IP-10 contributes to the inhibition of mycobacterial growth in an *ex vivo* whole blood assay

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A B S T R A C T

Interferon-γ inducible protein 10 (IP-10), is a potent chemoattractant that promotes migration of monocytes and activated T-cells to inflammation foci. IP-10 is elevated in serum of patients with chronic hepatitis C virus (HCV) and tuberculosis (TB) infections, although it remains to be determined the contribution of IP-10 in restricting *Mycobacterium tuberculosis* (*Mtb*) replication. Here, we investigated the impact of IP-10 on mycobacteria replication using the *ex vivo* model of human whole-blood (WB) assay. In particular, we compared the levels of IP-10 upon infection with different *Mtb* clinical strains and species of non-tuberculous mycobacteria (NTM) and evaluated how IP-10 may contain bacterial replication. Interestingly, we observed that the inhibition of the host enzyme dipeptidyl peptidase IV (DPP-IV), which inactivates IP-10 through cleavage of two amino acids at the chemokine N-terminus, restricted mycobacterial persistence in WB, supporting the critical role of full length IP-10 in mediating an anti-*Mtb* response. Addition of recombinant IP-10 expressed in eukaryotic cells enhanced the anti-mycobacterial activity in WB, although no differences were observed when IP-10 containing different proportions of cleaved and non-cleaved forms of the chemokine were added. Moreover, recombinant IP-10 did not exert a direct anti-mycobacterial effect. Our results underscore the clinical relevance of IP-10 in mycobacteria pathogenesis and support the potential outcomes that may derive by targeting the IP-10/CXCR3 pathway as host directed therapies for the treatment of *Mtb* or NTM infections.

1. Introduction

Interferon-γ-induced protein (IP)-10, also called CXCL10, is one of the interferon-inducible, Glu-Leu-Arg negative [ELR(-)] CXC chemokines, a group that includes CXCL9 and CXCL11 (Van et al., 2015). IP-10 binds and activates CXCR3, a G protein-coupled receptor, expressed on activated T cells, preferentially of the Th1 phenotype, but also on NK cells, epithelial cells, fibroblasts and even granulocytes (Metzemaekers et al., 2017). Activation of CXCR3 by IP-10 modulates migration, differentiation and activation of immune cells (Lacotte et al., 2009; Agostini et al., 2001). IP-10 is expressed by antigen-presenting cells in response to Interferon-γ (IFN-γ) and promotes the attraction of monocytes and activated T-cells to foci of inflammation, further enhancing Th1 responses (Azzurri et al., 2005; Petrone et al., 2015). Hence, IP-10 plays a key role at tissue level in the regulation of inflammatory responses, as for example delayed-type hypersensitivity (Kaplan et al., 1987). In line with these findings, altered levels of IP-10 have been associated with various Th1-type human inflammatory diseases of infectious and non-infectious origin (Flier et al., 2001; Sorensen et al., 1999; Lahrtz et al., 1997).

Interestingly, CXCR3 signaling can be inhibited by an antagonist form of IP-10 which lacks two amino acids (referred to as IP-10$_{3-77}$ or short form) in the N-terminal end of the mature protein (referred to as IP-10$_{1-77}$ or long form) (Casrouge et al., 2012). This post-secretion

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modification of IP-10 consists in the N-terminal cleavage of 2 amino acids by members of the X-prolyl dipeptidyl peptidase (DPP) family, the most characterized being dipeptidyl peptidase IV (DPPIV or membrane CD26) (Gorrell, 2005). Accumulation of higher levels of the antagonist form IP-10$_{3-77}$ have been observed in patients with chronic hepatitis caused by Hepatitis C Virus (HCV) not responding to therapy, suggesting that generation of high levels of the antagonism form of IP-10$_{3-77}$ may be a factor contributing to the inability of patients to clear HCV (Casrouge et al., 2011). These findings may have implications for other chronic/persistent infections such as for instance tuberculosis (TB).

Infection with *Mycobacterium tuberculosis* (*Mtb*) is controlled by the host in 90–95% of cases, with no signs or symptoms of the disease that is the hallmark of latent TB infection (LTBI) (Delogu and Goletti, 2014; Goletti et al., 2018a). LTBI is characterized by the persistence and a certain degree of replication of *Mtb* that remains in a dynamic equilibrium with the host immune response, which in most cases lasts for lifetime (Barry et al., 2009; Gengenbacher and Kaufmann, 2012; Chao and Rubin, 2010; Goletti et al., 2018a). Interestingly, increased levels of IP-10 were detected in active TB patients that were shown to decrease after therapy (Petrone et al., 2016; Azzurri et al., 2005; Hong et al., 2012; Cannas et al., 2010; Goletti et al., 2018b), suggesting the use of IP-10 as a biomarker to monitor response to TB therapy (Wergeland et al., 2015; Hong et al., 2014; Garcia-Basteiro et al., 2017), as well as in latent TB infection (La Manna et al., 2018; Petrone et al., 2018b; Kabeer et al., 2011).

To date, we lack a clear understanding of the potential role of IP-10, and primarily of the antagonist form IP-10$_{3-77}$, in TB pathogenesis, and we do not know whether the virulent *Mtb* can differentially modulate IP-10 compared to non-tuberculous mycobacteria (NTM), or whether *Mtb* strains belonging to different phylogeographic lineages (Gagneux, 2018) show a differential ability to elicit IP-10. In this study, we started to answer some of these questions by first implementing an *ex vivo* infection model based on the use of whole blood from healthy subjects.

