A synthetic thiol molecule releasing N-acetyl-l-cysteine and cysteamine drives early up-regulation of immunoproteasome subunits in the lymph nodes of mice infected with LP-BM5 leukemia retrovirus

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A synthetic thiol molecule releasing *N*-acetyl-L-cysteine and cysteamine drives early up-regulation of immunoproteasome subunits in the lymph nodes of mice infected with LP-BM5 leukemia retrovirus

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A B S T R A C T

Thiol molecules have been recently re-considered as drug candidates in viral infections because of their ability to induce redox changes which interfere with virus life cycle and modulate the host immune response. Little is known about the molecular mechanisms of their immunomodulatory properties.

Here we show that I-152, a thiol molecule metabolized to release *N*-acetyl-L-cysteine and cysteamine and acting as a pro-glutathione agent, causes early up-regulation of immunoproteasome subunits in the lymph nodes of murine leukemia virus infected mice.

This evidence suggests that the immunoproteasome may be modulated by thiol-based compounds with important implications in understanding redox-controlled immunoregulation.

The immune response is a complex process orchestrated by multiple cell types, tissues, and organs whose functioning is dependent on the extracellular and intracellular redox state. Redox reactions trigger and shape various aspects of this response including immune cell metabolic reprogramming, polarization, phagocytosis, cytokine production, chemotaxis, and pathogen sensing [1]. It has been established that several pathogens, including viruses, cause a redox imbalance towards a more oxidative state characterized by ROS overproduction, decreased activity of antioxidant systems and glutathione (GSH) depletion thus affecting the immune response. A pro-oxidant environment, on the one hand favours viral replication, on the other it promotes activation of redox-modulated pro-inflammatory signaling cascades which are involved in the activation of the innate immune response but, if uncontrolled, can cause systemic inflammation and tissue damage [2]. Therefore, redox modulating molecules, including low molecular weight thiols, have been proved effective both in decreasing viral load and in dampening the inflammatory response [3]. We have previously used C57BL/6 mice infected with LP-BM5 murine leukemia virus (MuLV) as an in vivo model to study whether thiol molecules may interfere with viral replication and modulate the immune response. LP-BM5 mice develop an immunodeficiency syndrome termed MAIDS, characterized by hypergammaglobulinemia, loss of $CD4^+$ T-cell function, increased susceptibility to infection and terminal B-cell lymphomas [4]. Notably, MAIDS is characterized by a prevalent Th2 immune response associated with an alternative pathway of macrophage activation and GSH depletion [5]. In this experimental model, it was possible to restore the intracellular redox state with a molecule, named I-152, able to boost intracellular GSH synthesis and release thiol species, i.e. *N*-acetyl-L-cysteine (NAC) and cysteamine (MEA) (Fig. 1A). I-152 treatment of LP-BM5-infected mice reduced the signs of the disease, the content of proviral DNA, and modulated the response/activity of different immune cells [5,6].

The molecular mechanisms fine-tuned by redox-modulating thiol molecules and thiol-based antioxidants in general in the context of the immune response, is poorly understood.

The aim of this study was to investigate whether redox manipulation by I-152 could affect the expression of a specialized form of the proteasome termed the immunoproteasome (IP) in the lymph nodes (LNs) of LP-BM5-infected mice. The hypothesis has been tested by conducting a follow-up analysis in samples obtained from uninfected and infected mice treated with I-152 or the vehicle [5,6]. LNs are essential in initiating and resolving immune responses; moreover, like other lymphoid tissues, they are significantly more "reduced" than the other tissues [7].

The immunoproteasome is particularly abundant in lymphoid tissues with important roles during viral infection. It contributes to MHC class I antigen production generating specific subsets of antigens; it also

