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ORIGINAL INVESTIGATION

Antimicrobial effects of murine mesenchymal stromal cells directed against *Toxoplasma gondii* and *Neospora caninum*: role of immunity-related GTPases (IRGs) and guanylate-binding proteins (GBPs)

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Abstract Mesenchymal stromal cells (MSCs) have a multilineage differentiation potential and provide immunosuppressive and antimicrobial functions. Murine as well as human MSCs restrict the proliferation of T cells. However, species-specific differences in the underlying molecular mechanisms have been described. Here, we analyzed the antiparasitic effector mechanisms active in murine MSCs. Murine MSCs, in contrast to human MSCs, could not restrict the growth of a highly virulent strain of *Toxoplasma gondii* (BK) after stimulation with IFN-γ. However, the growth of a type II strain of *T. gondii* (ME49) was strongly inhibited by IFN-γ-activated murine MSCs. Immunity-related GTPases (IRGs) as well as guanylate-binding proteins (GBPs) contributed to this antiparasitic effect. Further analysis showed that IFN-γ-activated mMSCs also inhibit the growth of *Neospora caninum*, a parasite belonging to the apicomplexan group as well. Detailed studies with murine IFN-γ-activated MSC indicated an involvement in IRGs like Irga6, Irgb6 and Irgd in the inhibition of *N. caninum*. Additional data showed that, furthermore, GBPs like mGBP1 and mGBP2 could have played a role in the anti-*N. caninum* effect of murine MSCs. These data underline that MSCs, in addition to their regenerative and immunosuppressive activity, function as antiparasitic effector cells as well. However, IRGs are not present in the human genome, indicating a species-specific difference in anti-*T. gondii* and anti-*N. caninum* effect between human and murine MSCs.

Keywords Mesenchymal stem cells · Interferon gamma · *Toxoplasma gondii* · *Neospora caninum* · IIRGs · GBPs

Introduction

Multipotent, mesenchymal stromal cells (MSCs) have been shown to differentiate along multiple mesenchymal lineages such as fibrinogenic, chondrogenic, myogenic and adipogenic pathways and are therefore an attractive tool to support tissue regeneration [1, 2]. An additional feature of

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human MSCs (hMSCs) is their capacity to inhibit the proliferation and the effector function of lymphocytes [2]. This immunosuppressive activity has been found in murine mesenchymal stromal cells (mMSCs) as well, but the immunosuppressive mechanisms induced seemed to be different in both species. For example, in hMSCs, the inhibition of T cell responses has been mediated by the IFN-γ-induced tryptophan-degrading enzyme indoleamine 2,3-dioxygenase (IDO) [3, 4], while in mMSCs, adenosine generation has been responsible for the growth control of T cells [5]. It has been shown that IDO is not induced in mMSCs by IFN-γ [6]. Nevertheless, other immunosuppressive effector mechanisms are comparably active in both species. For example, prostaglandin E2 has been described to modulate the immunostimulatory capacity of macrophages/monocytes of both, hMSCs and mMSCs [7, 8].

In addition to their immunosuppressive capacity, MSCs are potent antimicrobial effector cells. Again, species-specific differences in the antimicrobial effector mechanisms of hMSCs and mMSCs have been revealed. In 2011, we described that IFN-γ-induced IDO in activated hMSCs inhibited the growth of *Staphylococcus aureus*, *Toxoplasma gondii* (*T. gondii*) and human Cytomegalovirus [9]. In contrast, IFN-γ-stimulated mMSCs failed to mediate antibacterial or antiparasitic effects and IDO was not expressed. However, mMSCs co-stimulated with a cytokine cocktail consisting of IFN-γ, IL-1 and TNF-α expressed the inducible nitric oxide synthase (iNOS) and thereby controlled an infection with *T. gondii* [9].

Three major clonal strains of the parasite *T. gondii* have been identified until now. Historically, the differences in the virulence between these strains have been described in the murine system, but recently, different virulence factors could be revealed by genome analyses [10]. All three clonal strains infect humans and are therefore in the scope of research. Since *T. gondii* is an obligate intracellular pathogen which can infect nearly all nucleated cells, the cell-specific defense mechanisms that are active against this parasite are important. Since MSCs were suggested to be useful in the therapy of graft versus host reactions and host versus graft reactions [11] and clinical relevant *T. gondii* infections were mainly found in immunosuppressed patients [12], the capacity of MSCs to control *T. gondii* proliferation is of interest. We have recently shown that nitrogen radicals contribute to the defense against a high virulent *T. gondii* type I strain in mMSCs [9]. Additionally, using murine macrophages, another group has described that IFN-γ-induced, immunity-related GTPases (IRGs) are an essential defense mechanism against *T. gondii*, as well. Theses IRGs inhibited the growth of type II strains, but could not control the growth of type I strains [13].

The diverse and complex antiparasitic defense mechanisms directed against several apicomplexan parasites in mice [14] encouraged us to analyze additional effector mechanisms directed against these pathogens in mMSCs. We therefore analyzed the antiparasitic defense of mMSCs directed against *Neospora caninum* (*N. caninum*), an evolutionary close relative of *T. gondii* (7). Comparable to *T. gondii*, also, *N. caninum* has a complex life cycle. However, dogs instead of cats are the definitive hosts for *N. caninum* and cattle are important secondary hosts. *N. caninum* does not infect humans, but mice are sensitive to *N. caninum* and infections are comparable to infections with low virulent strains of *T. gondii* [15–17]. Therefore, we also analyzed the contribution of IRGs and GBPs in the defense against *N. caninum* in infected mMSCs.