2. Results

2.1. Setting of the experimental ex vivo model of infection

To investigate the consequences of mycobacteria infection on IP-10 secretion, we implemented the experimental model of whole-blood (WB) infection (Wallis et al., 2001, 2003; Fletcher et al., 2013; Berry et al., 2010), which warrant the interaction of the host immune cellular and humoral factors (Newton et al., 2011). For this aim, we used WB obtained from healthy donors and we firstly tested the experimental setting with an infection with the *M. bovis* BCG and *Mtb* H37Rv at a multiplicity of infection (MOI) of 5:1 and 1:1, respectively, calculated on the average blood monocyte composition. Following infection, at different time points, we evaluated the total colony-forming units (CFUs) on the cellular lysates and IP-10 and IFN-γ concentrations in the plasma. As expected for an avirulent strain, BCG did not replicate in the WB and a reduction in CFUs was observed between day 1 and day 3 post-infection, while *Mtb* H37Rv replicated as expected for a virulent *Mtb* strain (Fig. 1A). Both BCG and *Mtb* induced secretion of a similar pattern of IFN-γ over the three-days long experiment, although levels of IFN-γ, as expected, were lower for the BCG condition despite the fact that a 5 times higher MOI was used for BCG (Fig. 1B). *Mtb* induced endogenous secretion of IP-10 at day 1 and 3 post-infection, and similar levels of IP-10 were measured in BCG-infected WB (Fig. 1C), as expected for an IFN-γ inducible protein.

2.2. Mtb clinical strains induce varying levels of IP-10 in the WB model

To investigate the impact of *Mtb* genetic variability on IP-10 secretion, selected *Mtb* clinical strains, representative of the different lineages of modern and ancient strains of *M. tuberculosis* complex (MTBC) present at global level (Gagneux, 2018; Romagnoli et al.,

2018), were used to infect WB from healthy donors. *Mtb* strains belonging to the clades LAM, S and H3 belong to the modern Euro-American lineage and the clade CAS belong to the modern East-African Indian lineage. *Mtb* strains of the modern lineages were responsible for the largest and deadliest epidemics of the last centuries and appear to have enhanced pathogenic and transmission potential. *Mtb* strains belonging to the ancient lineage 1 cause TB mainly in the Indo-Oceanic regions and the Philippines, such as East African Indian (EAI_SOM or EAI_MAN). As shown in Fig. 2A, all modern *Mtb* strains replicated in the experimental model of WB, with an increase in total CFUs which was higher for the *Mtb* H3 and *Mtb* LAM. Conversely, the two *Mtb* clinical strains belonging to the ancient lineage EAI could not replicate, with total CFUs at day 6 significantly lower than those observed at day 1. The pattern of IP-10 secretion following infection with these *Mtb* strains was characterized by higher levels of IP-10 after infection with *Mtb* H37Rv and the *Mtb* S strains, while low levels were measured with the other clinical strains (Fig. 2B). Taken together these results indicate that *Mtb* strains of the modern lineages are characterized by enhanced virulence in this WB model of infection, that different *Mtb* strains may induce different levels of IP-10 and that there is no correlation between ability to replicate and IP-10 secretion.

2.3. Inhibiting DPP-IV results in a restriction of mycobacterial replication in the WB model of infection

It has been proposed that the truncated short form of IP-10 fails to exert the typical antimicrobial activity of the mature chemokine through defective lymphocyte trafficking (Decalf et al., 2016; Casrouge et al., 2012). To investigate the role of DPP-IV in this process, WB from healthy donors was collected in heparin tubes and in BD™ P800 tubes, containing spray-dried potassium EDTA anticoagulant and a proprietary cocktail of protease, esterase, and DPP-4 inhibitors (BD Biosciences) (Kishimoto and Noda, 2012), and then infected with the *Mtb* strains H37Rv, H3 and LAM of the modern lineage and *Mtb* EAI_SOM of the ancient lineage. As shown in Fig. 3A, inhibition of DPP-IV resulted in a reduction of total CFUs in WB, with a more remarkable reduction for the *Mtb* EAI-SOM strain. Interestingly, this reduction in the total CFUs for the ancient strain correlated with a dramatic increase in IP-10 (Fig. 3B). A less marked increase of IP-10 was also observed in WB treated with the DPP-IV inhibitor and then infected with the *Mtb* LAM, while no increase in IP-10 was observed for *Mtb* H37Rv and *Mtb* H3.

A similar experimental approach was used to evaluate the impact of DPP-IV inhibition on infection with NTM. As shown in Fig. 4A, using BD™ P800 tubes to inhibit the endogenous DPP-IV resulted in a significant reduction in total CFUs at day 1 post-infection for *M. abscessus* and *M. gordonae* compared to untreated WB. Inhibition of endogenous DPP-IV led to an increase in IP-10 levels (Fig. 4B). To exclude that the inhibition observed using BD™ P800 tubes was due to the presence of EDTA instead of heparin as anti-coagulant, an additional experiment was performed using also EDTA tubes. As shown in Fig. 4C, no differences were observed in total CFUs counts between the EDTA- and heparin- containing tubes, while a CFU reduction was observed using BD™ P800 tubes. Taken together, these results suggest that inhibition of endogenous DPP-IV enhances the anti-mycobacterial activity of WB cells resulting in a modulation of mycobacterial replication and endogenous IP-10 levels.

