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Short communication

Modulation of Th1/Th2 immune responses to HIV-1 Tat by new pro-GSH molecules

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ABSTRACT

We have previously demonstrated that in Ova-immunized mice the increase in intra-macrophage thiol pool induced by pro-GSH molecules modulates the Th1/Th2 balance in favour of a Th1-type immune response. We show now that the same molecules can support a Th1-type over Th2-type immunity against Tat, which is an early HIV-1 regulatory protein and a Th1 polarizing immunomodulator that is increasingly considered in new anti-HIV vaccination strategies. Our results indicate that Tat-immunized mice pre-treated with the C4 (n-butanoyl) derivative of reduced glutathione (GSH-C4) or a pro-drug of N-acetylcysteine (NAC) and beta-mercaptoethylamine (MEA) (I-152), have decreased levels of anti-Tat IgG1 as well as increased levels of anti-Tat IgG2a and IgG2b isotypes suggesting a Th1-type response. Moreover, Th1-(IFN-γ and IL-2) Ag-specific cellular responses were detected by ELISPOT assay in splenocytes of the same animals as well as an increase of IL-12 levels in the plasma. These findings suggest that the Th1 immune response to HIV-1 Tat could be further polarized by these molecules. These results together with those previously reported suggest that pro-GSH molecules could be used to modulate the immune response towards different antigens and may be further exploited for inducing specific Th1 immune responses against other HIV antigens as well as other intracellular pathogens in new Tat-based vaccination protocols.

1. Introduction

The redox state of antigen presenting cells (APC) may impact profoundly the immune responses to an antigen (Ag) by both influencing its processing and altering interleukin-12 (IL-12) secretion [1–3]. In particular, depletion of reduced glutathione (GSH) can impair the ability of APC to reduce Ag disulfide bonds required for Ag processing as well as to decrease the activity of thiol proteases important in Ag processing and cleaving of the invariant chain from major histocompatibility complex class II [1,3–5].

IL-12 is an important immunoregulatory cytokine that is produced mainly by APC. The expression of IL-12 during infection regulates innate responses and determines the type of adaptive immune responses; IL-12 induces IFN-γ production and triggers CD4+ T cells to differentiate into Th1 cells [6]. Upon stimulation, the alteration of intra-macrophage redox state causes either an increase or a decrease in IL-12 secretion due to an increased or a reduced intracellular GSH level, respectively [2,7]. GSH-C4 and I-152 are a derivative and a precursor of GSH respectively, that have been recently shown to be able to increase the intra-macrophage thiol content and to shift *in vivo* the immune response against Ova towards the Th1 type [8]. In this paper, we investigate the role of the same molecules in modulating the Th1/Th2 response in mice immunized with the HIV-1 Tat protein. Tat is a regulatory protein produced early after HIV-1 infection, which plays key roles in HIV replication and AIDS pathogenesis, and it possesses conserved immunogenic sequences among HIV clades [9]. These characteristics make it an excellent candidate for the development of both

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preventive and therapeutic vaccines against HIV-1 [9–11]. It has been demonstrated that Th1-type T cell response against Tat is predominant over a Th2-type B cell response, and that only when Tat is in its native conformation (partially reduced) is taken up and processed by monocyte-derived dendritic cells (MDDC) and macrophages driving Th1-type responses [12]. In fact, the biological activity of Tat is deeply influenced by its structural features, in particular by its region II (aa 22–39) which includes seven well conserved cysteins [13,14]. Moreover, it has been reported that the Tat uptake is less efficient in macrophages with altered intracellular redox state [15]. On the other hand, it is known that *in vitro* HIV-1 infection induces a significant decrease in intracellular GSH in human macrophages [16] and that high levels of intracellular GSH inside the APC support gene transcription that leads to secretion of IL-12, the major Th1-polarizing cytokine [2,7]. Thus, the macrophage redox state could affect Tat uptake [15], Tat biological activity [12], and the polarization of immune responses towards either a Th1 or a Th2 response by IL-12 production [2,7]. In consideration of the above facts and because Tat is presently in clinical trials as a preventive and/or therapeutic vaccine candidate [17], we explored the role of GSH-C4 and I-152 as tools to influence the immune response to Tat. Here, we report that the pro-GSH molecules induced *in vivo* specific immune responses against Tat, including significant IgG2a and IgG2b production as well as IL-12 secretion, characteristics of Th1-type responses. Moreover, cellular responses were induced more efficiently. Our findings suggest that the molecules investigated can be used to stimulate Th1 immune response in Tat-immunized animals and have the potential to improve the quantity and quality of the Tat vaccine-specific immune response.

2. Materials and methods

2.1. Animals

Four-week-old female BALB/c mice were purchased from Harlan Nossan (Milan, Italy), housed in a pathogen-free environment (Charles River Laboratories, France) and maintained on standard mouse chow and water *ad libitum*. Housing and treatment of mice were in compliance with the Guide for the Care and Use of Laboratory Animals by Ministero della Sanità D.L. 116, 1992 and approved by an appropriate institutional review committee.

