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Inhibition of myeloid-derived suppressor cell (MDSC) activity by redox-modulating agents restores T and B cell proliferative responses in murine AIDS

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ABSTRACT

The mechanisms by which myeloid-derived suppressor cells (MDSCs) mediate inhibition prominently include the production of reactive nitrogen species, in particular those generated by inducible nitric oxide synthase (iNOS), and reactive oxygen species. LP-BM5 murine retroviral infection results in a profound immunodeficiency, known as murine AIDS, as well as in increased numbers and activity of monocytic-type MDSCs (M-MDSCs) that suppress both T and B cell responses. While M-MDSCs suppress T cells *ex vivo* in a fully iNOS/NO-dependent manner, M-MDSC suppression of B cell responses is only partially due to iNOS/NO. This study preliminarily explored the role of two redox-modulating compounds in inhibiting the M-MDSC suppressive activity in LP-BM5 infection. The tested molecules were: I-152 consisting in a conjugate of N-acetyl-cysteine (NAC) and S-acetyl-cysteamine (SMEA) and C4-GSH that is the n-butanoyl glutathione (GSH) derivative. The results show that both molecules, tested in a concentration range between 3 and 20 mM, blocked the M-MDSC suppression of activated B and T cells *ex vivo* and restored their proliferative capacity *in vivo*. *Ex vivo* I-152 blockade of M-MDSC suppressiveness was more significant for T cell (about 70%) while M-MDSC blockade by C4-GSH was preferential for B cell responsiveness (about 60%), which was also confirmed by *in vivo* investigation. Beyond insights into redox-dependent suppressive effector mechanism(s) of M-MDSCs in LP-BM5 infection, these findings may ultimately be important to identify new immunotherapeutics against infectious diseases.

1. Introduction

Initial studies about MDSCs focused on their role in tumor biology, however, in the last years an important role for MDSCs in the control of immune responses to pathogens, including viruses, has been highlighted [1–3]. The functional role of MDSCs in viral diseases is to inhibit host defence and regulate inflammatory cytokines. Similar to the tumor microenvironment, MDSCs suppress antiviral host immunity by impairing the function of several immune cells, although most studies have been focused on the MDSC role in suppressing T-cell responses. Among the soluble factors allowing the functions of MDSCs are three key mediators: reactive oxygen species (ROS), inducible nitric oxide synthase (iNOS), and arginase-1, which play their role both independently and in concert with each other [4]. Upregulated iNOS in MDSCs metabolizes L-arginine into L-citrulline and nitric oxide (NO) which drives several molecular blockades in T cells [4–5]. ROS are directly involved in MDSC-mediated T cell suppression and inhibit MDSC differentiation. Arginase-1 contributes to L-arginine depletion, which is particularly important for the survival and function of T cells [6]. Interestingly, arginase-1 is regulated by Th1/Th2 cytokines, in fact, arginase-1 is induced by the Th2 cytokine IL-4, suggesting a cross-talk between MDSCs and T cells [7–8].

Murine MDSCs have been classified into two distinct groups: neutrophilic/granulocytic (PMN)-MDSCs and monocytic (M)-MDSCs with respect to their surface phenotype [9]. To shed light on the role and the suppressive mechanisms of MDSCs in retroviral infection, previous studies have been conducted on a murine retroviral immunodeficiency syndrome (MAIDS) model consisting of B6 mice infected with the LP-

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BM5 retroviral isolate. LP-BM5 infection induces M-MDSCs, which have been characterized with respect to cell surface phenotype, their suppressive activity, and the molecular mechanisms of inhibition [9]. In particular, an immunosuppressive M-MDSC (CD11b+; CD16/32+; GR-1+; Ly6G-/low; Ly6C+/high) population strongly inhibited proliferative and functional responses to polyclonal activators of both T cells and B cells. Suppression of T cell responses by LP-BM5-expanded M-MDSCs was found to be fully dependent on iNOS/NO, while suppression of B cell responses was only partially dependent on iNOS/NO [10–11]. Although most studies examined MDSC-mediated suppression of T cells, in the LP-BM5 retroviral system the additional mechanism(s) that are used by M-MDSCs to suppress B cells were further investigated. M-MDSCs from LP-BM5-infected mice suppressed B-cell responsiveness in part by a contact-dependent mechanism through the VISTA (variable immunoglobulin domain suppressor of T-cell activation) ligand [11], but also in a contact-independent manner by soluble mediators including NO, superoxide, peroxynitrite, and TGF-β [10].