Materials and methods

Cell culture and stimulation

Murine mesenchymal stromal cells (mMSCs) were prepared and characterized as described [5]. Cells were cultured in IMDM (Iscove's Modified Dulbecco's Medium, Cambrex, München, Germany), supplemented with 10 % fetal calf serum (FCS, Gibco, Karlsruhe, Germany), in the presence of 10 ng/ml basic fibroblast growth factor (bFGF, Sigma-Aldrich, Taufkirchen, Germany) and 10 ng/ml platelet-derived growth factor (PDGF, Sigma-Aldrich, Taufkirchen, Germany). In some experiments, mMSCs cultured in the absence of growth factors were used. Human mesenchymal stromal cells (hMSCs) were prepared and characterized as described previously [9]. In brief, primary human MSCs were generated from the mononuclear cell (MNC) fraction of bone marrow samples. The resulting MSC preparations exhibited the prototypical antigen expression profile of human MSCs (i.e. CD14, CD34 and CD45 negative; CD29, CD44, CD73/SH-3/4, CD90 and CD105/SH-2 positive) and under appropriate culture conditions differentiated into adipogenic, osteogenic and chondrogenic lineages. Human MSC lines from passages 3–8 were utilized for subsequent experiments.

Mouse embryonic fibroblast cell line 3T3 expressing recombinant, GFP-labeled mGBP2 or mGBP5 [18] was cultured in DMEM (Dulbecco's Modified Eagle Medium, Cambrex, München, Germany) supplemented with 10 % newborn calf serum (NCS). For stimulation experiments, recombinant murine or human IFN-γ (R&D Systems, Wiesbaden, Germany) was used. After 16 h of IFN-γ stimulation, the cells were harvested for preparation of RNA. Unstimulated cells served as control.

In vitro passage of *T. gondii* and *N. caninum*

T. gondii tachyzoites (type I strain BK and RH and type II strain ME49) and *N. caninum* tachyzoites [isolate Nc-1 and isolate Liverpool (Nc-Liv)] were grown on confluent monolayers of human foreskin fibroblasts (HFF).

Immunofluorescence analysis

For immunoassaying, cells were cultured on 12-mm circular cover-slips in 24-well tissue culture plates (BD Biosciences, Heidelberg, Germany). Non-confluent cell cultures were stimulated with 100 U/ml murine IFN-γ for 16 h, control cultures were left untreated. Thereafter, cells were infected with 1×10^6 freshly harvested RH, ME49, NC-Liv or NC-1 tachyzoites for 1–2 h at 37 °C. In order to remove free extracellular parasites, infected cells were washed with phosphate-buffered saline (PBS, Biochrom AG, Berlin, Germany). Cells were fixed for 20 min at room temperature with 3 % paraformaldehyde (Merck, Darmstadt, Germany) in PBS and after washing three times with PBS, parasites were permeabilized with 0.02 % saponin (Calbiochem, Darmstadt, Germany) in PBS for 15 min and blocked with 2 % normal goat serum (Daco, Glostrup, Denmark) in PBS for 20 min. Afterward, cover-slips were incubated with anti-Irga6 (mouse mab), anti-Irgd (rabbit antiserum), anti-Irgb6 (mouse mab), rabbit anti-mGBP1 or anti-mGBP5 (rabbit antisera) at 1:500 or 1:200 dilution or anti-Irgm (mouse mab, 1:500) in 0.2 % goat serum for 1 h. In order to visualize the outer membrane of *T. gondii* and *N. caninum* mouse, anti-*Tg*SAG1 mouse antiserum (Abcam, Cambridge, UK) and anti-*Nc*SAG1 mouse mab [19] were used at a concentration of 1:500. Cover-slips were washed three times with PBS and then incubated with AffiniPure goat anti-rabbit IgG cyanine dyes-Cy2-conjugated or AffiniPure goat anti-mouse IgG cyanine dyes-Cy3-conjugated (Dianova GMBH, Hamburg, Germany) at 1:500 dilution in 0.2 % goat serum in PBS for 45 min. After washing the cover-slips two times with PBS, the nuclei were stained with 4′6-diamidino-2-phenylindole in PBS (DAPI, Invitrogen Life Technologies, Karlsruhe, Germany) for 5 min and finally fixed onto microscope slides using Fluoromount-G (Biozol Diagnostica, Eching, Germany). Fluorescent images were analyzed with the Zeiss LSM 780 confocal microscope, and only parasites located in a visible PV were analyzed. Images were evaluated using the ZEN 2009 Light Edition software (Zeiss, Göttingen, Germany). Further experiments were performed in an analogous manner using 3T3 cells expressing recombinant GFP-labeled mGBPs.

