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C5aR contributes to the weak Th1 profile induced by an outbreak strain of *Mycobacterium tuberculosis*

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ABSTRACT

C5a anaphylatoxin is a component of the complement system involved in the modulation of T-cell polarization. Herein we investigated whether C5a receptors, C5aR and C5L2, modulate the cytokine profiles induced by *Mycobacterium tuberculosis* (*Mtb*). We analyzed the impact of both receptors on T helper cell polarization induced by the multidrug resistant outbreak strain named M, which is a poor IFN-γ inducer compared with the laboratory strain H37Rv. To this aim, we first blocked C5aR or C5L2 of peripheral blood monocytes (Mo) from patients with tuberculosis and healthy donors, then we stimulated the Mo either with H37Rv or the M strain, and finally we analyzed cytokine profiles of Mo/macrophages (MΦ) and $CD4^+$ T-cells. We found that: (i) *Mtb* modulated the expression of both C5a receptors, (ii) C5aR inhibited the expansion of $CD4^+IFN\text{-}\gamma^+$ lymphocytes stimulated by the M strain but not by H37Rv, (iii) both receptors modulated the Mo/MΦ cytokine expression induced by *Mtb*. We conclude that C5aR, but not C5L2, plays a role in T helper cell polarization induced by *Mtb* and that this effect is strain- and donor-dependent. We speculate that the epidemiologically successful M strain takes advantage of this C5aR-mediated inhibition of Th1 polarization to survive within the host.

1. Introduction

Tuberculosis (TB) is a major global health problem aggravated by the emergence of multi-drug resistant (MDR) forms of the disease [1]. Although the immunology of TB has been extensively studied for decades, it is only partially understood and there is still no effective vaccine to prevent the disease. Widening the focus of investigation to explore components of immunity that are usually overlooked might open new perspectives to approach the subject. In this regard, little attention has been paid to the contribution of the complement system, even though its activation products are elevated in tuberculous pleural effusions [2,3] and in sera from patients with active pulmonary TB [4].

The complement system, a crucial component of humoral innate immunity, has been recently found to be involved in the regulation of the adaptive immune response through the action of the anaphylatoxins produced as part of the proteolytic cascade of complement activation [5]. Indeed, C5a anaphylatoxin, which is well known for its role as a pro-inflammatory mediator, participates along with C3a in the regulation of T-cell profiles. This C5a function was mainly reported in murine models of asthma where it plays a dual role in Th1/Th2 polarization balance [6]. Moreover, dendritic cells from mice deficient in C5a receptor (C5aR) stimulated in vitro with OVA and/or Pam3CSK4 were found to bias cytokine

Abbreviations: TB, Tuberculosis; MDR, multidrug resistant; *Mtb*, *Mycobacterium tuberculosis*; PRR, pattern recognition receptors; C5, complement component 5; C5aR, C5a receptor; APC, antigen presenting cells; IL, Interleukin; Mo, peripheral blood monocytes; MΦ, macrophages; PPD+HD, protein purified derivate positive healthy donors.

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production towards Th17 and T regulatory profiles in detriment of Th1 polarization [7]. Conversely, C5a favoured the secretion of Th1 cytokines in LPS-activated human monocytes (Mo) [8]. Thus, the role of the C5a-C5aR axis in T-cell polarization is ambiguous and depends on the model analyzed. C5a exerts its effects through two seven-transmembrane receptors: C5aR (CD88) which is a G protein-coupled receptor [9], and C5L2 which is G protein-uncoupled [10]. Both receptors are expressed mainly on myeloid cells, although C5aR is also expressed on non-myeloid cells [10,11].