It has been previously demonstrated that alteration in the intracellular redox balance towards a more oxidative state occurs in the organs of LP-BM5 retrovirus-infected mice leading to unbalanced Th1/Th2 immune responses in favour of the Th2 [12]. In this context, treatment with N-(N-acetyl-L-cysteinyl)-S-acetylcysteamine (I-152) restored glutathione (GSH)/cysteine levels in multiple organs and a balanced Th response [12]. I-152 crosses the cell membrane, and inside the cell, it is metabolized providing high amounts of thiol species in the form of its dithiol derivative, cysteine, NAC, and cysteamine (MEA) which are partially used to enhance/restore GSH [13]. Moreover, I-152 was found to activate the Nrf2 pathway [13], known to play a pivotal role in controlling the expression of antioxidant genes, including those involved in GSH synthesis [14]. Interestingly, activation of Nrf2 and inhibition of ROS generation in MDSCs have been already proposed as therapeutic anticancer strategies [15]. Indeed, up-regulation of GSH synthesis or boosting GSH by NAC resulted in MDSC differentiation and improvement of immune responses [16]. Based on these observations, in this study, we evaluated whether I-152 affected M-MDSC suppressive activity in LP-BM5 infection. Moreover, to discern the effect of GSH on the suppressive MDSC activity, experimental conditions where modulation of intracellular redox state was achieved through C4-GSH (instead of I-152) were set up. C4-GSH is a hydrophobic GSH derivative with higher stability in circulation than GSH and able to easily cross the cell membrane [17]. Differently from I-152, redox changes induced by C4-GSH strictly depend on the increase of GSH and only partially by cysteine [17]. C4-GSH has been already employed as an antiviral and an immunomodulatory agent in different *in vitro* and *in vivo* models [17–18].

The activity and survival of MDSCs are under redox control and depend on ROS/RNS levels [19]. Although oxidative stress is one of the constituents by which MDSCs suppress T and B cell function, the relevance of how modulation of the redox state could influence MDSC activity and the impact on *in vivo* responses remained to be investigated.

2. Materials and methods

2.1. Redox-modulating agents

C4-GSH was synthesized by Gluos Srl (Urbino, Italy) [20]. I-152 was synthesized as described previously [21]. Characterization data for I-152, including ^{1}H NMR and ^{13}C NMR, are available on page S11 (as compound 6) in reference 21. The molecular structure of the two compounds is depicted in Fig. 1A.

2.2. Ex vivo studies

Mice: C57BL/6 mice, purchased from Charles River/NCI, USA, and housed in the Center for Comparative Medicine and Research, Geisel School of Medicine at Dartmouth, were either uninfected or infected by LP-BM5 mouse retrovirus at 8–10 weeks of age, via intraperitoneal injection as described [10].

M-MDSC suppressive activity tests: as sources of M-MDSCs, splenocyte suspensions from naïve or LP-BM5-infected mice (6–8 weeks post-infection) were enriched for M-MDSCs on the basis of CD11b- and Ly6C-positivity, and Ly6G-negativity, as published [10] and schematically depicted (Fig. 1B). M-MDSCs were pre-treated for 2 h with C4-GSH or I-152 at different concentrations (5, 10, 20 mM and 3, 6, 8 mM respectively) according to the experimental conditions (Fig. 1B). These concentrations were selected in preliminary *in vitro* activity and toxicity experiments performed in *ex vivo* MDSC suppression assays (not shown). In the experiments where iNOS/NO activity was evaluated, after washing off the molecules, 0.8 mM LNMMA (N^{G}-monomethyl-L-Arginine) was added for 1 h before the start of the co-culture with the responder cells as previously described [11]. Responder cell proliferation was measured by terminal ^{3}H-Thymidine pulse labelling of triplicate wells after 66 h activation of B and T cells through 10 μg/ml LPS or 2.5 μg/ml anti-CD3 and 1 μg/ml CD28 respectively (Fig. 1B). The mean CPM, that were statistically compared by the one-tailed, two-sample equal variance, Student T test, formed the basis for determining the inhibition by the M-MDSCs, and, subsequently, the percent blocking of MDSC activity by the two molecules (Supplementary Fig. 1). Five independent experiments were performed in M-MDSC suppressive activity tests where the inhibitory activity of the molecules was measured and two independent experiments were conducted with the iNOS/NO inhibitor.