Gel electrophoresis and western blot analysis

For cell lysate preparation, mMSCs were cultured in 75 cm^2 culture flasks (Greiner, Frickenhausen, Germany) and stimulated for 16 h with 100 U/ml murine IFN-γ, control cells were left untreated. After harvesting, cells were resuspended in 500 µl protease inhibitor-PBS cocktail (Roche Diagnostics GmbH, Mannheim, Germany). After three freeze/thaw cycles, cell lysates were centrifuged for 10 min at 9,700 rpm at room temperature. Proteins in the resulting supernatants were separated by SDS-PAGE on 10 % polyacrylamide gels (Invitrogen, Karlsruhe, Germany) and transferred to nitrocellulose membranes (Whatman GmbH, Dassel, Deutschland) by semi-dry blotting. The nitrocellulose membrane was saturated in 5 % skim milk (Oxoid, Wesel, Deutschland) in PBS for 1 h, and the membranes were then incubated with the appropriate anti-Irga6, anti-Irgd or anti-Irgb6 antibodies (1:1,000) for 16 h at 4 °C, followed by different washing steps and peroxidase-conjugated secondary IgG antibodies (1:7,500, Jackson Immunoresearch Laboratories, Suffolk, England). Signals were detected using the Supersignal ECL reagent (GE Healthcare, München, Germany).

Real-time RT-PCR analysis

Total RNA from IFN-γ-stimulated or unstimulated murine MSCs was isolated using TRIzol reagent (Invitrogen, Karlsruhe, Germany) based on the manufacturer's protocol. First-strand cDNA was generated from 1 µg RNA by oligo (dT) priming using Moloney murine leukemia virus reverse transcriptase (Invitrogen, Karlsruhe, Germany). Primers and probes used for PCRs are listed in Table 1. In order to avoid detection of genomic DNA, the primer pairs were selected to be intron overspanning. The results are expressed relative to expression in unstimulated cells, ß-actin served as internal control.

Table 1 Primers and probes for real-time RT-PCRs

Target gene	Forward primer	Reverse primer	Hybridization probe	Amplicon (bp)
ß-actin	TGACAGGATGCAGAAGGAGA	CGCTCAGGAGGAGCAATG	CTCTGGCT	75
mGBP1	CAGACTCCTGGAAAGGGACTC	CTTGGATTCAAAGTATTTTCTCAGC	GGCTGAAG	60
mGBP2	TGAGTACCTGGAACATTCACTGAC	AGTCGCGGCTCATTAAAGC	AGGAGCTG	75
mGBP3	GGCTGAGGACTGTCCCTGT	CATGGTCCACTCGGAAGC	CAGAGCCA	64
mGBP5	TCACTGAAGCTGAAGCAAGG	GCGTCAAAAACAAAGCATTTC	ACTGGGAA	107

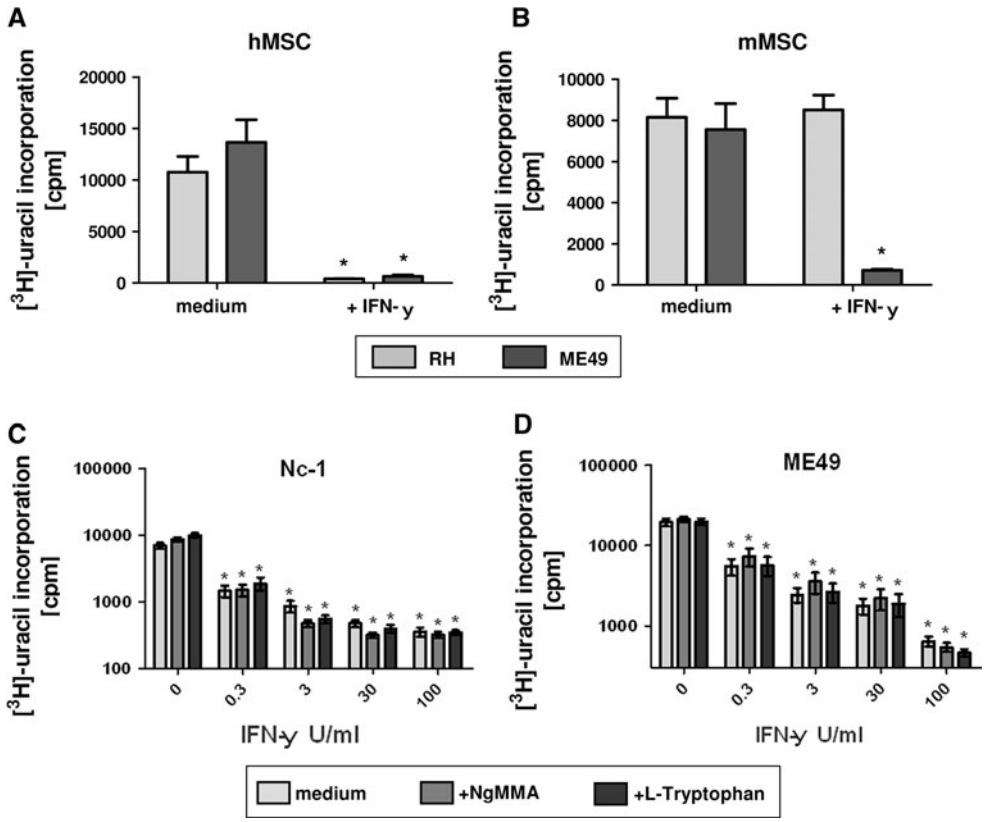

Fig. 1 Antiparasitic effect mediated by mesenchymal stem cells. **a**, **b** IFN-γ stimulated murine MSCs fail to inhibit the growth of *T. gondii* strain RH. Human MSC (hMSCs) (**a**) or murine (mMSCs) (**b**) were stimulated with IFN-γ (300 or 100 U/ml, respectively). Three days after activation cells were infected with *T. gondii* strain RH or ME49 (1×10^4/well) and parasite growth was determined using the [^{3}H]-uracil method 3 days later. Data are given as mean cpm ± SEM of three independent experiments, each done in triplicates. **c**, **d** IFN-γ mediated inhibition of *N. caninum* Nc-1 and *T. gondii* strain ME49 is independent of IDO- or iNOS activation. Murine MSC were stimulated with IFN-γ (0–100 U/ml). Three days after activation cells were infected with *N. caninum* isolate Nc-1 (**c**) or *T. gondii* strain ME49 (**d**) (1×10^4 tachyzoites/well). Parasite growth was determined using [^{3}H]-uracil method three days later. As a control some cells were cultured in the presence of L-tryptophan or NgMMA (100 μg/ml). Data are given as mean cpm ± SEM of four independent experiments, each done in quadruplicates. Statistical significant inhibitory effects are marked by *asterisks*