2.2. Reagents

GSH-C4 and I-152 were synthesized as previously described [18,19].

The monomeric biologically active Tat protein (86 aa) of HIV-1 (HTLVIII-BH10) was produced in *Escherichia coli*, purified as a good laboratory practice (GLP) manufactured product and provided by Diatheva (Fano, Italy). The biological activity of each batch of Tat protein was determined by means of a method based on Tat uptake by MDDC, as previously described [20]. Endotoxin content in Tat preparations was lower than 0.2 EU/μg protein. Tat protein was stored lyophilized at −80 °C and resuspended (2 mg/ml) in NaCl 0.9% immediately before use [21]. The VCF (VCFITKALGISYGRK) Tat peptide containing a K^d-restricted CTL epitope and a CD4+ T cell epitope [22] was synthesized by UFPeptides s.r.l. (Ferrara, Italy), resuspended in H_2O (10^{-2} M) and stored at −80 °C until use.

2.3. Treatment of mice with pro-GSH molecules

GSH-C4 or I-152 was injected intraperitoneally in BALB/c mice at the concentration of 32.5 μmoles/mouse in a final volume of 250 μl of NaCl 0.9%, 4 h before intraperitoneal (i.p.) injection of Tat, for a total of 3 administrations given at 21 day-intervals. Depending on the pre-treatment, the Tat-immunized mice were marked as follows: NaCl/Tat (receiving i.p. administrations of NaCl 0.9% 4 h before Tat injection); GSH-C4/Tat (receiving i.p. administrations of GSH-C4 4 h before Tat injection); I-152/Tat (receiving i.p. administrations of I-152 4 h before Tat injection).

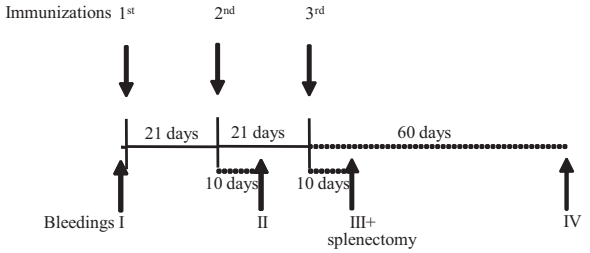

Fig. 1. Immunization schematic schedule. At the bleedings indicated as I, II, III, and IV, blood was drawn from all experimental groups to quantify plasma anti-Tat specific IgG and isotypes. At the bleeding III, the spleens were collected for analysis of the cellular responses by ELISPOT assay. At the bleeding IV, IL-12 levels were measured in plasma of Tat-immunized mice.

2.4. Tat protein immunization

A schematic representation of the immunization schedule is shown in Fig. 1. Mice were immunized i.p. with 10 μg/mouse of recombinant Tat protein and boosted with 5 μg of the protein 3 and 6 weeks later without any known adjuvant addition. Bleedings were performed 1 day before the 1st immunization (bleeding I), 10 days after the 2nd and 3rd immunization (bleedings II and III, respectively), and 60 days after the last immunization (bleeding IV). Ten days after the last immunization, at least three mice per group were sacrificed to collect spleens for the analysis of cellular responses by ELISPOT assays.

2.5. Analysis of anti-Tat IgG responses

The presence of plasma anti-Tat IgG was determined by ELISA. Ninety-six-well plates (Immulon IB M-Medical) were coated for 18 h at 4 °C with 100 ng/well of recombinant Tat protein dissolved in 0.05 M sodium carbonate–bicarbonate buffer pH 9.7. Plates were washed five times with PBS pH 7.0 containing 0.05% (v/v) Tween 20 (TPBS) and then blocked by addition of 200 μl/well of TPBS containing 1% BSA (Sigma–Aldrich) (w/v) (TPBSB) for 60 min at 37 °C. After five washes, 100 μl/well of serial dilutions (range 1:25–1:12,800 v/v) of murine plasma were dispensed and then incubated for 60 min at 37 °C. The plates were washed again with TPBS before the addition of 100 μl/well of HRP-conjugated goat anti-mouse IgG (Bio-Rad, Richmond, CA, USA) diluted 1:1000 in TPBSB. The plates were incubated for 60 min at 37 °C and then washed and coloured with 100 μl/well of ABTS as substrate (Roche Diagnostic Indianapolis, USA). The absorbance was measured at 405 nm with a microplate reader (Biorad) after 30 min of incubation at room temperature. Anti-Tat IgG concentration was determined on a standard curve previously generated with known concentration of purified mouse IgG (Sigma BioSciences, St. Louis, MO, USA).