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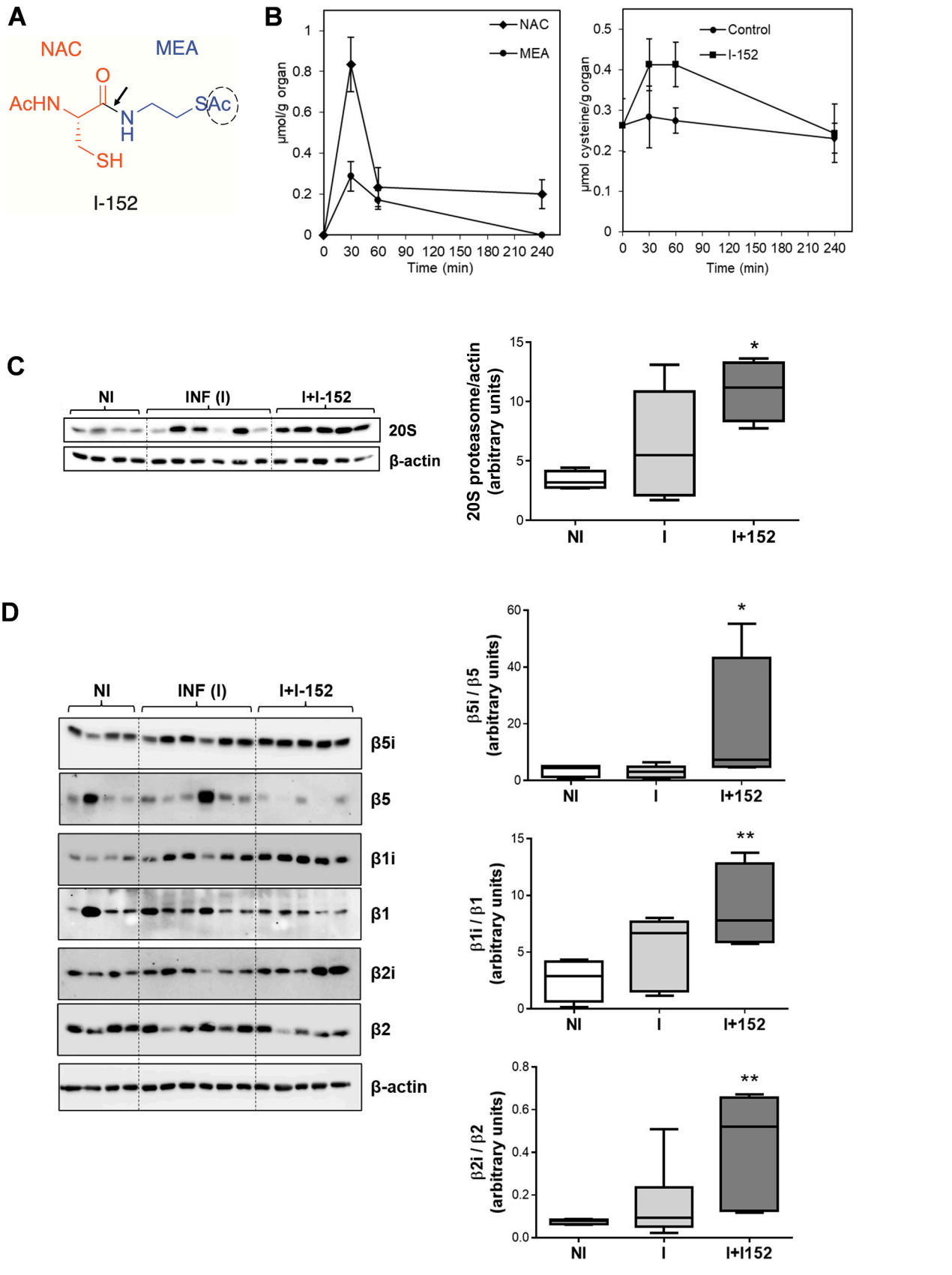

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Fig. 1. I-152 delivers thiol species and promotes immunoproteasome subunit up-regulation in the lymph nodes of LP-BM5 infected mice. (A) Chemical structure of I-152. The arrow indicates the bond which is hydrolyzed to release the parent compounds: NAC, *N*-acetyl-L-cysteine and MEA, cysteamine. The dotted circle highlights the acetyl group (Ac) which is removed to generate MEA. (B) Thiol content in mouse lymph nodes at different times after a single intraperitoneal injection of I-152 (30 μmol/mouse); thiol species were determined by an HPLC method [5]. Values represent the mean ± S.D. of 3 mice. (C, D) Western immunoblotting analysis of the proteasome and individual β subunits in the lymph nodes of uninfected (NI), infected/untreated (I) and infected/treated (I + I-152) mice. C57BL/6 mice were intraperitoneally injected with either NaCl 0.9 % (v/v) or I-152 (30 μmol/mouse) three times a week, every other day, and sacrificed at 2 weeks p.i, 18 h after the last injection. After tissue homogenization [6], lysates were separated on SDS-PAGE gels, transferred to membrane, and probed with specific antibodies. Immunoreactive bands were detected in a Chemidoc system and quantified with the Image Lab software. Protein levels were normalized on β-actin and the ratio between each immunosubunit and its constitutive counterpart was calculated and shown in the graphs of panel D. Four non infected, six infected/untreated and five infected/treated mice were analyzed. Mice were from one single experiment. Statistical analysis was performed using one-way ANOVA followed by the Dunnett's post-test. $^{*}p < .05$; $^{**}p < .01$.