Infection assays

Murine MSCs (1×10^4/well) were cultured in gelatine-coated (0.01 % in PBS) 96-well plates (Greiner, Frickenhausen, Germany) and stimulated with murine IFN-γ (0–100 U/ml) for 3 days. In some experiments, human MSCs (3×10^4/well) were stimulated with 300 U/ml IFN-γ. Thereafter, cultures were infected with *T. gondii* tachyzoites RH or ME49 or *N. caninum* Nc-1 (1×10^4/well). Parasite growth was examined microscopically and determined by the [^{3}H]-uracil method after 3 days of culture as described [20]. As a control, some cultures were supplemented with L-tryptophan (100 μg/ml, at the time point of infection) or NgMMA (100 μg/ml, at the time point of IFN-γ stimulation). All experiments were done in quadruplicate. The Students *t* test for unpaired probes was used to determine significant differences ($p < 0.05$).

Results

Interferon-γ-activated murine mesenchymal stromal cells inhibit growth of *N. caninum* and a *T. gondii* type II strain but fail to inhibit a type I strain

Murine mesenchymal stromal cells (mMSCs), in contrast to human MSCs (hMSCs), have been unable to control the growth of *T. gondii* type I strain BK [9]. We analyzed the antiparasitic defense of IFN-γ-activated mMSC against different apicomplexan parasites in more detail. In the control, hMSCs inhibited the growth of a type I (RH) and a type II (ME49) strain of *T. gondii* with a comparable efficiency (Fig. 1a). In contrast, mMSCs controlled *T. gondii* ME49, while the growth of *T. gondii* RH was not or only marginally influenced (Fig. 1b).

Further infection experiments revealed an antiparasitic effect of IFN-γ-activated mMSC against *N. caninum*

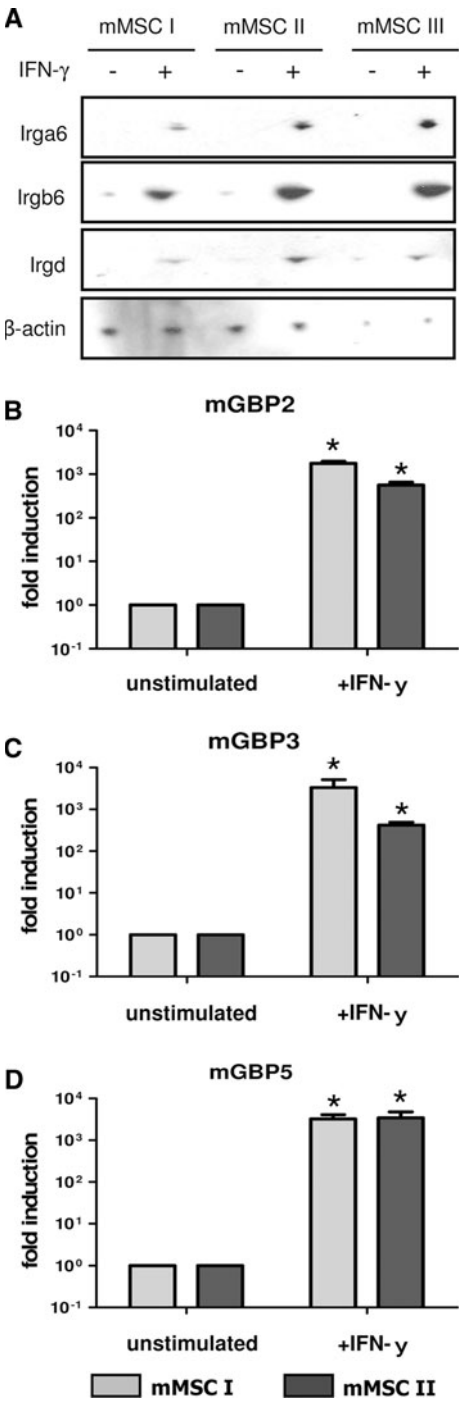

Fig. 2 IFN-γ induced expression of antimicrobial effector molecules in murine mesenchymal stem cells. **a** Expression of immunity-related GTPase proteins in murine MSCs. Three different murine MSC preparations (MSC I–III) were stimulated with IFN-γ (100 U/ml) for 16 h or left unstimulated. After cell lysis proteins were separated by SDS-Page. IRGs were detected with specific antibodies directed against Irga6, Irgb6 and Irgd. As loading control β-actin was used. **b–d** Induction of guanylate-binding proteins in murine MSCs. Two different MSC preparations were stimulated with IFN-γ (100 U/ml, 18 h). Thereafter, real-time PCR was performed and mGBP expression was calculated as fold induction in comparison with untreated cells. Data are given as the mean ± SEM of three independent experiments

(Nc-1) (Fig. 1c). In order to figure out the underlying defense mechanism, we added two well-known inhibitors of antiparasitic effectors, namely NgMMA (iNOS inhibitor) and supplemental tryptophan (functional IDO antagonist). Both inhibitors did not abrogate the inhibition of Nc-1 in mMSC (Fig. 1c). Comparable results were obtained with ME49 tachyzoites used as control (Fig. 1d). Therefore, iNOS and IDO were probably not involved in the mediation of the observed antiparasitic effect directed against low virulent parasites in mMSCs.