2.3. In vivo studies

Mice and infection: the mice were housed and treated in compliance with the recommendations of the Health Ministry of Italy, Law 26/2014, and the experiments were done upon authorization number 279/2015-PR by the Health Ministry of Italy.

Four-week-old female C57BL/6 (B6) mice were purchased from Charles River Laboratories Italy and housed in the Department of Biomolecular Sciences (University of Urbino) animal facility.

The LP-BM5 retrovirus was prepared as previously described [10] and mice were infected according to the protocol reported in ref. 12. Briefly, mice were infected by two successive intraperitoneal injections at 24-h intervals of LP-MB5 murine retrovirus stock, in which each injection contained 0.25 units of reverse transcriptase (RT).

The experimental groups were: the uninfected, the infected (I), the infected receiving either C4-GSH or I-152, and the infected receiving placebo (200 μl of 0.9% NaCl). At 1 h after each virus inoculation, the animals treated with the redox-modulating agents received i.p. injections of I-152 or C4-GSH (30 μmol/mouse in 200 μl of 0.9% NaCl). Treatments were repeated three times a week for a total of 9 weeks. The doses of the molecules were selected based on our previous data on mice where I-152 and C4-GSH were administered intraperitoneally without exerting any side effects [12,18].

At different times after virus inoculation, eight mice per group were sacrificed; the spleen weight of all animals was measured, while five mice of each group were randomly selected for evaluation of spleen cell proliferative index.

Lymphocyte proliferative index: splenocytes were prepared from all experimental groups and distributed in multiwell (96-well) plates at 5×10^{5} cells in a volume of 200 μl in quadruplicate, stimulated with 10 μg/ml PHA (T cells) or 50 μg/ml LPS (B cell) and incubated at 37 °C in a 5% CO_2 atmosphere. After 70 h, each well received 1 μCi of ^{3}[H]-Thymidine and after 8 h each sample was precipitated with 100 μl of 10% (w/v) trichloracetic acid, collected on glass microfibre filters, washed with 3% (w/v) of trichloracetic acid and the amount of acid insoluble radioactivity counted by liquid scintillation counting. Statistical analysis was performed by GraphPad Prism™ 6.0 software (GraphPad Software Inc., San Diego, California, USA) using a one-way Anova test.

Statistical analysis was performed on at least five animals.