To further investigate the consequences of DPP-IV inhibition on mycobacterial survival in WB, the drug sitagliptin, a well-known DPP-IV-inhibitor used in type-2 diabetes patients (Picatoste et al., 2013; Kishimoto and Noda, 2012), was added at different concentrations. As shown in Fig. 5A, sitagliptin decreased the CFUs of *M. gordonae* starting at 50 μM in heparin, and similar results were obtained when sitagliptin was added in WB with EDTA (Fig. 5B). Sitagliptin decreased the total CFUs also in WB infected with *M. abscessus* (Fig. 5C) and *Mtb* H37Rv (Fig. 5D), after a time course of 5 and 6 days post-infection and post-sitagliptin treatment, respectively. Interestingly, no reduction in total
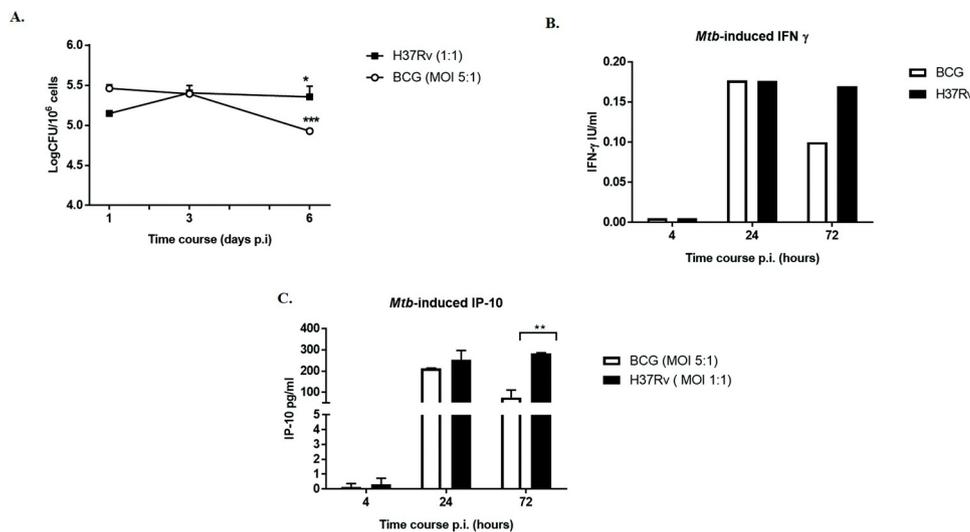
Fig. 1. **Definition of the experimental model based on WB from healthy subjects.** Whole-blood from different healthy donors were infected with virulent *Mtb* H37Rv and the vaccine strain *M. bovis* BCG at MOI 1:1 and 5:1, respectively. Numbers of colony-forming units (CFUs) of different strains were determined at 1, 3 and 6 days post-infection (p.i.) (A). Number of total mycobacteria are expressed as mean ± SD log CFUs for $1,2 \times 10^6$ cells, and statistical analysis was performed by two-way ANOVA followed by Bonferroni post-test (***p < 0.001; * p < 0.05). The results are the mean value of CFUs in triplicate from a representative experiment. Supernatants were collected at different time-points (4, 24 and 72 h p.i.) and assayed for IFN-γ (B) and IP-10 (C). Data were analysed by One-way ANOVA followed by Bonferroni post-test (**p < 0.005). No statistically significant differences were observed between *Mtb* H37Rv and BCG in terms of IFN-γ secretion.
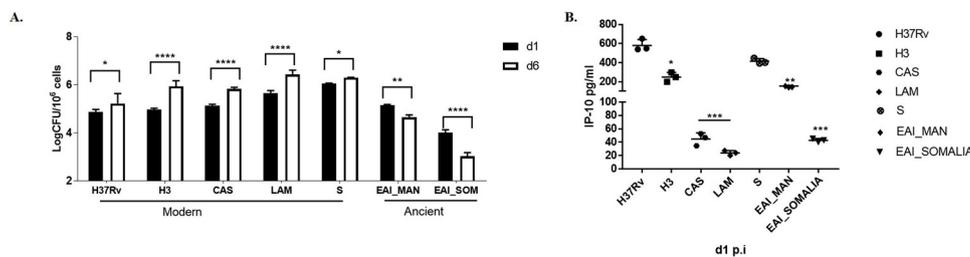

Fig. 2. **Impact of *Mtb* genetic variability on IP-10 secretion in WB:** To analyse the impact of *Mtb* genetic variability on the ability to persist (A) and to induce IP-10 (B), WB was infected with modern and ancient *Mtb* strains at MOI 1:1 and total mycobacteria replication was expressed as mean ± SD log CFUs/1,2 10^6 cells, and statistical analysis was performed by two-way ANOVA followed by Bonferroni's multiple comparisons test, comparing all the strains in the 2 different time points (*p < 0.05; ** p < 0.01, **** p < 0.001). The results for all *Mtb* strains, mean value of CFUs in quadruplicate from a representative experiment. Data for secretion of IP-10 were analysed by One- way ANOVA with Dunnett's multiple comparisons test against *Mtb* H37Rv. Values are expressed as a mean of three independent experiments.

CFUs were observed when sitagliptin was added to the *Mtb* infected WB at day 3 post-infection and CFUs counts evaluated at day 5 (Fig. 5E), suggesting that the anti-mycobacterial effect occurs only when sitagliptin is added early in the system. This seems the case for the virulent *Mtb* but not for avirulent mycobacteria as *M. gordonae*, where treatment with sitagliptin can still restrict intracellular replication when added later in the system (at day 3 p.i.).

2.4. Exogenous recombinant IP-10 inhibits mycobacterial growth

The results obtained upon inhibition of DPP-IV with BD™ P800 tubes and sitagliptin suggest that IP-10 cleavage may hinder the anti-mycobacterial activity of the chemokine. To further investigate the role of IP-10 isoforms, we expressed a recombinant form of IP-10 (rIP-10) in eukaryotic cells by transfecting rhabdomyosarcoma (RD) cells with the

plasmid pUNO1-hIP-10. Transfected cells were cultured in antibiotic-free media, in the presence or absence of sitagliptin, and supernatants collected at day 5. As shown in Fig. 6A, the concentration of total rIP-10 in the supernatants of these two preparations were similar, but the relative levels of the short and long forms were different, according with the literature (Decalf et al., 2016). Indeed, RD cells transfected with pUNO-hIP-10 and treated with sitagliptin led to an accumulation of higher levels of long rIP-10 compared to untreated transfected cells (Fig. 6A).