2.6. Analysis of anti-Tat IgG isotypes

Plasma IgG isotypes were determined by the same ELISA assay as described above with the difference that goat anti-mouse Ab directed against IgG1, IgG2a, IgG2b, or IgG3 isotype (Serotec) were used to detect anti-Tat antibodies. The specific antibodies were pre-diluted 1:400 (v/v) in TPBS and dispensed 100 μl per well. After incubation at 37 °C for 60 min, the immune complexes were

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detected by addition of an HRP-labelled rabbit anti-goat antibody (Santa Cruz) diluted 1:1000 (v/v) in TPBSB. After an incubation time of 60 min at 37 °C, 100 μl of ABTS substrate was added into the wells. The absorbance was read at 405 nm with a microplate reader (Biorad). Values of anti-Tat IgG isotypes were expressed as geometric mean antibody titers according to Perkins [23]. The antibody titers of anti-Tat IgG isotypes were calculated as the logarithmic values to base 10 of the reciprocals of plasma dilutions. The end point, or titer, is the highest dilution of plasma giving a positive test reaction. This was defined as any value above the cut off, represented by 10% of the maximum $OD_{405\,nm}$ [24].

2.7. Epitope mapping of Tat-specific Abs

Plasma samples were tested for IgG Abs to Tat peptides by ELISA. Peptides (synthesized by UFPeptides s.r.l., Ferrara, Italy) representing different regions of the BH-10 clone-derived Tat linear sequence (aa 1–20, aa 21–40, aa 36–50, aa 46–60, aa 56–70, aa 66–80, aa 73–86, aa 83–102), were plated in 96-well flat-bottom plates (Nunc-ImmunoTM Plate MaxiSorpTM Surface; Nunc, Kampstrup, Denmark). The wells were coated with 250 ng of synthetic peptides in 200 μl of PBS with calcium and magnesium. After overnight incubation at 4 °C, plates were washed five times with TPBS and blocked with TPBSB for 90 min at 37 °C. Each well was then incubated with 100 μl of serum diluted 1:100 in TPBSB and incubated at 37 °C for 90 min. Wells were then blocked again with TPBSB for 15 min at 37 °C. After washings, the same HRP-conjugated goat anti-mouse IgG (Bio-Rad, Richmond, CA, USA) diluted 1:1000 used above was added to the wells and peptide-bound Abs were revealed by the addition of the ABTS substrate (Roche Diagnostic SpA, Lecco, Italy) for 50 min at 37 °C. Absorbance was measured at 405 nm.

2.8. Analysis of cellular responses by ELISPOT assay

Ten days after the 3rd immunization, mice were sacrificed and cellular responses were analyzed using pools of at least three spleens per each experimental group. Splenocytes were purified from spleens squeezed on filters (Cell Strainer, 70 μm, Nylon, Becton Dickinson). Following lysis of erythrocytes with RBC lysis buffer (Sigma), cells were washed with RPMI 1640 (Cambrex) containing 10% FBS (Hyclone), spun for 10 min at 300 × *g*, resuspended in RPMI 1640 containing 10% FBS, 1% L-glutamine (BioWhittaker, Walkersville, MD), 1% penicillin/streptomycin (BioWhittaker, Walkersville, MD), 1% nonessential aminoacids (Sigma), 1 mM sodium pyruvate (Sigma) and 50 mM beta-mercaptoethanol (Gibco, Grand Island, NY). For ELISPOT analysis after *ex vivo* restimulation, splenocytes (3×10^6/ml) were cultured with the specific Tat peptide (3 μg/ml) for 5 days, extensively washed with RPMI 1640 containing 10% FBS, placed ($4–5 \times 10^4$ cells/well) on ELISPOT plates pre-coated with the anti-cytokine specific Ab and incubated with the VCF (VCFITKALGISYGRK) Tat peptide containing a K^d-restricted CTL epitope and a CD4+T cell epitope, for 16–20 h. ELISPOT assays were performed for Th1 cytokines (duplicate wells), using commercially available murine IFN-γ and IL-2 ELISPOT kits (BD, Pharmingen), as described [22]. Results are expressed as number of spot forming cells (SFC)/10^6 cells. Values at least 2-fold higher than the mean number of spots in the control wells (untreated cells) and ≥100 SFC/10^6 cells were considered positive.

2.9. IL-12 assay

Total mouse IL-12 protein, both monomeric p40 and p40 associated to p35 (p70), was measured in plasma of Tat-immunized mice using ELISA kits (GE Healthcare UK Limited Amersham Place, UK).

2.10. Statistical analysis

Statistical analysis of data was performed with the non-parametric Mann–Whitney test.

Analysis of cellular responses was done by the two-way ANOVA test and Bonferroni post test using the GraphPad software Prism 4 (El Camino Real, San Diego, CA). The criterion for statistical significance was $p \leq 0.05$.

