastatic lung colonization in NMRI-nu/nu mice

A pilot experiment with mice injected with untreated Mel showed about one-third surface metastases after 21 days (Fig. 4). In contrast, injection of Mϕ/Mel caused a much higher amount of surface metastases at this time. Interestingly, here the survival time after injection of Mϕ/Mel was also decreased, 4 of 7 mice did not survive the 21st day. Furthermore, in these mice besides to lung metastases, liver and extended peritoneal metastasis was noticeable (data not shown). Therefore, for further studies day 15 after post injection was used for investigation of *in vivo* metastasis. Here, increased metastatic lung colonization was observed in mice injected with Mϕ/Mel (surface metastases (%): 45 ± 9 day 15 p.i. and 88 ± 10 day 21 p.i) compared to mice injected with Mel (surface metastases (%): 31 ± 19 day 15 p.i. and 38 ± 25 day 21 p.i).

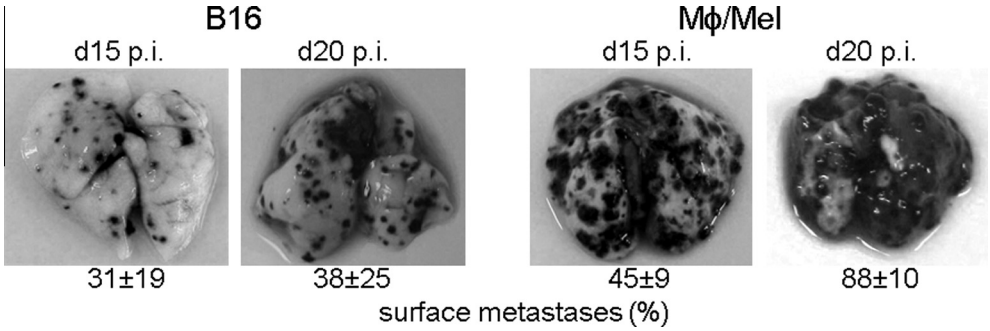

Fig. 4. Increased metastatic lung colonization after injection of Mϕ/Mel coculture in NMRI-*nu*/*nu* mice. The relative surface area of superficial metastases in proportion to the individual whole lunge area (% metastases) was determined by AutMessPlus software package and is given as mean ± SEM.

4. Discussion

In this work we report that mouse macrophages synthesize and secrete S100A4 to a high extent, and that macrophage-derived S100A4 is able to promote melanoma cell invasiveness *in vitro* and increased metastatic lung colonization *in vivo*.

A number of studies showed that S100A4 plays a key role in stimulation of metastasis including investigations in xenograft and genetically modified mouse models [22–24], but the exact mechanism how S100A4 interferes to metastasis, particularly, to interactions between tumor cells and TAM, are still unknown. Most studies are focused on the metastatic properties of intracellular S100A4 in tumor cells [25,26]. However, extracellular S100A4, predominantly found in stroma cells, also has been suggested to contribute to metastasis [27]. Therefore, in the present study we investigated macrophage-derived S100A4 protein in a syngeneic model by co-cultivating mouse B16-F10 melanoma cells (Mel) with RAW264.7 macrophages (Mϕ). In order to learn more about the hypothesized interactions, moderate dose X-ray irradiation was used as a pro-inflammatory stressor with certain clinical relevance to induce a regulative situation characterized by the generation of reactive oxygen species (ROS) and cellular oxidative stress [28]. From our results we suppose that in Mϕ the altered intracellular redox balance is responsible for the alterations in the S100A4 protein level. In line with this, recently the critical role of high ROS levels in enhancing the proinvasive effects of TAM in malignant melanoma was shown [29]. Additionally, up-regulation of protein synthesis post exposure to ionizing radiation has already been reported for S100A6 in colon and lung cancer cells. Likewise for S100A4 these authors reported protein modifications after exposing cells to ionizing radiation [30]. Accordingly, in this work irradiation induced an increased migration in Mel and Mϕ/Mel, and increased invasion in Mel. Moreover, Dukhanina et al. reported that the content of S100A4 measured by a self-developed enzyme immunoassay is increased in the medium after incubation of activated lymphocytes either with M3 melanoma cells or B16 cells. These authors suggested that S100A4 secretion in cancer cells is induced by activated lymphocytes, however, the underlying mechanisms were not investigated in detail [31].

