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Inhibition of Early Mouse Plasmacytoma Development in Case of *Plasmodium* Infection

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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Short Research Article

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ABSTRACT

Background: Inhibition of early cancer development, through enhancement of cancer immunosurveillance by innate cells has been reported after infection with different microorganisms. Since *Plasmodium* infection modulates the innate immune system, we examined the effect of *Plasmodium* infection on cancer immunosurveillance.
Methods: As a model, we used *Plasmodium yoelii 265 BY* infection of BALB/c mice and administration of TEPC.1033.C2 plasmacytoma cells.
Results: *Plasmodium* infection effectively inhibited the early development of plasmacytoma. The protective effect of infection was not due to a direct cytopathic destruction of cancer cells by the parasite. *Plasmodium* infection induced the production of proinflammatory cytokines such as interleukin-12 and interferon-gamma, known to be involved in cancer immunosurveillance. Depletion of $ASGM1^+$ cells *in vivo* largely suppressed the protective effect of the parasite.

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Conclusions: *Plasmodium* infection could influence on the inhibition of early mouse plasmacytoma. This observation suggest *Plasmodium* infections in humans might participate to the low incidence of multiple myeloma in countries where malaria is frequent and should be taken into account in public health policy of these countries.

Keywords: Plasmodium yoelii; plasmacytoma; interferon-gamma; interleukin-12; ASGM1+ cells.

ABBREVIATIONS

NK: Natural Killer; IFN: Interferon; IL: Interleukin; TNF: Tumor Necrosis Factor; LDV: Lactate Dehydrogenase-elevating Virus; TLR: Toll-Like Receptor; ASGM1: Asialoganglioside-GM1.

1. INTRODUCTION

Efficient control of early cancer development depends on immunosurveillance mediated by immune cells including Natural Killer (NK) and NKT cells and by their production of cytokines such as interferon-gamma (IFN-γ) and tumor necrosis factor (TNF) [1,2]. Although many cancers may be caused by infectious agents, some infections may enhance cancer immunosurveillance, leading to inhibition of early cancer development [3,4]. In mouse experimental models, infections with lactate dehydrogenase-elevating virus (LDV) or *Trypanosoma brucei*, and stimulation with Toll-like receptor (TLR) ligands similarly decrease early growth of tumors such as plasmacytoma/myeloma and mesothelioma [5-8]. This early inhibition of cancer development requires asialoganglioside-GM1 (ASGM1) positive cells and the production of proinflammatory cytokines such as IFN-γ and interleukin-12 (IL-12).

Multiple myeloma incidence is lower in developing countries where infections with a large array of viruses, bacteria and parasites, including *Plasmodium*, are higher than in industrialized countries [9,10]. Interestingly, similarly to LDV, *Plasmodium* infection results in activation of NK and NKT cells, and in increased IFN-γ production [11]. Therefore, we investigated whether mouse *Plasmodium* infection could also result in early inhibition of plasmacytoma development.

2. MATERIALS AND METHODS

2.1 Mice

Female BALB/c mice were bred at the Ludwig Institute for Cancer Research by P. Gomez Pinilla and used at the age of 7–10 weeks. For this observational study with control groups, a total number of 178 mice was used.

2.2 Plasmodium Infection

Blood-stage samples of *Plasmodium yoelii 265 BY* were kindly given by S. Pied (Lille, France). The *Plasmodium* stock was checked to be free of LDV contamination. Mice were infected by i.p. injection of 10^6 infected erythrocytes (iRBCs).

2.3 Tumor Cells

TEPC.1033.C2 plasmacytoma cell line, initially received from M. Potter, was cultured in supplemented Iscove's Modified Dulbecco's Medium. $4x10^4$ live cells in 500 µl PBS were injected intraperitoneally after being washed twice and counted with trypan blue as described previously [8]. 2 mg of anti-ASGM1 in 500µl of PBS were administered 8 days after *Plasmodium* infection, followed by 1 mg of anti-ASGM1 in 300 µl of PBS 2 days later.

2.4 Antibodies

Cell depletion with anti-asialoganglioside-GM1 (ASGM1) polyclonal antibody was performed as described previously [8].