A Th1 polarization of $CD4^+$ T-cells is crucial for a protective response against *Mycobacterium tuberculosis* (*Mtb*). These Th1 cells produce interferon gamma (IFN-γ), which is mainly secreted upon IL-12 production by APC [12]. In turn, IFN-γ activates bactericidal mechanisms that help controlling *Mtb* growth [13,14]. Increasing evidence has pointed to the importance of the *Mtb* genotype in the development of protective immunity [15–18]. Furthermore, we have previously shown that human immune cells respond differentially to *Mtb* depending not only on bacterial genotype but also on host immunological status [19,20]. For this reason, the traditional approaches using immune cells from healthy donors (HD) challenged with laboratory strains like H37Rv might fail to expose the involvement of certain regulatory mechanisms in the protective response and the pathophysiology of TB. In this regard, we have demonstrated that upon *Mtb* stimulation, $CD4^+$ T-cells from TB patients express less IFN-γ and more IL-4 than cells from healthy subjects, regardless of the *Mtb* strain used; however, the clinical strain M exacerbates this response in TB patients, and also biases the profile towards Th2 polarization in healthy subjects [19]. The M strain is a highly successful genotype that has been circulating as MDR since the '70s in Argentina [21], where it caused a large MDR-TB outbreak [22]. This strain accounted for 29% of MDR-TB and 40% of extremely-drug resistant (XDR)-TB cases between 2003 and 2009, and is still circulating in this country [23].

The objective of this work was to investigate the role of C5a receptors in the modulation of human T-cell polarization induced by *Mtb*. To this aim, we first analyzed the ex vivo expression of C5a receptors in peripheral blood from TB patients and healthy subjects and then assessed innate and adaptive cytokine profiles after blocking these receptors in monocytes-macrophages (Mo/MΦ) and challenging the cells with the laboratory strain H37Rv and the more pro-Th2 M strain.

2. Materials and methods

2.1. Patients and healthy donors

Blood samples were obtained from TB patients who were hospitalized in the Posadas Hospital, Buenos Aires, Argentina. All patients were diagnosed by the presence of recent clinical respiratory symptoms, abnormal chest radiography, a sputum smear positive for acid-fast bacilli and, if necessary, the identification of *Mtb* in culture. Sputum smear examination and mycobacterial culture were performed according to standard procedures. A total of 36 patients were included (26 men, 10 women; 18–66 years old, median age 25 years). All patients showed radiological signs of advanced pulmonary disease (65% had bilateral cavities, 35% had unilateral cavities). The exclusion criteria were a positive test for human immunodeficiency virus and the presence of concurrent infectious diseases or noninfectious conditions such as cancer, diabetes, or steroid therapy. Sera and buffy coats from healthy blood donors (HD) with unknown protein purified derivative (PPD) skin test status were obtained from the Regional Hemotherapy Center-Blood Bank at Garrahan Hospital, Buenos Aires city. For some experiments, blood from PPD-positive healthy laboratory personnel was used.

Informed consent was obtained from patients and donors. Ethical approval for the study was obtained from the Ethic Committees of Hospital Posadas and of Academia Nacional de Medicina, according to the principles laid down in the Declaration of Helsinki recommendations guiding physicians in biomedical research involving human subjects.

2.2. Bacteria

The M strain was obtained from the collection kept at the Mycobacteria Service at the INEI-ANLIS "Carlos G. Malbrán" in Buenos Aires. I.N. de Kantor (former head of TB laboratory, INPPAZ PAHO/WHO) kindly provided the H37Rv strain. All strains were grown in Sauton medium at 37 °C. Mycobacteria were harvested at the logarithmic growth phase and washed three times. Bacterial pellets were inactivated by gamma-irradiation at the National Atomic Energy Commission (CNEA, Argentina). Thereafter, the pellets were suspended in pyrogen-free PBS, sonicated and suspended at an optical density of 1 at 600 nm ($\approx 10^8$ bacteria/ml). The inactivated bacteria were stored at −20 °C until use.

2.3. Sera

Sera from TB patients and HD were obtained from fresh non-heparinized blood. Blood samples were incubated for 60 min at 37 °C and centrifuged twice for 15 min at 330 g to separate the serum from the clot. Unless immediately used, sera were aliquoted, conserved at −80 °C, and thawed only once.

2.4. Cell cultures

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by Ficoll-Triyosom gradient centrifugation. Adherent cells (85–95% Mo) were obtained from PBMC by plastic adherence. Briefly, PBMC (2×10^6 cells/well) suspended in RPMI 1640 (HyClone; Thermo Scientific, USA) were seeded in 48-well polystyrene plates (Corning, USA) in a 5% CO_2 humidified atmosphere at 37 °C for 2 h. Afterwards, non-adherent cells were removed, washed, suspended in complete media and reserved until use in autologous assays. Complete media consisted of RPMI 1640 containing 100 U/ml penicillin, 100 μg/ml streptomycin and 20% autologous serum as a source of complement. The adherent cells were washed twice with warm saline solution, and cultured in complete medium. Cell purity and viability were tested by flow cytometry and trypan blue exclusion.