In contrast, in our work incubation/coculture with Mϕ did not induce the synthesis and secretion of S100A4 in Mel (B16-F10 cells). The replacement of the medium by conditioned medium evoked a down-regulation of S100A4 and RAGE mRNA expression as well in Mel and Mϕ as in Mϕ/Mel. Mel also revealed enhanced migration properties after incubation with CM1 and CM2, whereas Mϕ/Mel showed an increased migration after incubation with CM1 and increased invasion after incubation with CM2, respectively. Here, we suppose para- and autocrine effects via the S100A4-RAGE signaling cascade, and an increased RAGE expression has been found in Mel and in Mϕ/Mel, respectively. To further support the paracrine effect of S100A4 we treated cells with recombinant human S100A4, and observed an enhanced invasion. Similar effects have been reported for S100A4-positive epithelial ovarian carcinoma cells in which migration was induced after incubation with recombinant S100A4 [5]. Schmidt-Hansen et al. demonstrated invasive growth in immortalized mouse microvascular endothelial cells after stimulation with S100A4 [8]. The importance of S100A4 referring to cell motility and migration after transfection or silencing of S100A4 has been extensively analyzed [32–34]. Grum-Schwensen et al. reported that tumors of highly metastatic carcinoma cells did not metastasize in S100A4 knockout mice, but the metastatic phenotype was partly restored by co-injecting the cancer cells with S100A4-expressing stromal cells [35]. Based on our experimental data and the data from literature we suppose that extracellular S100A4, and, particularly, macrophage-derived S100A4 has a great impact in forwarding the invasiveness of tumor cells. High expression of S100A4 in macrophages and regulation of motile and invasive capabilities initiated through S100A4 has further been demonstrated [11]. S100A4 is also known to induce the activation of the transcription factor NFκB in several cell systems [36], thereby provoking cell proliferation and differentiation. For this purpose Mel were stable transfected with S100A4 and cocultivated with Mϕ. Here, a strong activation of NFκB was seen, with pronouncing effects in both ^{mS100A4}Mel and ^{mS100A4}Mel/Mϕ-cocultures. NFκB activation is a well-known downstream event of RAGE signaling, and our data support the hypothesis that in melanoma interaction of S100A4 with RAGE is a prominent pathway resulting in NFκB activation. Consistently, NFκB activation induced by S100A4-RAGE interaction was already shown in human melanoma cells by us [18] and in chondrocytes by others [37]. However, contribution of other molecules present in a prooxidative/proinflammatory tumor microenvironment, e.g., cytokines or eicosanoids also has to be considered.

Our pilot animal experiment indicated that the intravenous administration of Mϕ/Mel induces high metastatic lung colonization compared to injecting Mel alone. Moreover, the animals revealed a shorter survival rate after injection of cocultivated cells. Consistent with our observation Zhang et al. found that co-inoculation of RAW macrophages and Lewis lung carcinoma (LLC) cells enhance tumor growth, promote lymphangiogenesis and lymph node metastasis in LLC tumors [15].

In summary, the results suggest that the metastatic potential of B16 melanoma cells is enhanced by S100A4 derived from RAW macrophages thereby promoting melanoma cell invasiveness *in vitro* and increased metastatic lung colonization *in vivo*. The data strongly support the hypothesis that in a prooxidative tumor microenvironment S100A4 plays a role as proinflammatory mediator independent of the cellular origin of this protein. The results also indicate that targeting S100A4 secretion and S100A4-RAGE interaction potentially offer therapeutic options for prevention of melanoma metastasis.

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