2.5 Cytokine Assays

Cytokines were measured in sera by ELISA. IL-12 levels were measured as described previously [12]. For TNF and IFN-γ, Maxisorb ELISA plates (Nunc, UK) were coated with 4 µg/mL of anti-TNF-α (ref. 14-7325-85) and 2 µg/mL of anti-mouse IFN-γ (ref. 14-7311-85, eBioscience Inc.), respectively. After blocking in PBS with 10% FCS, samples were incubated for 2hr at 37°C, followed by detection antibodies (4 µg/mL of biotinylated TNF- α Antibody Cocktail (ref .13-7326-85) and 4 µg/mL of biotinylated IFN- γ Monoclonal Antibody (ref. 13-7311-85, eBioscience Inc.), by avidin-HRP (1:2000 dilution; ref. 405103, Biolegend), by 1-Step™ Ultra TMB-ELISA (ref. 34028, Thermo Fisher Scientific), and by 20 µl of stop solution

(2M H_2SO_4). The absorbance reads were made at 450 nm, using a 96-well plate spectrophotometer (VERSAmax, Molecular Device).

2.6 Statistical Analysis

Statistical analysis was performed with Prism 6 (GraphPad Software, La Jolla, CA) using a non-parametric test (Mann–Whitney), and a Log-rank test (survival curve).

3. RESULTS

3.1 Effect of *Plasmodium Yoelii* 265 by Infection on Plasmacytoma Development

The impact of *Plasmodium yoelii* infection on early plasmacytoma growth was examined by administration of TEPC.1033.C2 cells into control BALB/c mice or into animals that had been infected 10 days earlier. This time was chosen as the beginning of parasitemia and anemia increase after *Plasmodium yoelii* inoculation (data not shown). The majority of uninfected mice quickly developed tumors and died around 20 days after tumor cell administration (Fig. 1A). In contrast, plasmacytoma development in mice infected with *Plasmodium* was significantly delayed (Fig. 1A, $p<0.0001$, three pooled independent experiments). Although most animals finally developed tumor and died, some of them were still free of clinical signs of tumor for more than six weeks in some experiments.

To determine whether the alteration of plasmacytoma development after *Plasmodium* infection was caused by direct effect of the parasite on cancer cells, we cultured TEPC.1033.C2 cells in the presence of *Plasmodium*. *In vitro* tumor cell replication was measured on day 3, 5, and 7. No difference in cell proliferation was found between with and without *Plasmodium* (Fig. 1B, representative of two independent experiments).

3.2 Involvement of ASGM1+ Cells in the Protective Effect of Plasmodium Infection on early Plasmacytoma Growth

ASGM1+ cells have been found previously to be responsible for the protective effect of LDV and of TLR ligands on early cancer growth [5,7,8]. To determine whether these cells were also involved in the protective effect of *Plasmodium*, mice were treated with anti-ASGM1 polyclonal antibody two days before, and the day of tumor cell inoculation. As shown in Fig. 1C, this treatment completely suppressed the protective effect of *Plasmodium* protection ($p=0.0002$ for TEPC + *P. yoelii* versus TEPC + *P. yoelii* + anti-ASGM1, three pooled independent experiments), demonstrating the role of ASGM1+ cells.

3.3 Cytokine Production after *Plasmodium yoelii* 265 by

Activation of immune innate cells involved in cancer immunosurveillance results in production of proinflammatory cytokines. Some of these cytokines, such as IL-12 and IFN-γ have been shown to play a major role in the inhibition of plasmacytoma growth after LDV infection [5]. We therefore analysed the effect of *Plasmodium yoelii* infection on their secretion. At day 10 after *Plasmodium* infection, we observed a significant increase of IL-12 (Fig. 2A, $p=0.0294$), as well as a moderate, although non significant, increase of IFN-γ ($p=0.0797$) and of TNF ($p=0.2676$). Similar results were obtained in two independent experiments. To determine whether this cytokine production originated from ASGM1+ cells, we treated infected mice with depleting anti-ASGM1 antibody. As shown in Fig. 2B, depletion of ASGM1+ cells did not result in a decrease of IFN-γ ($p=0.2436$) nor TNF ($p=0.4277$) but induced a decrease in IL-12 production ($p=0.0594$).