For the experiments with Mo isolated from buffy coats, the same procedure was followed but cells were cultured in complete medium supplemented with 10% fetal calf serum (Natocor, Argentina) instead of autologous serum. Buffy coats were used only to determine the impact of irradiated *Mtb* strains on the surface expression of C5a receptors. H37Rv or M strain bacilli were added at a *Mtb* to Mo ratio of 2:1 and cultured in 5% CO_2 humidified atmosphere at 37 °C for 18 h, 48 h, or 6 days (144 h).

The role of C5a receptors on cytokine expression was evaluated by adding or not 5 μg/ml of anti-C5aR (clone S5/1) or anti-C5L2 (clone 1D9-M12) blocking antibodies (Biolegend, USA) to the adherent cells for 30 min. Then, the medium was washed and replaced with fresh medium and Mo were stimulated with H37Rv or M strain at a ratio of 2:1. Afterwards, the cultures were incubated for 18 h in 5% CO_2 at 37 °C. For the evaluation of Mo cytokine secretion, we harvested and used the supernatants. The intracellular expression of cytokines in $CD4^+$ T-cells was determined by adding autologous non-adherent cells at this point and co-culturing them with the adherent cells for 5 days in 5% CO_2 at 37 °C.

2.5. Immunofluorescence staining

The following monoclonal antibodies were used to analyze surface phenotypes: PE-labeled anti-C5aR (CD88), PE/Cy5-labeled anti-CD14, and unconjugated C5L2 as primary antibody with a FITC-labeled goat anti-mouse IgG as secondary antibody (all from Biolegend). Approximately 1×10^6 lymphocytes or 2×10^5 Mo were seeded into tubes and washed once with PBS. The cells were stained for 30 min at 4 °C and washed twice. Adherent cells were detached by incubating at 4 °C for 20 min and gently scrapping before surface-staining.

For intracellular cytokine expression in $CD4^+$ cells, Brefeldin A (5 μg/ml; Sigma Chemical Co., USA) was added to the co-cultures for the last 4 h to block cytokine secretion. Then, the cells were surface-stained with PE/Cy5-anti-CD4 (Biolegend), fixed and permeabilized according to the manufacturer's instructions (Perm2, BD Bioscience). Subsequently, FITC-anti-IFN-γ (Biolegend), PE-anti-IL-4 (Biolegend), PE-anti-IL-17 (R&D System, USA), or the corresponding isotype matched controls were added for 20 min at 4 °C. The stained cells were washed, fixed with 0.5% paraformaldehyde and suspended in Isoflow™ (BD Bioscience, USA).

Thirty thousand events were acquired on Mo or lymphocyte gates according to forward and side-scatter parameters using a FACSCan flow cytometer (BD Bioscience). Cell debris and apoptotic cells were excluded. FCS Express software (De Novo Software, USA) was used for the analysis. The results were expressed as percentage of positive cells or Median Fluorescence Intensity (MFI).

2.6. ELISAs

The C5/C5a serum levels were evaluated in samples from TB patients and HD by ELISA using C5a ELISA kit (R&D Systems), following the manufacturer's specifications.

The amounts of IL-10, TNF-α and IL-12p40 were measured in culture supernatants of adherent cells at 18 h by ELISA commercial kits according to the manufacturers' instructions (Biolegend).

2.7. Statistical analysis

The results were expressed as medians and 25th to 75th percentiles. The nonparametric Kruskal-Wallis test was used to compare data from TB patients and HD, followed by Mann–Whitney *U* test to compare two groups. The Friedman test was performed to compare data within each group, followed by the Wilcoxon rank sum test. All statistical analyses were two-sided and the significance level adopted was $P < 0.05$.