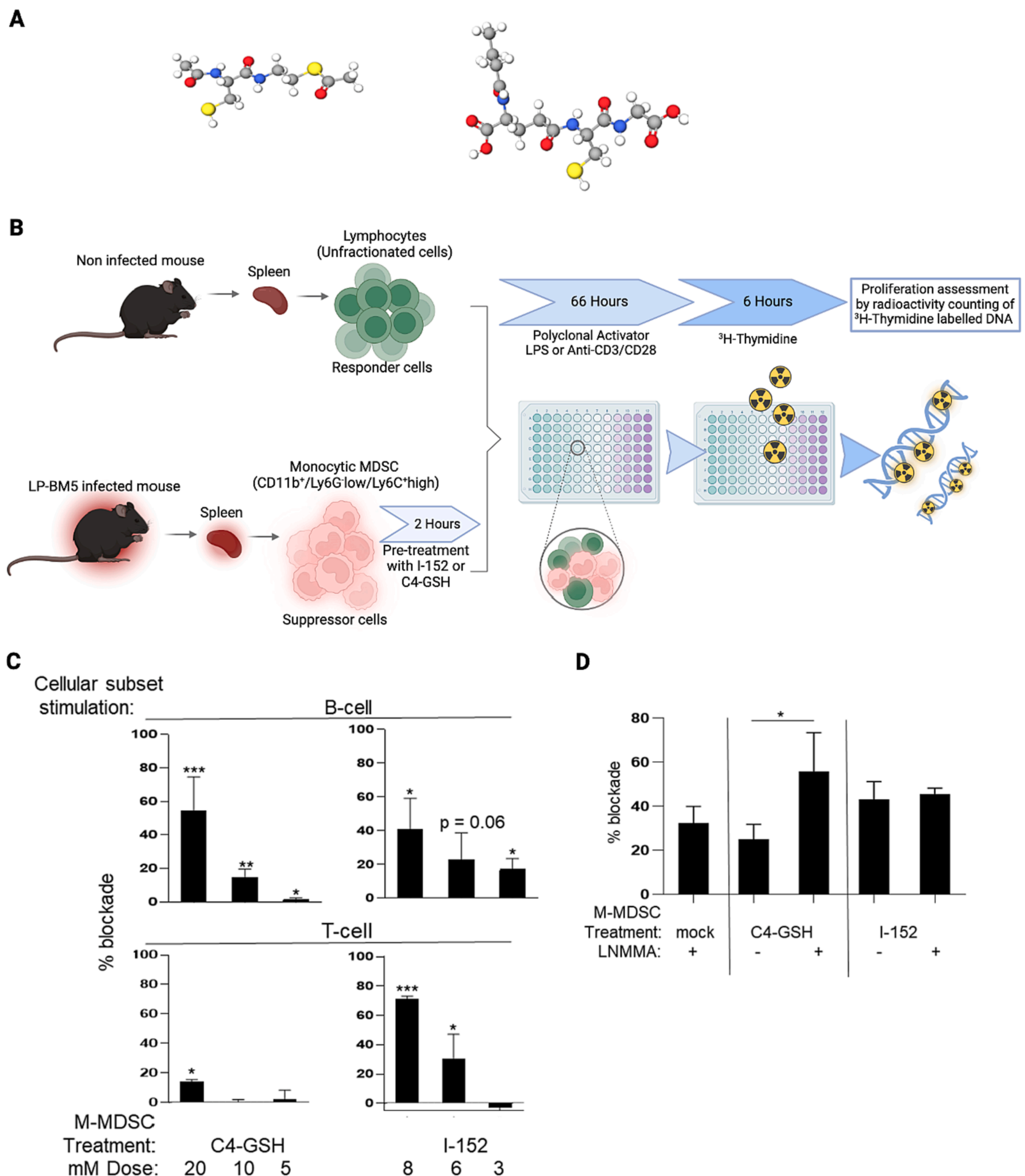

Fig. 1. **(A)** Chemical structure of the molecules used in this study: I-152 (left) and C4-GSH (right). I-152 is a conjugate of N-acetyl-cysteine (NAC) and S-acetyl-cysteamine (SMEA). C4-GSH is the n-butanoyl GSH derivative with an aliphatic chain bound to the α-NH_2 group of Glu. White spheres: hydrogen; grey spheres: carbon; blue spheres: nitrogen; yellow spheres: sulphur; red spheres: oxygen. **(B)** Schematic setup testing blockade of monocytic MDSC (M-MDSC) inhibition of activated B- or T-cell proliferation (induced through 10 μg/ml LPS or 2.5 μg/ml anti-CD3 and 1 μg/ml CD28 respectively), as assessed by the metabolic incorporation of ^{3}H-Thymidine into cellular DNA. **(C)** M-MDSC suppression assays, using a responder cell to suppressor cell ratio of 4:1, demonstrate overlapping but distinct blocking activities by C4-GSH vs. I-152. The percentage of blockade by C4-GSH or I-152 was normalized to the residual response from co-cultures of responder cells and sham-treated M-MDSCs. The pattern of results depicted is representative of five total experiments. Error bars represent the standard deviation of the means. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. **(D)** M-MDSC suppression assays in the presence of iNOS inhibitor LNMMA resulting in additive blocking of MDSC suppression of B-cell proliferation only by C4-GSH. M-MDSCs were pre-treated with C4-GSH (20 mM) or I-152 (8 mM) (2 h-pre-treatment with washout), and then with LNMMA (0.8 mM). Using a 4:1 ratio of the responder to suppressor cells, M-MDSC suppressive activity and blockade percentage were evaluated. The same pattern of results was seen in a total of 2 experiments. *$p < 0.05$.