The supernatants containing rIP-10, treated or not with sitagliptin, were used to condition the WB in different experimental settings to reach a final concentration of exogenous administered rIP-10 of 2 ng/ml. Addition of recombinant rIP-10 to WB decreased the total CFUs counts following infection with the *Mtb* H37Rv and the selected modern and ancient *Mtb* strains, regardless of the presence of sitagliptin and of
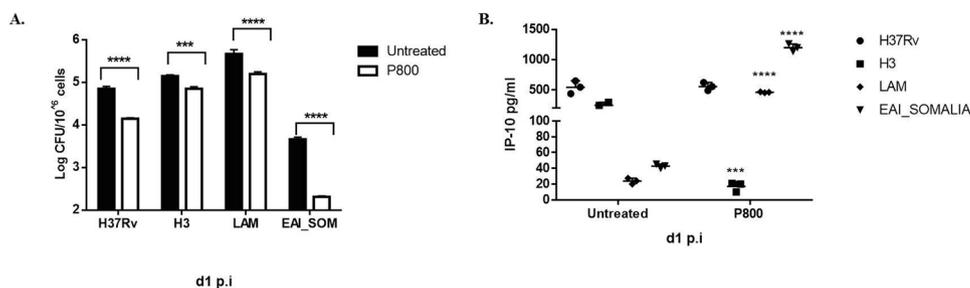

Fig. 3. **Inhibition of DPP-IV restricts mycobacterial growth in WB.** WB collected in tubes containing heparin (untreated condition) and in BD™ P800 tubes containing DPP-IV inhibitor (P800 condition), were infected with the *Mtb* strains of the ancient (EAI_SOM) or modern (H3, LAM) lineages and with reference strain *Mtb* H37Rv, at MOI 1:1. At day 1 post-infection, cells were lysed to measure the number of viable bacteria by plating serial dilutions to determine CFUs (A). The results represent the mean ± SD of representative of three independent experiments and statistical analysis was performed by two-way ANOVA followed by Bonferroni post-test (***p < 0.001). Data obtained from single independent infections are reported in Fig. S2. Cell culture supernatants were collected at day 1 post-infection. The production of IP-10 was measured by ELISA Quantikine Human IP-10 (Aviva systems biology) (B). Values are expressed as a median of three independent experiments, and statistical analysis was performed by One- way ANOVA with Dunnett's multiple comparisons test against untreated conditions (***p < 0.005, ****p < 0001).
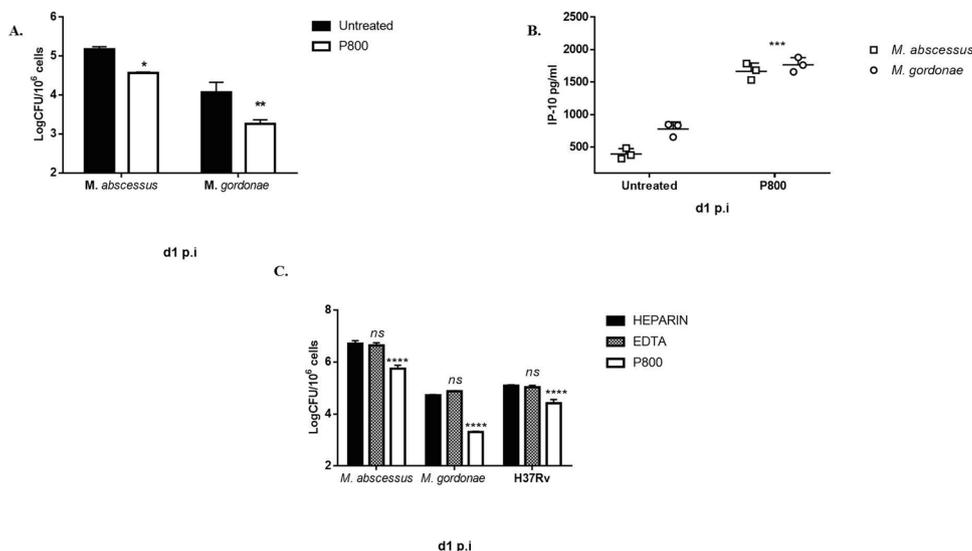
Fig. 4. Inhibition of DPP-IV enhances the anti-mycobacterial activity of WB cells. WB harvested in tubes containing or not DPP-IV inhibitor (P800) were infected with NTM strains (*M. abscessus*, *M. gordonae*) at MOI 1:1. At day 1 post-infection, mycobacterial load was measured by CFU counting (A). The results represent the mean ± SD of representative of three independent experiments and statistical analysis was performed by two-way ANOVA followed by Bonferroni post-test (*p < 0.05, **p < 0.001) between untreated and treated strains. Data obtained from single independent infections are reported in Fig. S2. Cell culture supernatants were used to measure IP-10 concentrations (B). Values are expressed as a mean of three independent experiments, and statistical analysis was performed by One-way ANOVA with Dunnett's multiple comparisons test against untreated conditions (***p < 0005). To rule out that the observed anti-mycobacterial effect was not dependent on the different anti-coagulants, WB harvested tubes with heparin, EDTA and in BD™ P800 tubes, infected with NTM and CFUs counted as described above (C). Statistical analysis was performed by two-way ANOVA followed by Bonferroni post-test (**** p < 0.001, and not significant).

the long-/short-IP-10 relative levels (Fig. 6B), with a CFUs reduction higher than 50% for the *Mtb* LAM and *Mtb* EAI_SOM strains (Fig. 6C). Treatment of *Mtb* infected WB 1 h post-infection resulted in a reduction in total CFUs, with the only exception of WB infected with the *Mtb* EAI and conditioned with the supernatant containing rIP-10 and sitagliptin (Fig. 6D and E). In addition, conditioning of WB infected with the *Mtb* strains at day 1 post infection did not result in a reduction of total CFUs at day 2 (Fig. 6F and G).