Expression of interferon-inducible GTPases in activated murine mesenchymal stromal cells

It has been shown that IRG-proteins are involved in the defense against *T. gondii* in several murine cell types. We therefore analyzed the expression of immunity-related GTPase proteins (IRGs) and guanylate-binding proteins (GBPs) in IFN-γ-activated mMSCs. Western blot analysis revealed that Irga6, Irgb6 and Irgd proteins were detectable in lysates of IFN-γ-activated (100 U/ml) mMSCs but not, or only in low amounts, in lysates of unstimulated cells (Fig. 2a). Additional quantitative PCR demonstrated that mGBP2, mGBP3 and mGBP5 are upregulated in mMSCs after IFN-γ stimulation as well (Fig. 2b–d). These data confirmed that IFN-γ-activated mMSC, like many other murine cell types, are capable of expressing GTPases that could contribute to the antiparasitic defense.

IRGs and GBPs are involved in the IFN-γ-induced resistance of murine mesenchymal stromal cells against *T. gondii* and *N. caninum*

In order to clear cells from *T. gondii,* IRGs as well as mGBPs have to accumulate at the parasitophorous vacuole (PV) of the parasite. To explore the role of interferon-inducible GTPases in the defense of mMSCs, we analyzed the targeting of IRGs and GBPs to the PV of different apicomplexan parasites.

While IRGs were suggested to target to the PV of ME49 tachyzoites, the PV of RH tachyzoites is usually not targeted. Figure 3a illustrates exemplarily that Irga6 is targeted to the PV of ME49 tachyzoites, replicating in IFN-γ-activated murine MSCs while the PV of RH tachyzoites is not decorated. In addition, we found that Irga6, Irgb6 and Irgd are targeted to the PV of *N. caninum* (Nc-1) (Fig. 3b).

Comparable data were obtained by analyzing the behavior of GBPs in mMSC. For example, in IFN-γ-activated mMSCs, mGBP1 is targeted to the PV of *N. caninum* Nc-1 and Nc-Liv. However, mGBP5 was not targeted to the PV of Nc-1(Fig. 4a) in mMSCs.