3. Results

3.1. Effect of pro-GSH molecules on anti-Tat IgG responses

The ability of GSH-C4 or I-152 to modulate the Th1/Th2 responses against Tat was investigated by measuring the total anti-Tat IgG as well as IgG isotypes. Mice treated with GSH-C4 or I-152 were immunized with Tat as reported in Fig. 1 and at different time points, anti-Tat specific IgG levels were determined. Treatment with pro-GSH molecules was performed 4 h before i.p. injection of Tat, based on the results showing that the highest levels of thiols in the peritoneal macrophages of mice treated with the above molecules can be obtained between 2 h (42 ± 4 pmoles of thiols/μg protein in the untreated control vs 55 ± 1 in the GSH-C4-treated mice and 79 ± 1 in the I-152-treated mice) and 5 h (35 ± 4 pmoles of thiols/μg protein in the untreated control vs 59 ± 2 in the GSH-C4-treated mice and 92 ± 3 in the I-152-treated mice) after the pro-GSH molecules administration [8]. At 10 days after the 2nd immunization (bleeding II in Fig. 1), plasma IgG were barely detectable and differences among the immunized animals were not found (data not shown). At 10 days after the 3rd immunization (bleeding III in Fig. 1), high levels of anti-Tat-specific IgG were measured and significant differences among the different experimental groups became apparent (Fig. 2); in agreement with our previous results [8], significantly lower IgG levels were observed in mice pre-treated with GSH-C4 or I-152 as compared with mice immunized with Tat receiving NaCl 0.9% (NaCl/Tat). However, at 60 days after the 3rd immunization (bleeding IV in Fig. 1), the amount of anti-Tat IgG decreased in all groups and the differences were no longer appreciable.

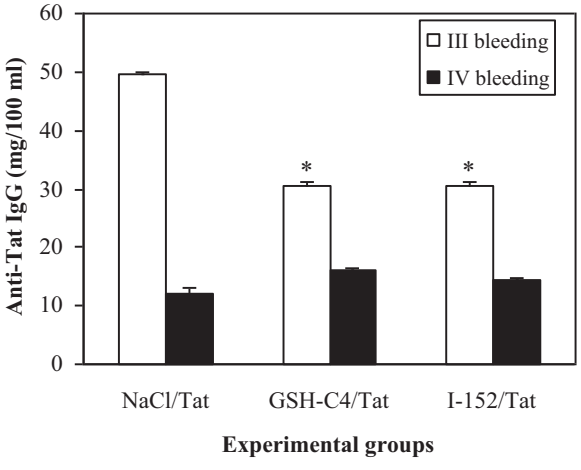

Fig. 2. Anti-Tat IgG in plasma of Tat-immunized mice. Bleedings III and IV were performed at 10 and 60 days after the 3rd immunization respectively, as indicated in Fig. 1. The concentration of anti-Tat IgG was evaluated by ELISA as described in Section 2. The experimental groups were marked as follows: NaCl/Tat (Tat-immunized mice receiving i.p. administrations of NaCl 0.9% 4 h before Tat injection); GSH-C4/Tat (Tat-immunized mice receiving i.p. administrations of GSH-C4 4 h before Tat injection); I-152/Tat (Tat-immunized mice receiving i.p. administrations of I-152 4 h before Tat injection). Values are the mean ± S.D. of 5 animals. $^*p < 0.01$ vs NaCl/Tat at the bleeding III.

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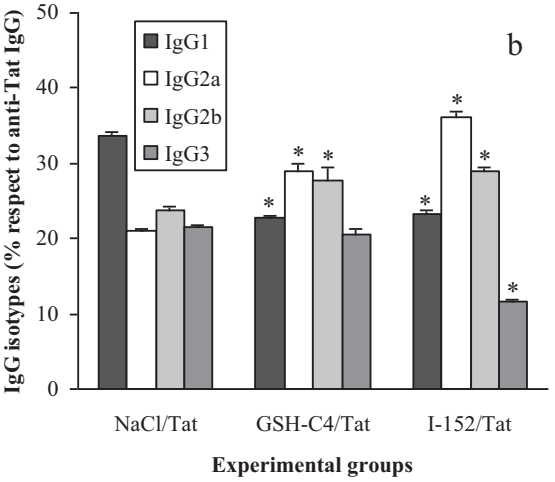
a

Anti-Tat IgG Isotype	NaCl/ Tat	GSH-C4/ Tat	I-152/ Tat
IgG1	6039	1585	1585
IgG2a	1585	3162	6039
IgG2b	1585	3162	3162
IgG3	1585	1585	795



c

Anti-Tat IgG Isotype	NaCl/ Tat	GSH-C4/ Tat	I-152/ Tat
IgG1	6309	3162	795
IgG2a	1584	6309	6309
IgG2b	795	795	3162
IgG3	1584	795	795