2.5. Recombinant IP-10 does not have a direct antimicrobial activity against mycobacteria

Recent reports indicated that IP-10 may have a direct antibacterial effect on a variety of pathogenic bacteria such as *Bacillus anthracis* (Margulieux et al., 2016), *Staphylococcus aureus, Listeria monocytogenes, Chlamydophila pneumoniae* and *Escherichia coli* (Cole et al., 2001; Balogh et al., 2011; Crawford et al., 2009). To test whether IP-10 may have a direct activity on mycobacteria, the supernatants of transfected RD cells
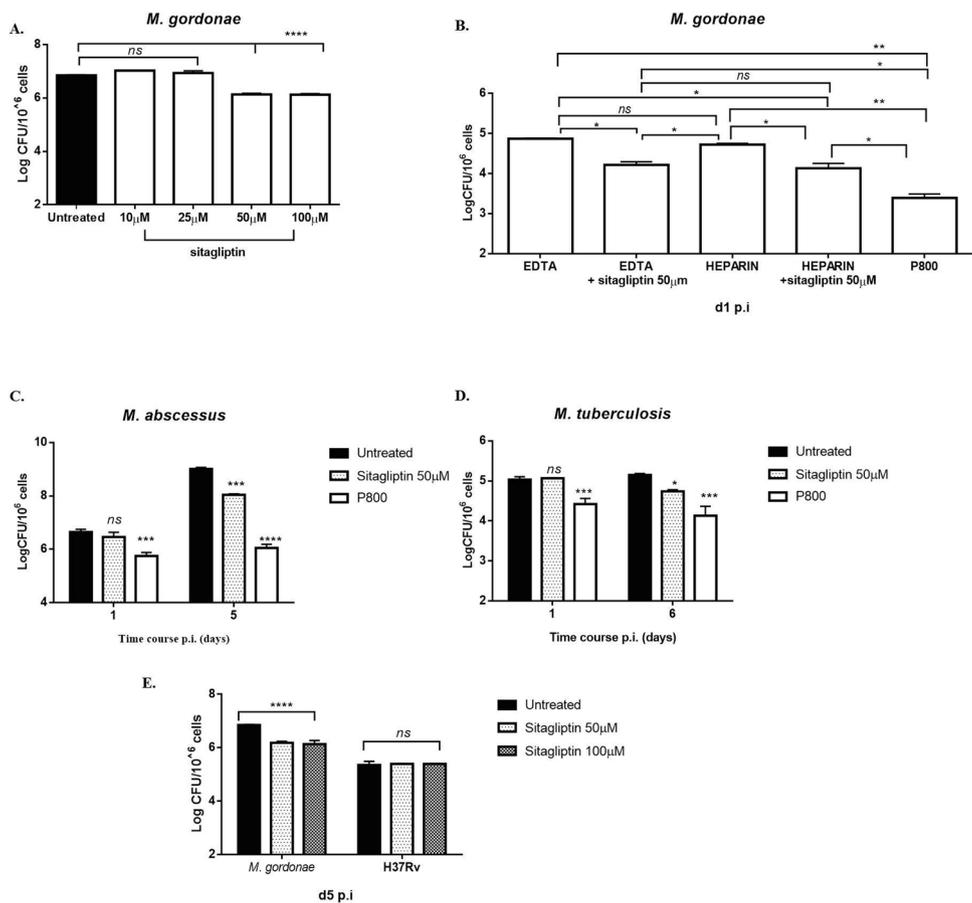