regulates $CD8^{+}$ T cell expansion, promotes the differentiation of pro-inflammatory T-helper type 1 (Th1) cells and regulates the production of cytokines from monocytes and T cells [8]. Recently, the IP has been shown to have a more pleiotropic role in maintaining protein homeostasis [9]. In the immunoproteasome, β1, β2, and β5 catalytic subunits of the constitutively expressed proteasome (CP) are replaced by the so-called immunoproteasome subunits β1i (LMP2), β2i (MECL-1), and β5i (LMP7).

Here we provide evidence that redox modulation by I-152 induces early up-regulation of immunoproteasome subunits in infected mice at the post-transcriptional level.

We had already demonstrated that I-152 restored GSH and cysteine contents in the lymph nodes of C57BL/6 mice where LP-BM5 infection had caused GSH depletion [5]. In this work, biodistribution studies conducted in mice showed that, after a single intraperitoneal injection of I-152, its metabolites could be found in the lymph nodes. In details, at 30 min high NAC and MEA levels were detected; after 240 min NAC levels declined and MEA disappeared (Fig. 1B). Moreover, high cysteine levels were detected both at 30 and 60 min. These results are consistent with the knowledge that I-152 supplies GSH precursors and promotes the de novo synthesis of the rate limiting enzyme in GSH biosynthesis through nuclear factor E2-related factor 2 (Nrf2) activation [10].

To study immunomodulatory properties of I-152, the molecule was injected three times a week and analyses were conducted after 2 weeks p.i. (p.i.) where the immune response has been established and 5 weeks p.i. where the signs of the disease were evident [5,6]. Proteasome expression in the LNs of uninfected and infected mice treated or not with I-152 was preliminary assessed by western immunoblotting analysis with a pan antibody which recognizes a pool of constitutive proteasome and immunoproteasome subunits. Although less informative, analysis of the whole tissue rather than of cell types was preferred to avoid possible changes in phenotype and/or redox state which may occur during isolation procedures. As shown in Fig. 1C, proteasome subunits were basally expressed in the LNs of non-infected mice (NI) and up-regulated at 2-weeks post infection in infected mice, however only in I-152-treated animals (I + I-152) the difference compared to uninfected mice was statistically significant. At 5-weeks p.i., infected (I) and I + I-152 animal groups both displayed increased proteasome content compared to NI controls (Fig. S1A), suggesting that I-152 anticipated rather than enhanced proteasome induction.

The upregulation of the immunoproteasome subunits in mouse LNs after infection has also been demonstrated by others [11]. The authors concluded that naturally occurring infections, which induce IFN-γ, can dramatically activate immunoproteasome expression in vivo. However, mRNA expression analysis demonstrated that PSMB9 (β1i) gene transcription was significantly up-regulated both in the I and I + I-152 groups with no significant difference between them (Fig. S1B). By contrast, β5i mRNA levels were unchanged. Thus, although previous findings indicated that cultured spleen cells obtained from I + I-152 mice produced 2-fold more IFNγ compared to cells obtained from infected mice [5], higher IFNγ levels do not seem to be the direct cause of early IP induction. Since discrepancies between proteasome subunit mRNA and protein levels have been reported previously both in vitro and in vivo [11], we next sought to evaluate protein expression of individual immunoproteasome subunits compared to constitutive counterparts. Immunoblotting analysis revealed that immunosubunit expression, and β5i/β5, β1i/β1 and β2i/β2 ratio increased after infection but they were significantly up-regulated compared to uninfected mice only in the I + I-152 group (Fig. S1C and Fig. 1D, respectively). A parallel decrease of constitutive subunit content was also observed but it was not significant suggesting that proteasome replacement during infection occurred at very low levels (Fig. S1C).

Taken together, these observations suggest that in infected mice I-152 anticipates immunoproteasome induction possibly through a post-transcriptional mechanism which primarily up-regulates IP protein subunits without concomitantly downregulating CP counterparts.