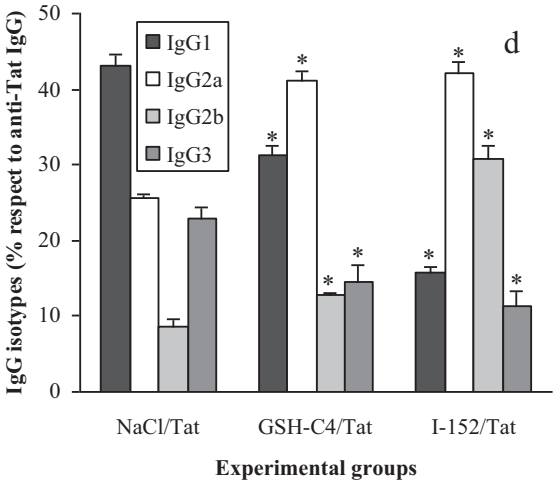


Fig. 3. Isotype analysis of anti-Tat IgG at 10 days (above) and 60 days (below) after the 3rd immunization (bleedings III and IV respectively in Fig. 1). IgG isotypes were determined by ELISA as described in Section 2. Values are expressed both as geometric mean antibody titers for each IgG isotype (a and c) and as the percentage respect to total anti-Tat IgG (b and d). The description of the experimental groups is reported in the legend to Fig. 2. Values are the mean ± S.D. of 5 animals. $^{*}p < 0.01$ vs NaCl/Tat.

3.2. Effect of pro-GSH molecules on anti-Tat IgG isotypes

To establish whether GSH-C4 or I-152 could induce changes in the Th1/Th2 balance and thus in the type of antibody subclass induced, IgG isotype determination was performed 10 and 60 days after the 3rd immunization (bleedings III and IV in Fig. 1). In mice, IgG1 is associated with a Th2-type immune response, while a Th1 response is associated with the induction of IgG2a, IgG2b and IgG3 antibodies [25].

IgG isotype titers were determined in samples obtained at 10 days after the 3rd immunization (bleeding III in Fig. 1) when the animals possessed the highest plasma IgG levels. As reported in Fig. 3a, animals pre-treated with the pro-GSH molecules had a preponderant IgG2a and IgG2b response with titers higher than IgG1 titers, the opposite of what observed in the NaCl/Tat control group. In contrast, IgG3 levels were comparable in the GSH-C4/Tat and NaCl/Tat groups and decreased in I-152/Tat. As shown in Fig. 3c, similar results were obtained at 60 days after the 3rd immunization (bleeding IV in Fig. 1). The predominant induction of antibodies associated to a Th1 response by pre-treatment of Tat-immunized mice with either GSH-C4 or I-152 was also evident when the percentages of the different IgG isotypes respect to total anti-Tat IgG were determined. At 10 days after the 3rd immunization both Th1 and Th2 types of immune responses were detected in Tat-immunized mice (NaCl/Tat) with a prevalence of Th1 response (IgG1: about 34%; IgG2a, IgG2b and IgG3: 66%) (Fig. 3b). This Th1 polarization was further increased in Tat-immunized mice pre-treated with GSH-C4 and I-152, in which anti-Tat IgG1 represented 23% of all anti-Tat IgG with the sum of IgG2a, IgG2b and IgG3 representing 77% of total anti-Tat IgG. At 60 days after the 3rd immunization, even though the levels of total anti-Tat IgG were declining (Fig. 2), the IgG subtypes associated with a Th1-type immune response were still prevalent in all immunized groups and in particular in those pre-treated with either GSH-C4 or I-152; in fact, the sum of IgG2a, IgG2b and IgG3 was about 57%, 70% and 85% in NaCl/Tat, GSH-C4/Tat and I-152/Tat groups respectively while IgG1 represented about 43%, 31% and 16% of the total, respectively (Fig. 3d).

3.3. Effect of pro-GSH molecules on Tat-specific antibody epitope mapping

The epitope reactivity of plasma from Tat-immunized mice was analyzed by using eight synthetic peptides representing different regions of the BH-10 clone-derived Tat linear sequence. At 10 days after the second immunization (bleeding II in Fig. 1), the epitope reactivity of the Abs was broad, with two major sites mapped at residues 1–20 and 46–60 in all Tat immunized mice; a third reactive epitope was identified at residues 73–86 in plasma of Tat immunized animals pre-treated with GSH-C4 or I-152 (Fig. 4a). At 10 days after the third immunization (bleeding III in Fig. 1) the epitope