Fig. 5. Pharmacological inhibition of DPP-IV enhances the anti-mycobacterial activity only when administered in the early steps of infection. WB from different donors harvested in tubes containing or not sitagliptin, were infected with different mycobacterial species (*M. abscessus*, *M. gordonae* and *Mtb*) at MOI 1:1. A) WB were pre-treated with increasing concentrations of sitagliptin (10, 25, 50 and 100 µM) and then infected with *M. gordonae;* bacterial load was measured by CFU counting. Values are expressed as a mean of three independent experiments, and statistical analysis was performed by One-way ANOVA with Dunnett's multiple comparisons test against untreated conditions (****p < 0.01 and not significant). B) WB was harvested in a tube with heparin or with EDTA, with or without the addition of 50 µM of sitagliptin and infected with *M. gordonae* as described above. Statistical analysis was performed by One-WayANOVA followed by Tukey's multiple comparisons test (*p < 0.05, ** p < 0.01 and not significant). Panels C and D show the bacterial loads at different time point post-infection in WB infected with *M. abscessus* and *M. tuberculosis*, pre-treated or not with sitaglitpin; or when sitagliptin was added at day 3 post-infection (E). Statistical analysis was performed by two-way ANOVA followed by Bonferroni post-test (*p < 0.05, ***p < 0.01, **** p < 0.005 and not significant).
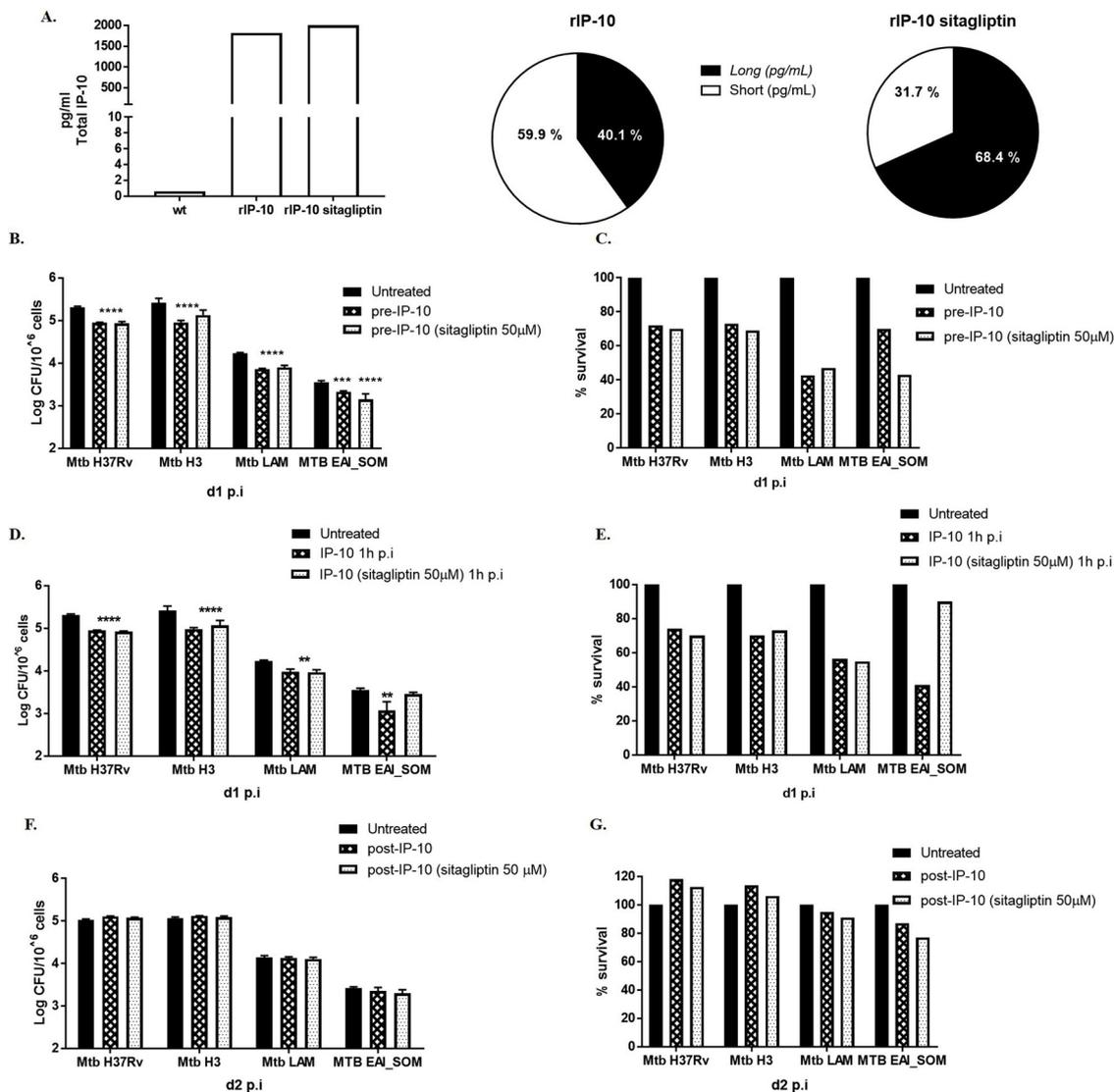
Fig. 6. Addition of rIP-10 increases the antimicrobial activity of WB: Human recombinant IP-10$_{1-77}$ and its truncated form IP-10 $_{3-77}$ obtained as a mix from RD cells transfected with pUNO-hIP-10 and grown in presence or absence of sitagliptin, were quantified with SIMOA (Casrouge et al., 2012; Meissner et al., 2015) (A). The two mixes of agonist and antagonist forms of IP-10 were used to condition the WB assay, by adding rIP-10 1 h before *Mtb* infection (B), 1 -h post-*Mtb* infection (D) and finally 1 day after *Mtb* infection (F). To highlight the inhibition of mycobacterial survival following conditioning with rIP-10, we expressed the results as percentage of survival of treated vs untreated cells in panels C, E and G for the CFUs shown in panels B, D and F, respectively. Bacterial loads were determined by CFU counting of total WB lysates. Values are expressed as a mean of three independent experiments, and statistical analysis was performed by One- way ANOVA with Dunnett's multiple comparisons test against untreated condition (**p < 0.01, ****p < 0001).

containing rIP-10, cultured in the presence or not of sitagliptin, were evaluated against different species of mycobacteria (*M. smegmatis*, *M. gordonae* and *M. abscessus*), in liquid and solid media (supplementary data). Mycobacteria were exposed at concentrations of rIP-10 up to 2 ng/ml, similar to those commonly found in biological fluids from healthy donors (see Fig. 6A). No anti-mycobacterial effect was detected in all the experiments performed (supplementary data), indicating that the rIP-10 expressed in eukaryotic cells does not exert any direct activity against mycobacteria.

Taken together these results indicate that IP-10 can have an anti-mycobacterial activity and that this activity is not directed against bacterial cells but requires the presence of host cells, as expected for a chemokine.

3. Discussion

IP-10 is a chemokine known to play a key role during inflammation and in chronic persistent infections as TB (Wang et al., 2012). Blood IP-

10 concentrations are found to be higher in patients with active TB and LTBI compared to healthy subjects, suggesting a potential role for this chemokine as a TB biomarker (Wang et al., 2012; Armand et al., 2014). However, the role of IP-10 in TB pathogenesis remains to be determined (Blauenfeldt et al., 2018), as whether *Mtb* can modulate or manipulate IP-10 secretion, or the associated downstream signaling, to promote persistence in host tissues and disease. For instance, it is not clear whether *Mtb* strains with different virulence and pathogenetic properties are able to differentially elicit IP-10 or whether IP-10 concentrations affect mycobacterial survival in host cells. To investigate the role of IP-10 during *Mtb* infections, we set up an experimental protocol based on the infection of human whole blood (WB) (Fletcher et al., 2013), which ensures the combined presence of multiple cells that can express and secrete IP-10 and its receptor CXCR3 (Berry et al., 2010).

Infection with a panel of *Mtb* clinical strains belonging to different phylogeographic lineages showed that "modern" *Mtb* strains replicate in WB during the 6 days of incubation, with subtle differences between the different clades. Conversely, *Mtb* strains of the "ancient" lineages

could not replicate. These results confirm that modern and ancient strains have different virulence properties, as recently shown by our group in human monocyte derived macrophages (Romagnoli et al., 2018) and by others in similar *ex vivo* systems (Fletcher et al., 2013). However, concentrations of IP-10 were different between the *Mtb* clinical strains evaluated and did not directly or inversely correlate with virulence in WB. Further studies shall be performed by including groups of healthy donors and *Mtb*-infected subjects, so to assess the correlation between *Mtb* growth and IP-10 in WB in conditions including an *Mtb*-specific immune response (O'Shea et al., 2018). In these systems it might also be possible to further evaluate the impact of the *Mtb* genetic diversity by using modern or ancient strains, or generalist and specialist sub-lineages (Stucki et al., 2016; Gagneux, 2018). The lack of correlation between the ability to persist in WB and secretion of IP-10 was also confirmed following infection with three NTM species, although the genetic and immunological differences between these species is far greater than what observed within MTBC complex and the host defense mechanisms poorly understood (Chan et al., 2010).