4. DISCUSSION

Although the oncogenic capacity of many infectious agents has been demonstrated, some of them have been reported to enhance cancer immunosurveillance, both in humans and in animal models [3-8,13]. Moreover, *Plasmodium* infection occurring at the time or after cancer cell inoculation in mouse models of Lewis lung cancer, breast cancer, and hepatoma inhibits tumor development through different mechanisms, including enhancement of antitumoral immune responses [14-19]. These observations have led to the approval of clinical trials of malaria immunotherapy in China. In our study, we found that the enhancement of innate immune responses after *Plasmodium* infection may also result in a more efficient cancer immunosurveillance, leading to further prevention of plasmacytoma growth. This preventive effect of *Plasmodium*, as well as of LDV infection [5,7] is mediated by $ASGM1^+$ cells that include NK cells, NKT cells and a subpopulation of $CD8^+$ T

cells that share the capacity of early non cognate response, including IFN-γ production [20]. A similar enhancement of cancer immunosurveillance has been reported after ligation of TLR receptors, and especially of TLR9 [21] and was also shown to require ASGM1+ cells [8]. Interestingly, *Plasmodium* parasites activate the innate immune system through ligation of TLR9 by their byproduct hemozoin [22], and therefore this mechanism might be at least partly responsible for the parasite preventive effect. However, it remains to be identified which cell population, among the ASGM1+ cells, is responsible for this prevention of tumor growth.

IL-12, activating NK and NKT cells, and IFN-γ, produced in response to IL-12 by those cells are strongly involved in antitumoral responses and have been shown to be involved in the preventive effect of infections on tumor growth [5-8,13,23]. In addition, although TNF is known to alter tumor progression, it has been reported to sometines exert the opposite effect [24]. Although the production of these three cytokines was enhanced after *Plasmodium* infection, we were not able to demonstrate their role in the prevention of plasmacytoma growth in our model. Moreover, in our model, neither IFN-γ nor TNF were produced by ASGM1+ cells that were however required for prevention of cancer growth. This contrasts with LDV infection that induces prevention of plasmacytoma growth through IFN-γ production by NK and NKT cells [5]. It may be hypothesized that in our model of *Plasmodium* infection, IFN-γ is produced mostly by T lymphocytes, while TNF might be produced by

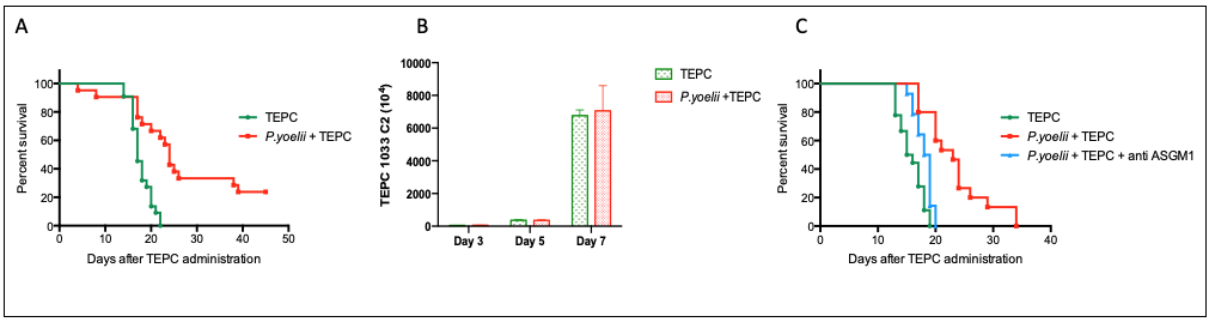

Fig. 1. Effect of *Plasmodium* infection on TEPC.1033.C2 plasmacytoma cell early growth