3. Results and discussion

MDSC activity and survival are tightly linked to oxidative stress being dependent on ROS/RNS levels [19]. However, the influence of redox modulation on MDSCs is still elusive. In order to dissect whether redox-active compounds, such as I-152 and C4-GSH (Fig. 1A), could exert their action on MDSC suppressive activity, we measured the *in vitro* proliferative responses to the T-cell polyclonal stimulator anti-CD3/CD28 and the B-cell mitogen LPS, considered routine measures of the degree of unresponsiveness of MAIDS [10]. As reported, the suppressive CD11b + CD16/32 + GR-1 + Ly6C+/Ly6G- monocytic M-MDSC subset obtained from B6 mice previously infected with LP-BM5 caused marked suppression of both activated B-cell and T-cell proliferative responses, compared to either no suppressor cell addition or addition of spleen cells from non-infected B6 mice [10]. In this study, M-MDSCs were pre-treated for 2 h with the molecules before addition to the responder cells in suppression co-cultures (Fig. 1B). As shown in Fig. 1C and Supplementary Fig. 1 both molecules blocked the suppression of polyclonally-activated lymphocytes by LP-BM5-augmented M-MDSCs in a dose-dependent manner. C4-GSH blockade of M-MDSC suppressiveness was more significant for B cell (~60%), rather than T cell (only ~ 15%) responses at the highest concentration, whereas M-MDSC blockade by I-152 (8 mM) was significant for both responses, but preferential for T cell responsiveness (~70% and ~ 40% for T- and B-cell responses, respectively).

Although MDSCs, that are present in most cancer patients, inhibit T-cell-mediated antitumor immunity by the production of arginase, ROS, iNOS, interleukin-10, and through depletion of cysteine [22], iNOS/NO has been previously described as the main, if not sole, mechanism involved in M-MDSC-mediated suppression of activated T cell proliferation and IFN-gamma production in the LP-BM5 retroviral system. On the other hand, M-MDSC suppression of B-cell proliferation and IL-10 production in response to LPS was only partially iNOS-dependent [10]. To further characterize the mechanisms(s) by which C4-GSH and I-152 block M-MDSC activity, we focused on B-cell activation/proliferation, where approximately half of the M-MDSC suppression was due to iNOS-dependent mechanisms [9–11]. To this aim, M-MDSCs were i) untreated; or ii) treated with only the iNOS inhibitor LNMMA, as we previously reported [11]; or iii) pre-treated with C4-GSH or I-152 after which a portion of these cells was also treated with LNMMA, all before their addition to responder cell cultures polyclonally stimulated with LPS (Fig. 1D). The blockade of M-MDSC activity by C4-GSH and LNMMA was clearly additive, suggesting that C4-GSH inhibited iNOS-independent mechanism(s) of M-MDSC suppression of B cells. Indeed, there are several mechanisms by which M-MDSCs hinder B cell responses, including contact-dependent ones and those mediated by soluble factors [9–11]. In sharp contrast, the blocking by I-152 was not augmented by LNMMA co-treatment. These dichotomous results implied that for B-cell responses: 1) the mechanisms of action by C4-GSH vs I-152 on M-MDSC function are different, and 2) I-152 blocks M-MDSC activity in an iNOS-dependent fashion, most likely via interruption of the generation of downstream reactive compounds such as NO, and/or peroxynitrites, etc.

Collectively, these results suggest that both compounds, although with different effectiveness, block the suppression of T cell responses by inhibiting iNOS/NO. Hypoxia-inducible factor α (HIF-1α) has been demonstrated to have an important role in the impairment of M-MDSC *ex vivo* suppression of T cell proliferation and function, including a possibility that it regulates iNOS/NO production in LP-BM5 infection too [23]. Regarding I-152 inhibitory mechanism(s), we hypothesize that inhibition of iNOS/NO could be linked to its capacity to down-modulate gene expression of HIF-1α as demonstrated in other experimental conditions where I-152-dependent modulation of cellular redox status was used as a strategy to modulate the cellular response to hypoxia [24]. Moreover, in that ROS release is one of the major mechanisms through which MDSCs suppress T cells [19,25], ROS level reduction likely induced by I-152, as recently demonstrated [24], may be an additional mechanism by which the compound can modulate MDSC suppressive activity.

In addition, iNOS/NO has been identified as a main target for I-152 in blocking M-MDSC suppression of B-cell proliferation. This is different from C4-GSH treatment, which seems to have additional targets, among those involved in the suppression of B-cell responsiveness by LP-BM5-expanded M-MDSCs [11]. We can hypothesize that, although C4-GSH treatment provides both cysteine and GSH [17–18], its effect is mostly mediated by its capacity to increase intracellular GSH since there was no evidence that LP-BM5-expanded M-MDSCs utilized cysteine depletion as a substantial mechanism to suppress B cells [10].