Seminal studies, performed in *M. bovis BCG*-infected epithelial cells (Mendez-Samperio et al., 2009), demonstrated that expression of IP-10 is regulated via the NF-kB signaling pathway that induces transcriptional activation of the *ip-10* gene (Cheeran et al., 2003) by direct interactions between mycobacterial components (Mendez-Samperio et al., 2009) and in a paracrine and autocrine mode by the release of type 1 IFN from *Mtb*-infected cells (Lande et al., 2003). IP-10 has been proposed to participate in destroying *Mtb*-infected cells through the recruitment and selective homing of activated/effectors cells (Lande et al., 2003). *In vitro* and *in vivo* (Decalf et al., 2016) studies have shown that DPP-IV can cleave IP-10 at N-terminus leading to the generation of an antagonist form (IP-10$_{3-77}$) (Proost et al., 2001; Casrouge et al., 2011). Interestingly, elevated levels of IP-10$_{3-77}$ were associated with an increased activity of the serine protease DPP-IV, both being negative predictors for viral clearance in chronic and acute HCV patients (Casrouge et al., 2011; Ragab et al., 2013). Moreover, agonist and antagonist IP-10 have been detected in plasma and urine from patients with active TB (Blauenfeldt et al., 2018; Petrone et al., 2018a). To investigate whether, following *Mtb* infection, inhibition of DPP-IV would impact IP-10 secretion and *Mtb* survival, WB was infected in the presence of a DPP-IV inhibitor contained in the BD™ P800 tubes. Interestingly, infection of WB in the presence of the DPP-IV inhibitor did not consistently result in higher concentration of total IP-10, which instead differed depending on the *Mtb* clinical strain used or the mycobacterial species, although we could not distinguish the truncated form (IP-10$_{3-77}$) from the non-truncated one (IP-10$_{1-77}$). However, inhibition of DPP-IV in infected WB consistently enhanced the killing of *Mtb* or other NTM, though the extent of the anti-mycobacterial activity differed depending on the *Mtb* strain or the NTM species. In line with these findings, WB infected in the presence of the DPP-IV inhibitor sitagliptin, a clinically approved drug for the treatment of type 2 diabetes (Barreira da et al., 2015), showed enhanced and dose-dependent anti-mycobacterial activity. However, when sitagliptin was added three days following infection, a reduction of CFUs was observed for WB infected with *M. gordonae* but not with *M. smegmatis* or *Mtb*, supporting the critical role of DPP-IV at the early events following infection (Lande et al., 2003).

To investigate the contribution of IP-10 in restricting mycobacterial replication in WB, we expressed a recombinant form of IP-10 (rIP-10) in eukaryotic cells, in the presence or not of the DPP-IV inhibitor sitagliptin. Surprisingly, addition of rIP-10 enhanced mycobacterial killing in WB regardless of the differences in the ratio between the truncated and non-truncated forms of rIP-10. It is possible that the amount of IP-10$_{1-77}$ is sufficiently high even in the untreated supernatants to exert the anti-mycobacterial activity or, as described by Casrouge *et al* (Casrouge et al., 2012), that inhibition of IP-10$_{1-77}$ activity can be achieved only when the concentration of the truncated form (IP-10$_{3-77}$) is 5–10 times than non-truncated one (IP-10$_{1-77}$).

Again, addition of rIP-10 enhanced the anti-mycobacterial activity of WB only when added early during infection, while it had no effect when added later on (1-day post-infection), in line with the results obtained with sitagliptin. Moreover, we could not measure any direct activity of rIP-10 against *Mtb* or a panel of NTM. This is different from recent reports, where IP-10 was found to exert a direct antimicrobial activity against *B. anthracis* (Margulieux et al., 2016). The exact mechanism by which IP-10, and more in general a group of other chemokines belonging to the interferon-inducible ELR⁻CXC family (Cole et al., 2001), mediate a direct antibacterial activity remains unclear, though the membrane protein FtsX emerged as a potential target (Schutte et al., 2016). It is possible that we could not observe a direct activity against *Mtb* or NTM because we used IP-10 concentrations lower compared to those employed in previous studies. We preferred to use concentrations of rIP-10 (max concentration of 2 ng/ml) which is closer to the physiological conditions, ranging from 10 to 100 pg/ml in plasmas (Decalf et al., 2016) and 100–1000 pg/ml in *Mtb* infected WB as in this study. Conversely, higher concentrations of rIP-10 were used in the studies showing a direct activity against bacteria (ranged between 0.1 to 100 µg/ml) (Cole et al., 2001). Moreover, we used a rIP-10 expressed in eukaryotic cells rather than a protein expressed in *E. coli* (Schutte et al., 2016; Margulieux et al., 2016). Nonetheless, we trust that the experimental system we used to test the direct activity of IP-10 against mycobacteria resembles the physiological conditions *in vivo* (Joosten et al., 2018). Moreover, we exclude that the antimicrobial activity observed in infected WB following addition of IP-10 is the result of a direct anti-mycobacterial activity of the chemokine.