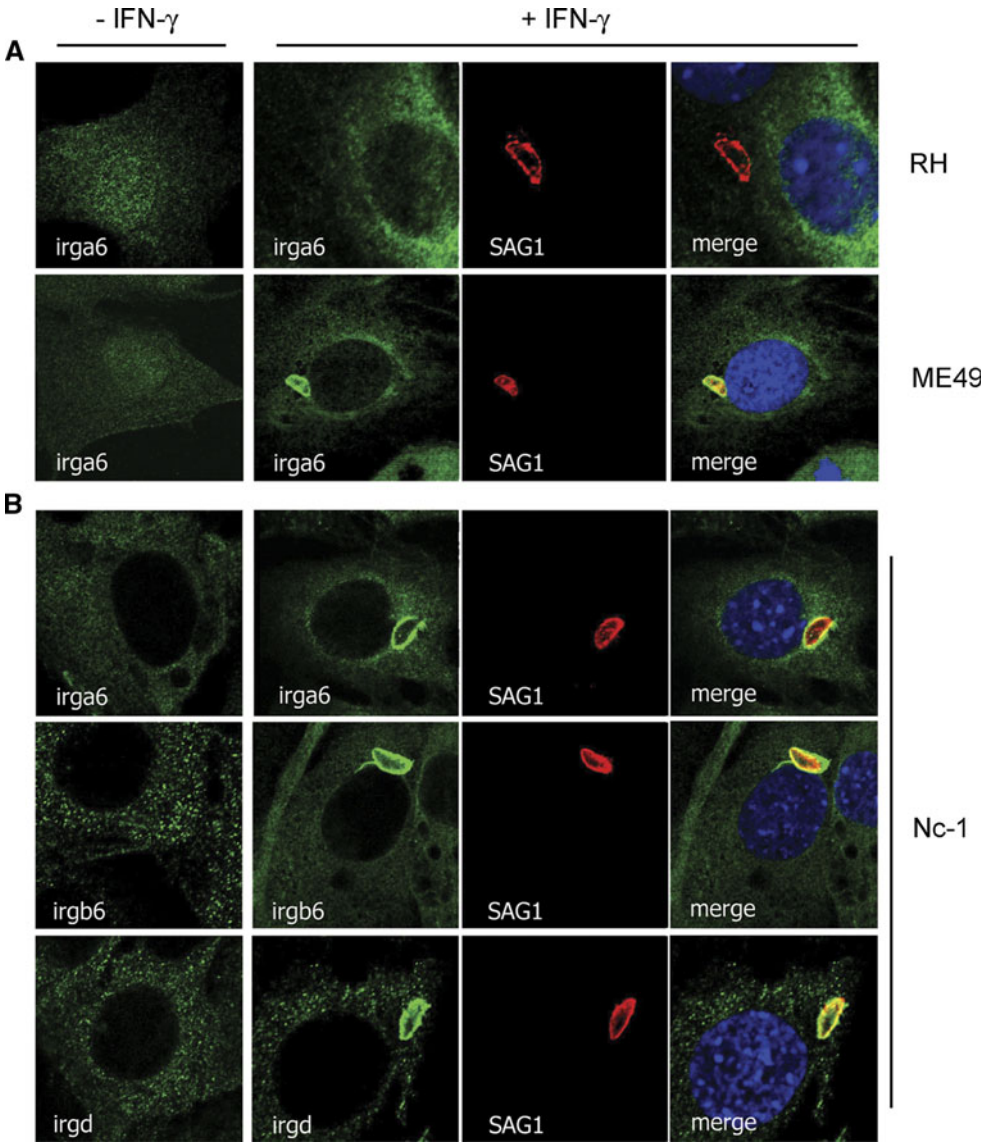

Fig. 3 Targeting of immunity-related GTPase proteins to the parasitophorous vacuole of *T. gondii* and *N. caninum* in murine mesenchymal stem cells Murine MSCs were cultured in the absence or presence of IFN-γ for 16 h and thereafter infected for 2 h with *T. gondii* strain RH or ME49 (**a**) or *N. caninum* Nc-1 (**b**). Intracellular parasites were stained in *red* with an antibody directed against a parasite surface antigen (SAG1), IRGs were detected in *green* with specific mouse mab. In addition DNA was stained with DAPI

In further experiments, we used murine (3T3) fibroblasts constitutively expressing recombinant GFP-labeled mGBP2 or mGBP5 as host cells for parasites. The advantage of this experimental setting is that specific antibodies as well as secondary antibodies were not necessary to test the targeting behavior of GFP-labeled mGBPs, which might result in the reduction of background staining by crossreactive antibodies in the antisera used. Figure 4b confirms that GFP-labeled mGBP5 is not targeted to the PV of Nc-1 tachyzoites in detectable amounts. As a control, GFP-mGBP2 expressing cells were used and after IFN-γ stimulation, mGBP2 is clearly targeted to the PV of Nc-1. In sum, our data indicate that IRGs as well as GBPs might participate in the defense against *N. caninum.*

Discussion

IFN-γ is a well-known inducer of antimicrobial effector molecules that frequently synergizes with other pro-inflammatory cytokines. In particular, the IFN-γ induced production of toxic oxygen or nitrogen radicals in macrophages has been intensively investigated. These toxic nitrogen radicals are involved in the control of the protozoan parasite *T. gondii* in in vitro and in in vivo studies. However, nitrogen radicals are effective not only against *T. gondii* strains of different virulence, but also against *N. caninum* and other pathogens as well [21, 22].

In 2011, we revealed that the inducible nitrogen synthase (iNOS) is expressed in mMSCs after co-stimulation with IFN-γ, IL-1 and TNFα and that only this