Redox switch induced by these molecules may also interfere with the specific energy metabolic program used by MDSCs to support their differentiation and functions [26]. Although further experiments are necessary to shed light on the inhibitory effects exerted by I-152 and C4-GSH as well as on the mechanisms/signalling pathways involved in their immunomodulatory action, these preliminary results clearly show that MDSC suppressive activity is influenced by intracellular redox state and that one or more factors influencing B cell suppression by LP-BM5-expanded M-MDSCs can be affected by increased GSH level rather than by modifications of the whole redox state. GSH is not only the most important antioxidant in cells but also mediates specific effects in immune cells. For instance, GSH influences IL-12 production and antigen processing in macrophages [27] and it is involved in the reprogramming of effector T cells during inflammation [28]. In MDSCs, increased levels of GSH induced their differentiation mainly through the neutralization of ROS, but other mechanisms could not be excluded [15–16]. Most studies have examined MDSC-mediated immune suppression mainly directed versus T cells, while its role in modulating B cell responses is still poorly understood. Moreover, MDSCs have been predominantly studied in the tumor microenvironment [29], hence these results may provide insight into new mechanisms by which MDSCs may regulate B cell immune responses during a viral infection.

The similar but distinct effects of I-152 and C4-GSH on M-MDSC activity could be due to the different thiol-based regulatory switches induced by the two molecules in redox-dependent signalling pathways impaired by the virus, as already observed in other viral infections [30]. This assumption is also supported by previous results showing that other reducing agents, such as beta-mercaptoethanol (2ME) and NAC, did not block B-cell suppression by M-MDSCs [10]. Usually virus-induced oxidative stress plays a critical role in the viral life cycle as well as in the host immune system. For this reason, antioxidants have been proposed as effective strategies against viral infections. However, several factors should be considered, such as the type of virus, the redox modulator, and the cell control of the oxidative stress response during viral infection, that altogether make it difficult to foresee the final effect of the different compounds.

It has been previously reported that the response to mitogens of T and B lymphocytes isolated from C57BL/6 mice infected with the LP-BM5 retrovirus is significantly impaired [31]. To evaluate whether C4-GSH and I-152 blockade of MDSC activity observed in *ex vivo* suppression assays could affect T and B cell proliferation during the disease process, LP-BM5-infected mice were treated with either C4-GSH or I-152 for 9 weeks following the protocol described in the Methods section. At 2, 5, and 9 weeks post-infection, spleen cells were isolated from uninfected, infected/untreated (Infected, I) and infected/treated mice, and proliferative responses of T and B cells were determined. No appreciable differences were observed at 2 and 5 weeks post-infection between uninfected mice and the infected groups (not shown). While at 9 weeks post-infection, B and T cell response impairment was partially alleviated by the compounds (Fig. 2). In agreement with the results obtained in *ex vivo* experiments (Fig. 1), C4-GSH blockade of M-MDSC suppressiveness was consistently more significant for B cell than T cell responses, while I-152 restored mitogen responsiveness for both B and T cell responses. Taken together, these results suggest that the two molecules may