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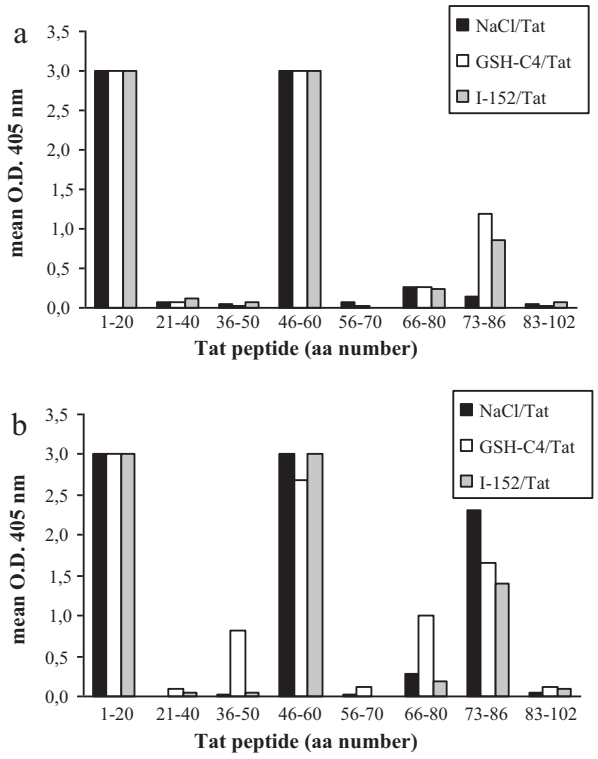

Fig. 4. Epitope mapping of anti-Tat antibodies. It was determined by ELISA as described in Section 2 at 10 days after the 2nd immunization (a) and at 10 days after the 3rd immunization (b). The description of the experimental groups is reported in the legend to Fig. 2. Values represent the mean absorbance values (405 nm) of three immunized mice.

mapping at amino acids 36–50 was recognized by mice immunized with Tat pre-treated with GSH-C4 and the epitopic site mapping at amino acids 73–86 was recognized also by mice immunized with Tat (Fig. 4 b).

3.4. Effect of pro-GSH molecules on cellular immune response

Ten days after the 3rd immunization (bleeding III in Fig. 1), the cellular responses against the VCF Tat peptide, containing both a CD4+ and CTL epitope, were evaluated on splenocytes of immunized mice by ELISPOT. As shown in Fig. 5, Th1-type cellular responses (IFN-γ, IL-2) were generally increased in mice pre-

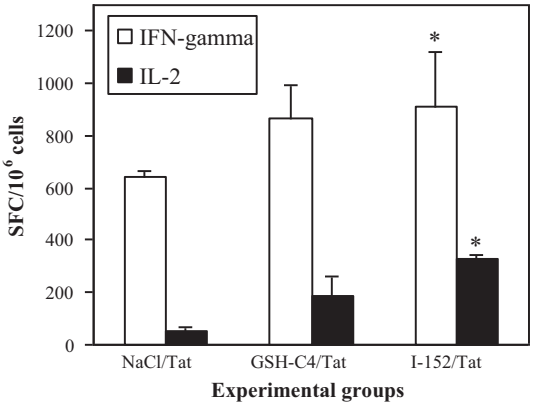

Fig. 5. Analysis of anti-Tat cellular responses. Ten days after the 3rd immunization (III bleeding in Fig. 1), IFN-γ and IL-2 secretion was measured on mice splenocytes (pool of 3 spleens/experimental group) by ELISPOT as described in Section 2. Results are expressed as number of spot forming cells (SFC)/10^6 cells. The description of the experimental groups is reported in the legend to Fig. 2. $^*p < 0.05$ vs NaCl/Tat.

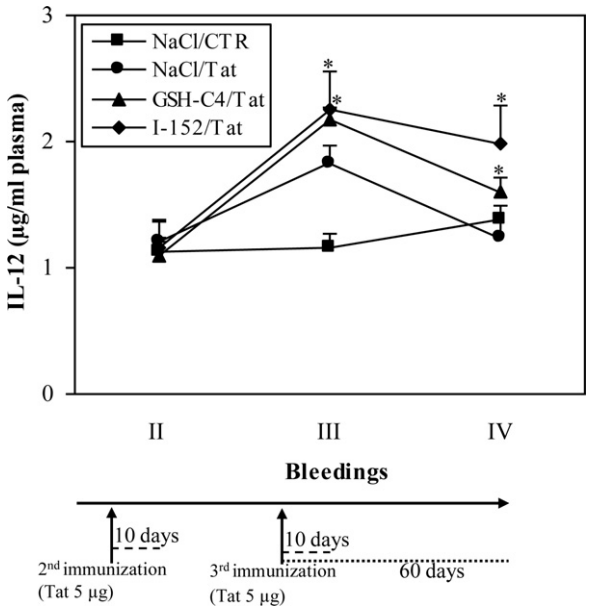

Fig. 6. Kinetics of IL-12 levels in plasma of Tat-immunized mice. IL-12 concentration was determined in the plasma of each experimental group as described in Section 2. The experimental groups are described in the legend to Fig. 2; NaCl/CTR = control, receiving i.p. administration of NaCl 0.9%. The arrows indicate when animals received the 2nd and the 3rd injection. Values are the mean ± S.D. of 5 animals. $^*p < 0.05$ vs NaCl/Tat.

treated with the pro-GSH molecules (GSH-C4/Tat and I-152/Tat) as compared to the control mice (NaCl/Tat). Remarkably, the increase was statistically significant in the I-152/Tat group.