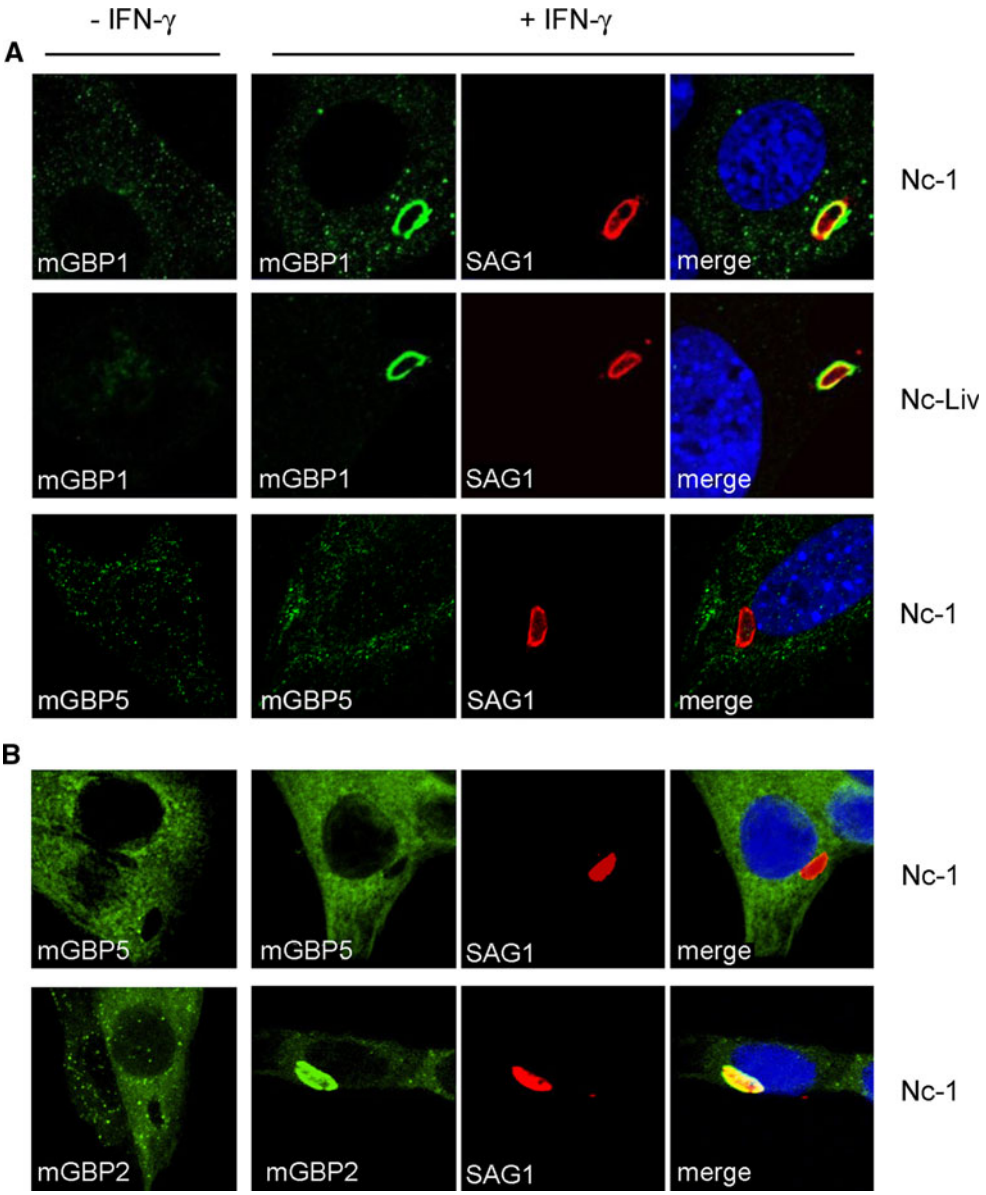

Fig. 4 Targeting of guanylate-binding proteins to the parasitophorous vacuole of *N. caninum* in murine cells. **a** Murine MSCs were cultured in the absence or presence of IFN-γ for 16 h and thereafter cells were infected for 2 h with *N. caninum* isolate Nc-1 or Nc-Liv. Intracellular parasites were stained in *red* with an antibody directed against a parasite surface antigen (SAG1), mGBP1 and mGBP5 were detected in green with specific rabbit antisera and DNA was stained with DAPI (*blue*). **b** Targeting of mGBP2 to the parasitophorous vacuole of *N. caninum*. Murine fibroblasts expressing GFP-labeled mGBP2 or mGBP5 were stimulated with IFN-γ and infected with *N. caninum*. An anti-SAG1 antibody was used to detect *N. caninum* (*red*) and DNA was stained with DAPI (*blue*)

co-stimulation resulted in a growth inhibition of virulent *T. gondii* BK tachyzoites. This antiparasitic effect was antagonized by NgMMA, an inhibitor of iNOS activity [9]. At the same time, we also showed that mMSCs were unable to inhibit the growth of *T. gondii* BK tachyzoites after stimulation with IFN-γ alone. Now, we analyzed the defense mechanisms against other Toxoplasma strains and, as well, Neospora isolates in mMSCs.

IFN-γ-activated mMSCs inhibited tachyzoite growth of *T. gondii* ME49 parasites (type II strain). Therefore, mMSCs behaved like other murine cells like, for example, murine fibroblasts [23]. This is in obvious contrast to data obtained with human MSCs which could restrict the growth of *T. gondii* type I and type II after stimulation with IFN-γ. An IFN-γ stimulation of human and bovine cells results in the induction of the tryptophan-degrading enzyme indoleamine 2,3-dioxygenase (IDO) that was responsible for antiparasitic effect directed against *T. gondii* and *N. caninum* [9, 20], while IDO was not or only marginally expressed in murine MSCs [5, 6, 9].

In this manuscript, we show that neither iNOS nor IDO contributed to the defense of IFN-γ-activated mMSCs directed against *T. gondii* type II strains, evidenced by the fact that a functional blockade of IDO or iNOS could not abrogate the observed antimicrobial effect. Therefore, different antimicrobial effector mechanisms must be active in mMSCs.

Pioneered by Taylor et al. [24], a new group of IFN-γ induced molecules termed "immunity-related GTPases" (IRG-proteins, also known as p47 GTPases), have been

recognized to contribute to resistance against *T. gondii* in murine cells. Several IRGs, for example, Irgd, Irgm3 and Irga6 have been identified as anti-toxoplasma defense molecules [14, 25].

In this manuscript, we present data that confirm the expression of IRG-proteins in mMSCs after IFN-γ stimulation. Furthermore, several IRGs expressed in mMSCs like Irga6, Irgb6 and Irgd were targeted to the parasitophorous vacuole (PV) of *T. gondii* ME49 and to the PV of *N. caninum* isolate Nc-1. In contrast, the same IRGs did not decorate the PV of type I RH tachyzoites. Therefore, mMSCs were, in this respect, comparable to other murine cell types [25, 26]. While IRG-proteins, at least in part, mediated their antiparasitic effect via disruption of the PV of type II Toxoplasma [27], type I Toxoplasma avoid the targeting and subsequent disrupture of the PV by phosphorylating IRG-proteins via ROP18 [28]. Since the IRG system was differentially active against different *T. gondii* strains, it was interesting to analyze the efficiency of IRGs in the defense against *N. caninum* isolates of different virulence. The Nc-1 isolate has been shown to be less virulent than the *N. caninum* isolate Liverpool (Nc-Liv) [17]. Recent data from Reid et al. [29] showed that Irg6a decorates the PV of *N. caninum* isolate Liverpool in IFN-γ-activated murine embryonic fibroblasts. In this manuscript, we demonstrate that the Irg6b and Irgd were also targeted to the PV of *N. caninum* Nc-1 in IFN-γ-activated mMSCs. Furthermore, Reid et al. [29] have performed extensive genomic and transcriptomic analyses and have described that ROP18 is pseudogenized in *N. caninum*. Since ROP18 is a major defense molecule of *T. gondii* type I strains in murine cells [30, 31], the obvious absence of functional ROP18 in *N. caninum* might explain the high sensitivity of this parasite to the activation of the IRG system [29]. The IRG system has been extensively analyzed in mice, and homologous proteins are present in many vertebrates. However, in humans, only one IRG homologous protein called IRGM has been described. In comparison with murine IRGs, human IRGM is truncated and there has been no evidence for an involvement of IRGs in antiparasitic defense in human cells [32].