The results of this study support the contribution of IP-10 in restricting *Mtb* replication in host tissues, though no direct activity of IP-10 against mycobacteria was observed. However, the relative concentrations of IP-10, and of the truncated and non-truncated forms, in the intercellular microenvironment in granulomas and small lesions, where the interactions between *Mtb*-infected cells and other host cells take place, may be much higher than those commonly found in biological fluids which may represent an average of quite different conditions. Hence, the differential activity of the agonist and antagonist forms of IP-10 can still be critical to control the *Mtb* infection in the intercellular environment. This is important considering the recent experimental evidences on TB pathogenesis obtained in the non-human primate model of TB, indicating that animals latently infected with *Mtb* have a significant number of replicating bacilli in lung lesions and that animals with active TB can sterilize certain granulomas but not others due to the heterogeneity in the innate and adaptive responses (Lin et al., 2014). Interestingly, each lesion in monkeys with active TB appears to originate from a single bacterium and the local events in each lesion, resulting from the complex host-pathogen interactions and the ensuing chemokine and cytokine milieu, will shape the fate of each lesion, thus contributing to dictate the clinical outcome of *Mtb* infection (Lin et al., 2014). In this scenario, the role of IP-10 in modelling the cellular composition and the inflammatory responses at local level in each lesion or granulomas may be critical and can contribute in defining the ability of the host to contain or not *Mtb* infection. The recent observation that restriction of mycobacterial growth in PBMCs is associated with trained immunity mediated by IP-10 produced primarily by the no classical CD14^{dim} monocyte subsets (Joosten et al., 2018) support our findings on the clinical relevance of IP-10 in mycobacterial pathogenesis and suggest that targeting the IP-10/CXCR3 pathway may open the way for new host directed therapies for the treatment of TB or other NTM infections (Palucci and Delogu, 2018). Future studies are needed to elucidate the cells involved in the execution of the IP-10-mediated anti-mycobacterial activity and we anticipate that the use of *ex vivo* experimental models, such for instance the use of PBMC to establish the granuloma-like structures or experiments carried out in transwell settings, may allow monitoring of the types of cells being recruited by infected macrophage, thus contributing to get insights on the mechanism responsible for the observed anti-mycobacterial activity

of IP-10.

4. Materials and methods

4.1. Bacterial strains

All mycobacteria both NTM and *Mtb* complex clinical strains (MTBC) used in this study were selected from a collection of clinical strains isolated at the Fondazione Policlinico Gemelli IRCCS, Università Cattolica del Sacro Cuore (Romagnoli et al., 2018; Camassa et al., 2017); *Mtb* H37Rv was obtained from the Trudeau Culture Collection. All mycobacteria were grown in Middlebrook 7H9 (Difco BD, NY) medium supplemented with 0.2% glycerol, 10% ADC (Microbiol, Cagliari, Italy) and 0.05% Tween 80 (Sigma-Aldrich, St. Louis, MO) at 37 °C. Mycobacterial cultures were harvested at late log phase, glycerol was added at 20% final concentration and 1-ml aliquots stored at −80 °C.

4.2. Patients and recruitment

The WB derived from healthy donors. Participants were recruited among people who had recently tested negative for HIV, not vaccinated with BCG, male, Caucasian and aged between 30–35 years. Written informed consent was obtained from each donor. The study was approved by the INMI Ethical Committee (pareri n° 29/2014; 34/2010; 28/2014).

4.3. Whole-blood (WB) assay

Whole blood was obtained from healthy donors, added in tubes with heparin or containing EDTA or with specific BD™ P800 tubes with inhibitor of DPP-IV (BD biosciences), as previously described (Wallis et al., 2001), nucleated cells counted by Nucleoconter (Chemotec) and plated at concentration of 1.2×10^6 cell/ml in 48 well plates. WB were incubated in rocking incubator at 37 °C, 20 rev/min. Mycobacteria *inocula* at a MOI of 1:1, based on the average blood monocyte composition, were directly added to cell culture and different time point post-infection total mycobacterial CFUs were enumerated. WB at different time points after infection especially on day 5 or 6 each time we tested sitagliptin as a treatment against mycobacterial infection, were lysed and resuspended in PBS with 0.05% Tween 80, then total lysates were serially diluted and plated on 7H11/OADC agar medium (Camassa et al., 2017). Results are expressed as $LogCFU/10^6$ nucleated cells to allow normalization and comparison between different experiments. Plasma (supernatants) from WB at day 1 post-infection were collected and stored a −80 °C until tested by ELISA (INF-γ ELISA was from Invitrogen).

4.4. IP-10 detection

Supernatant from mycobacteria–infected cell cultures were collected at day 1 post infection, and chemokine IP-10 was evaluated by ELISA Quantikine Human IP-10 (Aviva systems biology). Concentrations of total IP-10, agonist IP-10 (IP-10$_{1-77}$) and antagonist IP-10 (IP10$_{3-77}$) was been measured in protease-inhibited plasma using in-house SIMOA ultrasensitive digital ELISA (Quanterix) as previously described (Casrouge et al., 2012; Meissner et al., 2015).

4.5. Cell transfection

The pUNO1-hIP-10 (Invivogen, USA) has been used to transfect rhabdomyosarcoma (RD) cells with Lipofectamine 2000 (Life Technologies, USA) according to the manufacturer's instructions. The transfected cells were grown in RPMI media with blasticidin (2.5 μg/ml) (Invivogen, USA), a selective antibiotic, for 5 days, in the presence or not of sitagliptin (50 μM) (Luxembourg/Luxemburg *MSD)*.

Supernatants of the selected transfected RD cells were harvested, and aliquots stored at −80 °C.

4.6. Direct antimicrobial assay

Live mycobacteria was quantified after addition of rIP-10 (2 ng/ml) via the generation of a fluorescent signal from the reduction of the AlamarBlue oxidation-reduction dye by measuring the fluorescence (Margulieux et al., 2016).

4.7. Statistical analysis

Data were analyzed using the GraphPad Prism software, version 7.02 for Windows (GraphPad Software, San Diego, CA). For all the experiments shown, with the exception of the cytokine results, the data were evaluated by analyzing the median and standard deviation. The statistical significance of the differences between 2 groups was determined by using two-way ANOVA followed by Bonferroni post-test. For the cytokine results, data were analyzed evaluating the media and p-values were calculated using the One- way ANOVA with Dunnett's multiple comparisons test against referent strain. Differences were considered significant if p values were ≤ 0.05.

Declarations of interest

DG has been a consultant and received fees for presentations for QUIDEL, JENSSEN and QIAGEN. The other authors do not report any conflict of interest

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijmm.2019.05.005.

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