3.5. Effect of pro-GSH molecules on in vivo IL-12 production

It has been widely reported that the ability to generate a Th1- or Th2-type response is partly dependent on the intracellular thiol redox state of APC, influencing IL-12 secretion [2,7,26,27]. IL-12 is a cytokine that promotes the development and the activation of Th1 cells and induces antibody production of the IgG2a, IgG2b and IgG3 subclasses [28]. In order to assess whether the effect of GSH-C4 or I-152 on the immune response to Tat was associated with IL-12 increase, IL-12 levels were measured in plasma of Tat-immunized mice 10 days after the 2nd immunization (bleeding II in Fig. 1) as well as 10 and 60 days after the 3rd immunization (bleedings III and IV in Fig. 1) (Fig. 6). At the 1st time point studied (bleeding II), differences among the experimental groups were not revealed while at the bleeding III, IL-12 levels were significantly higher in animals pre-treated with the pro-GSH molecules as compared to control animals (NaCl/Tat). Also at the bleeding IV, IL-12 was still significantly higher in mice pre-treated with GSH-C4 and I-152 even if IL-12 levels were reduced respect to bleeding III in all immunized animals.

4. Discussion

GSH-C4 and I-152 are molecules able to replenish intracellular GSH, that have gained our interest because of their antiviral activity and ability to modulate the immune response towards Th1 type in Ova-sensitized mice [8,18,19]. The results reported here strengthen the data previously obtained in Ova-immunized mice showing that both GSH-C4 and I-152 are able to potentiate the immune response of another Ag, i.e. Tat, which is in clinical trials for the development of anti-HIV/AIDS vaccine [9–11,29]. It has been reported that thiol content is essential for Ag processing and for cleaving the invariant chain from the major histocompatibility

complex class II necessary for peptide loading [1,3–5]. Low levels of intracellular GSH correlate with defective Ag processing [1]. This appears to be even more important for HIV/AIDS vaccines based on the HIV-1 regulatory protein Tat, a key molecule in HIV/AIDS pathogenesis, which is highly conserved in most functional and immunogenic domains, and can induce both Th1-type T cell and Th2-type B cell responses [9]. In fact, a number of evidence suggests that the redox state of both Tat and APC strongly influence the immunomodulatory properties of the protein: both uptake of Tat by dendritic cells and its capacity to induce Th1-associated cytokine production are affected by its oxidation [12]; the redox state of the protein is essential for its internalisation and/or translocation to cell compartments in macrophages and more precisely, Tat trafficking into human macrophages occurs only when three of the Tat SH groups are free [15]; oxidation of the intra-macrophage sulphydryl reservoir inhibits the uptake of reduced Tat [15]; oxidation induces the formation of inter- and intra-molecular disulfide bonds leading to the loss of its biological activity [13,15]. Moreover, it is important to keep in mind that HIV-infected macrophages have low SH levels [16]. The increase in the SH content induced by GSH-C4 or I-152 may be of importance to optimize the development of Tat-based vaccine; in fact, increasing and/or restoring the redox state of APC is necessary for both the uptake and processing of the Tat antigen, and to stimulate IL-12 secretion, favouring the mounting of Th1 immune response. This hypothesis has been validated *in vivo* in mice immunized with Tat. We found that in these animals, both GSH-C4 and I-152 could induce changes in the Th1/Th2 balance and thus in the antibody subclass generated; in fact, in these animals both molecules promoted the induction of Ag-specific antibodies mainly of the IgG2a and IgG2b isotypes, which are associated with a Th1 response, while suppressing the induction of the Th2-associated isotype IgG1. Further, the higher number of IFN-γ and IL-2 secreting cells detected in mice pre-treated with the pro-GSH molecules as compared to untreated (i.e. NaCl/Tat-immunized mice), indicates that also cellular immune responses were induced more efficiently. In agreement with these data, alteration of the redox state of APC by GSH-C4 or I-152 resulted in significantly higher levels of IL-12 in the plasma of animals treated with the pro-GSH molecules. The highest values of IL-12 were found 10 days after the 3rd immunization. Similar results were previously obtained in Ova-immunized mice (8). Of note, a prevalence of IgG isotypes associated with a Th1-type immune response was detected at the same time point. Two months after the 3rd immunization (bleeding IV), IL-12 decreased in all immunized animals and in NaCl/Tat mice IL-12 levels were similar to those measured in control animals (NaCl/CTR). Notably, significant differences were still evident between Tat-immunized animals pre-treated with the pro-GSH molecules and Tat-immunized mice (NaCl/Tat) (Fig. 6). Higher levels of IL-12 in Tat-immunized mice pre-treated with GSH-C4 and I-152 may be important for both initiation and maintenance of cell-mediated immunity to Tat.