A. Survival of BALB/c mice uninfected or infected with Plasmodium yoelii 265 BY 10 days before administration of 4x10^4 TEPC.1033.C2 cells. Pooled data from three independent experiments, 22 mice per group
B. Cell number after different times of culture of 4x10^4 TEPC.1033.C2 cells in the presence of 2x10^6 iRBCs. Results as means ± SEM for triplicate cultures, representative of two independent experiments
C. Survival of BALB/c mice uninfected or infected with Plasmodium yoelii 265 BY 10 days before administration of 4x10^4 TEPC.1033.C2 tumor cells, without or with anti-ASGM1 antibody treatment. Pooled data from three independent experiments, 18 mice per group

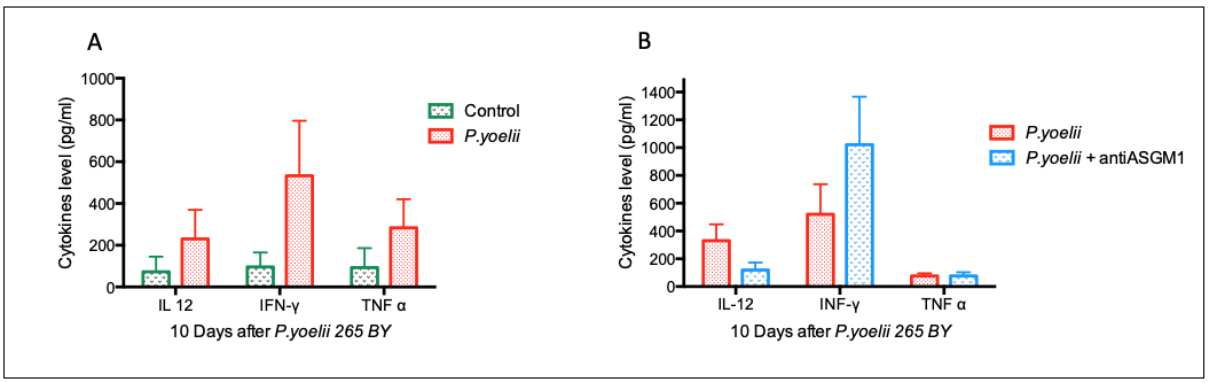

Fig. 2. Cytokine analysis after *Plasmodium yoelii* infection

A. IL-12, IFN-γ and TNF were measured in sera of BALB/c mice uninfected or infected for 10 days with Plasmodium yoelii 265 BY. Results as means ± SEM for 8 mice per group
B. IL-12, IFN-γ and TNF were measured in sera of BALB/c mice infected with Plasmodium yoelii 265 BY obtained 2 days after the second anti-ASGM1 antibody treatment and in control infected but untreated animals. Results as means ± SEM for pooled data from two independent experiments, 8 mice per group

macrophages. Surprisingly, IL-12 production was partly suppressed after depletion of ASGM1+ cells. It should be determined whether this reflects production of this cytokine by one ASGM1+ cell subset, or an indirect effect of these ASGM1+ cells on IL-12 production by other cells such as dendritic cells. The possible role of these cytokines in *Plasmodium*-induced prevention of plasmacytoma growth would require most extensive investigation.

5. CONCLUSION

Whatever the molecular mechanisms responsible for the protective effect of *Plasmodium* infection on plasmacytoma early growth, our results strongly suggest that common infections may have an incidence on the prevalence of cancers that are sensitive to immunosurveillance. This effect may be to decrease this incidence or to enhance it. Indeed, infectious agents that can inhibit NK cell activation and IFN-γ production, such as *Schistosoma*, rather enhance the early growth of plasmacytoma in mice and might increase the incidence of multiple myeloma in exposed populations (M. Mandour, manuscript in preparation). It may nevertheless be suggested that in low and middle income countries, the global effect of infections enhancing cancer immunosurveillance exceeds factors that promote tumor growth, resulting in a global lower incidence of some cancers, including multiple myeloma.

HIGHLIGHTS

- *Plasmodium* infection inhibited early mouse plasmacytoma development;
- This effect was mediated by ASGM1+ immune cells;
- It was correlated with an overproduction of IL-12, TNF and IFN-γ

CONSENT

It is not applicable.

ETHICAL APPROVAL

Experiments were approved by the Comité d'Ethique facultaire pour l'Expérimentation Animale - Secteur des Sciences de la Santé - Université catholique de Louvain (ref. 2018/UCL/MD/007).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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