The p65 guanylate-binding proteins (GBPs) (also known as p65 GTPases) are highly conserved within vertebrates and are involved in the defense against *T. gondii* [18]. Up to now, the functional importance of IFN-γ-regulated GBPs is not completely understood. Murine GBP2 (mGBP2) and human GBP1 (hGBP1) have been implicated in the regulation of cell growth [33, 34] and hGBP1 may have a weak antiviral effector function [35]. Recently, murine GBPs have been implicated in the control of *Listeria monocytogenes* and *Mycobacterium bovis* infections and have been described to promote oxidative killing and the delivery of antimicrobial peptides to autophagosomes [36]. In addition, using GBP deficient animals, it has been described that GBPs are involved in the targeting of Irgb6 to the PV of *T. gondii* [37]. Our experiments illustrate that mGBP1 is expressed in IFN-γ-activated mMSCs and was targeted to the PV of *N. caninum* Nc-1. Furthermore, mGBP1 was also targeted to the PV of tachyzoites of the higher virulent isolate Nc-Liv. In addition, mGBP5 was expressed in IFN-γ-activated mMSC, but did not target to the PV of *N. caninum* (Nc-1). The negative result for mGBP5 was confirmed in experiments with IFN-γ-activated fibroblasts expressing GFP-labeled mGBP5.

The targeting behavior of mGBP1, mGBP2 and mGBP5 to the PV of *N. caninum* was identical to the distribution of these mGBPs in IFN-γ-stimulated, *T. gondii* (ME49)-infected murine fibroblasts and macrophages [18]. In addition, a targeting of mGBP1 to the PV of *T. gondii* (Pru) has been proven by Virreira-Winter et al. [38]. In contrast to our results, this group has described a targeting of GFP-mGBP5 to the PV of this *T. gondii* type II strain. However, in comparison with the data obtained with GFP-mGBP2, the PV staining by GFP-mGBP5 was less intense. They explained the different findings concerning mGBP5 targeting by the use of different methods. In fact, while in our previous work [18], we used an mGBP5-specific antiserum and Virreira-Winter et al. detected GFP fluorescence of recombinant proteins. Using a comparable experimental setting, we were unable to detect mGBP5 targeting to the PV of *N. caninum* in IFN-γ-activated GFP-mGBP5 over expressing cells. This discrepancy might be explained by a different sensitivity of the detection methods or the use of different parasites and host cells. However, we have been unable to detect a targeting of endogenous GBP5 to the PV in *T. gondii* infected fibroblasts (18) or murine mesenchymal stromal cells.

In sum, the data, despite some differences in details, indicate that mGBPs, in addition to IRGs, might contribute to the defense against *T. gondii* in murine cells. In contrast to IRGs, mGBPs are expressed in mice and man, and therefore, additional studies are necessary to define the antiparasitic effector mechanisms of GBPs in human cells [36].

Although the IRG system is a potent antiparasitic effector mechanism against *T. gondii* in mice, it is not involved in the defense against apicomplexan parasites in general. Liesenfeld et al. [14] found that while Irga6-deficient animals, in comparison with wild type animals, are susceptible to *T. gondii* infection, the defense against the apicomplexan parasite *Plasmodium berghei* (*P. berghei)* was not influenced. Additionally, further analyses confirmed that Irga6, as well as Irgb6, Irgd, Irgm1 and Irgm3, did not target to the PV of *P. berghei*.

However, IRGs targeted to the PV of *N. caninum*, an evolutionary close relative to *T. gondii*. The same has also

been observed for the targeting of native GBP1 to the PV of *N. caninum*. We suggest that mice, and probably other rodents, in contrast to humans, successfully exploited the ancient vertebrate IRG system during co-evolution with coccidian apicomplexa. The IRG system defends these animals against the evolutionary closely related parasites *T. gondii* and *N. caninum*, whereas *P. berghei,* more distantly related to *T. gondii,* can circumvent the antiparasitic effect of IRGs by unknown mechanisms. The high effectiveness of the IRG system against *N. caninum* might be responsible for the observation that mice are relatively resistant against an infection with *N. caninum*. Mice do usually not suffer from neosporosis, although mice get into contact with *N. caninum* in their natural habitat [15]. Even though *N. caninum* has been able to replicate well in unstimulated murine cells in vitro, experimental infection of mice with up to 1×10^6 tachyzoites from different *N. caninum* isolates has not resulted in enhanced mortality. Furthermore, less than 2 % of these animals have developed clinical signs compatible with neosporosis [16], which might be explained by the high efficiency of IRGs and GBPs against this parasite.

In sum, our data indicate that mMSCs, like hMSC, in addition to their impressive immunosuppressive capacity, are endowed with highly efficient antiparasitic effector mechanisms. However, effector mechanisms used to control apicomplexa parasites are species-specific. This must be taken into account, especially if mMSCs were used in in vivo experiments to mimic the human situation.

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