The epitope mapping analysis revealed that Abs in the animals immunized with Tat and pre-treated with the pro-GSH molecules reacted with a broad spectrum of linear Tat epitopes, mapping at residues 1–20, 46–60, 73–86 at both bleedings studied (II and III corresponding to 10 days after the 2nd and the 3rd immunization respectively), while in mice immunized with Tat receiving NaCl i.p. fewer epitope reactivities were detected at bleeding II, while they substantially overlapped with those detected in pro-GSH molecules-treated animals at the bleeding III. So, we can conclude that GSH-C4 or I-152 treatment does not alter the pattern of Tat epitopes recognized but makes two immunizations sufficient to obtain a broad immune response.

The results indicating that pre-treatment with GSH-C4 and I-152 elicits lower levels of anti-Tat specific IgG responses are in agreement with previous data showing a lower concentration of anti-Ova antibodies in mice pre-treated with GSH-C4 and I-152 as compared to immunization with Ova alone (8). At present it is unclear how down-modulation of Ag-specific IgG responses mediated by pro-GSH compounds vs an optimal shaping of Th responses may affect the efficacy of a vaccine treatment. Vaccine efficacy cannot be addressed in the present experimental model, but future efficacy studies using adequate preclinical challenge models will likely address this question. Also, it is presumable that the immunomodulatory activity of pro-GSH molecules should be evaluated case by case depending for example on their preventive or therapeutic use, the type of diseases to be treated, etc. Depending on the disease setting, the induction of a balanced and broad immune response (i.e. humoral and cellular of both Th1 and Th2 type) may be more important than the induction of high antibody titers. Although it cannot be excluded that in some disease settings the overall reduction in antibody titers observed using this approach might lead to impaired vaccine efficacy (e.g. even in the presence of the "right" response pattern, the absolute "values" might be too low to confer adequate protection), it is important to note that in this study the concentration of anti-Tat IgG was still very high in animals treated with GSH-C4 and I-152, and remarkably, in these groups two months after immunization IgG concentration persisted at levels similar to those present in control animals receiving only Tat (NaCl/Tat) (Fig. 2), suggesting that adequate memory B cell responses had been induced.

The considerable progresses attained in the development of CpG-ODN as Th1 immunotherapeutic agent have been recently reviewed [30]. Both CpG-ODN and pro-GSH molecules induce secretion of IL-12 by activated macrophages and promote the induction of the Th1-associated IgG2a isotype shifting the immune response from a Th2 towards a Th1-dominant environment. These effects may be important in suppression of Th2-type response *in vivo*. Further comparable studies are warranted to determine whether pro-GSH molecules, as already demonstrated with CpG-ODN, may be employed in the treatment of Th2 diseases such as asthma and allergic disorders.

The results reported in the previous paper [8] showed that GSH-C4 or I-152 could shift a prevalent Th2 immune response against Ova towards Th1 type and we concluded that these molecules could be used as immunomodulators in those pathologies where GSH depletion can contribute to a shift from the typical Th1 profile towards a Th2 response, such as AIDS and autoimmune and allergic diseases. The present data suggest that by modifying the redox state of APC it is possible to further polarize the immune response induced by an adjuvant-free Tat-based vaccine towards a Th1 type, a feature considered of importance to efficiently combat intracellular pathogens such as HIV-1.

An additional advantage linked to the use of pro-GSH molecules in Tat-based vaccines derives from a large body of evidence showing that the redox state of the protein greatly influences its immunomodulatory properties. It has also been hypothesized that Tat can be an optimal co-antigen in the development of new vaccination strategies since it is able to expand *in vivo* epitope-specific IFN-γ T cell responses directed to different HIV-1 antigens [31]. In this respect, an additional advantage of using the pro-GSH molecules is that they can be exploited to drive or to increase Th1 immune responses and CTL activity against other HIV Ags in HIV vaccine candidates combining different HIV proteins, to enhance existing or to generate new anti-HIV immune response. GSH-C4 and I-152, thanks to their antiviral activity [18,19], may be considered useful components of new therapeutic vaccines whose ultimate goal is to generate anti-HIV immune responses able to clear the infection. We think that the data reported in this paper are necessary and preliminary to further investigations. The i.p. route of vaccination used here was chosen to validate the effects of the pro-GSH molecules when using another Ag, that is Ova [8]; however, the

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poor feasibility of such type of administration clearly represents a limit of the study and further investigations adopting more conventional vaccination routes, such as subcutaneous or intramuscular, are warranted to confirm the effect of the pro-GSH molecules on the immune response against Tat and other antigens before advancing their use to clinical trials. In particular, new studies in which the adjuvanticity of our pro-GSH molecules will be evaluated upon administration by different routes, and compared to that afforded by CpG ODN are planned. Along the same vein, future studies will address whether GSH-C4 and I-152 act as Th1 immunomodulators also when co-administered with the antigen.

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