Other than the generation of antigenic peptides for presentation on MHC class I molecules, the role of the immunoproteasome in viral infection is poorly understood. Accumulating evidence suggests that the immunoproteasome serves proteostatic functions being involved in the degradation of oxidized proteins in a ubiquitin- (Ub) and ATP-dependent or Ub-independent manner, thus protecting cells from cytokine-induced proteotoxic stress [9]. In our experimental model, accumulation of Ub-conjugated proteins, which occurs when proteasome activity is limiting, was observed neither in infected mice nor in I-152-treated animals (Fig. S2A), suggesting that immunoproteasome induction may be necessary for maintaining proteostasis in infected tissues. LP-BM5 infection is characterized by proliferation and polyclonal activation of B cells resulting in abnormal production of immunoglobulins (Ig) and development of late lymphomas. In this model we have previously demonstrated that I-152 treatment causes Ig accumulation within the endoplasmic reticulum at 2 weeks p.i., probably by interfering with Ig oxidative folding, as we recently provided evidence for SARS-CoV-2 Spike protein [6,12]. Moreover, data obtained in RAW 264.7 cells demonstrated that I-152 is per se sufficient to activate all the three unfolded protein response (UPR) branches [10] (unpublished results). Interestingly, immunoproteasome inhibition has been found to activate the UPR and induce apoptosis in plasma cells (PC) obtained from kidney allotransplanted rats, suggesting a role for the immunoproteasome in protecting plasma cell from ER stress [13]. Therefore, it could be speculated that immunoproteasome induction in LP-BM5 infection may be necessary to maintain a balance between load (Ig synthesis) and capacity of the proteolytic machinery. In this context, ER perturbance induced by I-152 may cause this response to occur earlier resulting in anticipated IP induction at 2 weeks p.i., while causing plasma cell death and/or inhibition of PC maturation occurring at later times (i.e. 5 weeks p.i.) [6]. Activation of the UPR is indeed essential for B cells to handle proper folding of Ig, but also for differentiation [14].

Moreover, since the immunoproteasome stimulates production of cytokines such as TNFα and IFNγ, anticipation of IP induction in I + I-152 mice may promote the activation of the innate immunity. Most of these inflammatory cytokines are expressed under the control of NF-κB. However, in the LNs of I-152-treated mice we found repression of NF-κB phosphorylation which is part of the NF-κB signaling cascade of activation (Fig. S2B). Whether the immunoproteasome may influence cytokine production by modulating NF-κB signaling is a matter of debate. In our experimental model, I-152 reduced basal NF-κB activity in the LNs, which is known to be essential for innate immune cell

development, survival, and activation but that is also involved in the growth of some types of B-cell lymphomas [15]. This observation would partially explain the reduction of plasma cell maturation and lymphadenopathy at 5 weeks p.i. [5,6] and would rule out an involvement of NF-kB in modulating cytokine expression and/or immunoproteasome up-regulation. In agreement with these observations the mRNA expression levels of tumor necrosis factor-α and interleukin-6 were found not different between the experimental groups (Fig. S2C).

In conclusion, we have shown that I-152 treatment can deliver thiol species to the lymph nodes; in LP-BM5-infected mice this condition promotes early induction of immunoproteasome subunit expression, although the underlying molecular mechanisms are not yet known. Anticipation of IP up-regulation by I-152 may be associated with disturbed ER proteostasis, especially in plasma cells whose maturation and functioning relies on an active UPR. Due to the multiple implications of the immunoproteasome in the immune response and immune cell functioning, this effect could partially explain some of the immunomodulatory properties of I-152. Compounds that can modulate immunoproteasome expression may be useful in boosting the innate cell-mediated immune response thus acting as broad-spectrum antivirals. The ability of I-152 to cause ER stress in cells particularly sensitive to limitation in proteasome function may be exploited to interfere with viral replication which causes ER overload due to sustained viral protein synthesis, thus providing an opportunity to arm the host's immune system and target the virus at the same time.

CrediT authorship contribution statement

Rita Crinelli: Conceptualization, Formal analysis, Investigation, Data curation, Writing original draft, review & editing, Visualization, Funding acquisition, Manuscript revision. Francesca Monittola: Investigation, Data curation, Manuscript revision Sofia Masini: Investigation, Data curation. Aurora Diotallevi: Investigation, Data curation, Manuscript revision. Francesca Bartoccini: Resources, Visualization. Michaël Smietana: Resources, Review & editing. Luca Galluzzi: Formal analysis, Data curation, Review & editing, Manuscript revision. Mauro Magnani: Resources, Review & editing. Alessandra Fraternale: Conceptualization, Formal analysis, Investigation, Data curation, Review & editing, Visualization.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbadis.2023.166918.

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