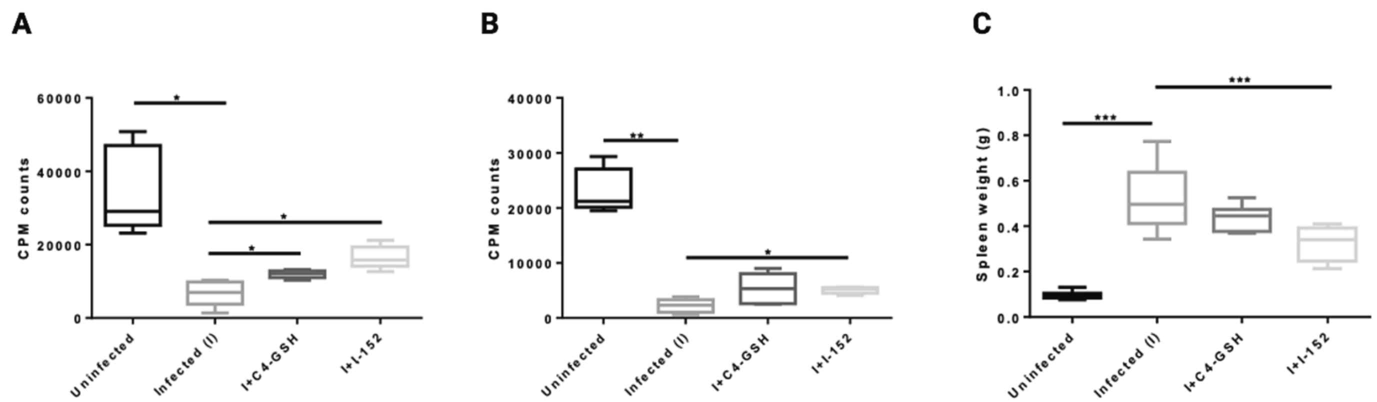

Fig. 2. Splenocyte responses to polyclonal stimuli and splenomegaly demonstrating inhibitory activity by C4-GSH and I-152 in LP-BM5-infected mice. Lymphocyte proliferative response to LPS (50 μg/ml) of B cells (**A**) and to PHA (10 μg/ml) of T cells (**B**) in LP-BM5-infected mice not treated (Infected, I) or treated with C4-GSH or I-152 three days a week for a total of 9 weeks. Spleen lymphocytes were obtained 9 weeks after infection and were assayed for ^{3}[H]-Thymidine incorporation after 3 days of culture with mitogens. The values are means ± S.D. of five animals. (**C**) Spleen weights at 9 weeks post-infection of Uninfected and Infected mice, untreated (I) or treated with C4-GSH or I-152. The results referring to the group Infected having received placebo have not been shown since they were comparable with the infected animals. The values are means ± S.D. of eight animals. *p < 0.05; **p < 0.01; ***p < 0.001.

differentially affect T and B cell proliferation during the disease process in infected mice. These results were in accordance with those obtained by evaluation of splenomegaly, a useful parameter to follow the disease over time. A reduction in spleen weight was observed in all the treated animals at 9 weeks post-infection, with significant effects obtained through I-152 treatment. These data suggest that T and B cell responses may have an important role in disease progression.

We have previously shown that the immune response of LP-BM5-infected mice was characterized by a prevalence of Th2 cytokines and that I-152 treatment re-established Th1/Th2 balance as well as induced the switch from M2- to M1-like macrophages [12]. We can speculate that modulation of cytokines influencing Th1/Th2 response, e.g., reduction of IL-4 induced by I-152 treatment in LP-BM5-infected mice may also play a role in orchestrating the crosstalk between MDSC and T cells. In fact, suppression of T cell function is strongly enhanced upon exposure to IL-4 via Arginase1-dependent mechanism [5–8].

On the other hand, C4-GSH treatment affected viral replication and induced antiviral Th1-type immune response in influenza-infected senescent mice [18]. Based on these data and the results from other laboratories [32], we had concluded that modulation of GSH content in antigen-presenting cells could influence Th1/Th2 balance, making stronger antiviral responses. Indeed, this work suggests that redox-modulating small molecules may exert their immunomodulatory/antiviral activity by different mechanisms and act on different cell targets, including MDSCs, contributing to the regulation of the intense crosstalk between the different immune cell populations. As a precedent, in the LP-BM5 retroviral system employed here, M-MDSC activity is crucial to the initiation/progression of the virus-induced immunodeficiency syndrome. Our very recent studies, utilizing selective *in vivo* depletion of M-MDSCs, have shown a substantially significant reduction of both the early activation events, as well as the subsequent profound B-cell and T-cell non-responsiveness of the LP-BM5 induced syndrome (unpublished data). These results, taken together, suggest possible targeted strategies for therapeutic approaches to down-regulate MDSC activity, potentially in a variety of settings involving B- or T-cell functions in viral infections, as well as in other disease states (e.g. anti-tumor immunity). Collectively the present study highlights that changes in the redox state can affect MDSC activity and that redox-modulating compounds could be potential immunotherapeutic candidates for targeting MDSCs in viral infection. Immunotherapeutic approaches have been increasingly investigated for infectious diseases to overcome the limitations linked to the use of classical therapies such as lack of adequate efficacy, drug toxicity, and the emergence of drug resistance. Although most immunotherapeutics have found wide application against cancer, progress made in the treatment of human immunodeficiency virus, tuberculosis, malaria and, most recently COVID-19 support their use in the broader field of infectious disease control.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

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The graphical abstract and Figure 1B were created using BioRender.com.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2023.110882.

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