3. Results

3.1. Mtb modulates the C5a receptors in a strain-dependent fashion

In previous works, we described an altered expression of surface molecules and receptors of Mo and T-cells from TB patients [20,24]. Herein, we first analyzed whether the C5a-C5a receptor axis is also altered in TB patients. To this end, we measured C5/C5a serum levels and assessed Mo surface expression of C5aR and C5L2 receptors. No differences were detected between TB patients and HD in either C5/C5a serum levels (Fig. 1, upper panel) or in the surface expression of C5aR in $CD14^+$ cells (Fig. 1, lower left panel). C5L2 surface expression was elevated in Mo from TB patients compared to HD (Fig. 1, lower right panel), whereas the percentage of Mo expressing this receptor did not differ between both groups (data not shown). Finally, we found negligible expression of both receptors on lymphocytes (data not shown), which is in line with previous reports [11].

We then explored whether H37Rv and the M strain could modulate the expression of C5aR and C5L2 along Mo differentiation to MΦ. To this aim, we exposed or not Mo from HD to *Mtb* strains for 18 h, 48 h or 6 days and assessed receptor expression in $CD14^+$ cells. C5aR was expressed by almost all freshly isolated Mo and fully differentiated MΦ, although the percentage of positive cells decreased during spontaneous differentiation ($p < 0.05$, % C5aR 0 vs. 6 days). A concomitant increase in its expression level was observed ($p < 0.05$, C5aR MFI 0 vs. 6 days) (Fig. 2a, c). Both *Mtb* strains were able to further reduce the percentage of $C5aR^+$ cells but the reduction induced by H37Rv occurred earlier (Fig. 2a). Transient and slight alterations in C5aR MFI were evident upon differentiation induced by both *Mtb* strains but no significant differences were detected after 6 days (Fig. 2b). In contrast, we only detected C5L2 expression in a Mo subpopulation which declined after 48 h ($p < 0.05$, % C5L2 24 vs. 48 h) but reverted to initial percentages (Fig. 2b), with a marked increase in its expression levels in fully differentiated MΦ ($p < 0.05$, C5L2 MFI 48 h vs. 6 days) (Fig. 2d). This transient drop was not observed in the presence of H37Rv, while the M strain had no effect on spontaneous C5L2 modulation.

Collectively, these results demonstrate that circulating Mo from TB patients express higher levels of C5L2 on their surface and that both anaphylatoxin receptors can be modulated by *Mtb* in a strain-dependent fashion throughout Mo differentiation to MΦ.

3.2. C5aR inhibits the expansion of $CD4^+IFN\text{-}\gamma^+$ lymphocytes upon stimulation with the M strain

Considering that in several models C5a receptors were found to modulate T-cell polarization, we aimed to establish if these receptors are also involved in the polarization of human $CD4^+$ T-cells induced by *Mtb*. To assess this, we blocked C5a receptors of Mo from HD or from TB patients prior to stimulating the cells with the *Mtb* strains and co-incubating them with autologous lymphocytes. After 5 days, we measured the intracellular expression of IFN-γ, IL-4 and IL-17 in $CD4^+$ T-cells as representatives of Th1, Th2 and Th17 profiles, respectively. We then evaluated the effect of C5a receptor neutralization on the response elicited by the laboratory strain H37Rv and the more pro-Th2 strain M (Fig. 3).

C5aR neutralization led to a significant expansion of $CD4^+IFN\text{-}\gamma^+$ T cells induced by the M strain in HD and TB patients (Fig. 3, upper panel), whereas this treatment had no effect on H37Rv-induced IFN-γ or IL-4 (Fig. 3, upper and middle panels). Moreover, when C5aR was blocked, the percentage of $CD4^+IL\text{-}4^+$ cells from HD stimulated with the M strain decreased, although not significantly, in 7 of the 9 studied cases (see Supplementary Fig. 1). Finally, the blockage of C5aR had no effect on $CD4^+IL\text{-}17^+$ cells from HD but resulted in an increased percentage of $CD4^+IL\text{-}17^+$ T-cells from TB patients when stimulated with H37Rv.

Anti-C5L2 treatment did not affect the Th profile of $CD4^+$ cells in any of the study conditions.

Altogether, these observations demonstrate that C5aR, but not C5L2, plays a role in T helper cell polarization induced by *Mtb* and this effect is strain- and donor-dependent.

3.3. C5aR and C5L2 modulate Mo/MΦ cytokine production induced by Mtb

C5a can influence T cell polarization by modulating APC cytokine expression [25]. Taking this into account, we analyzed if the C5a receptors had an effect on the induction of these cytokines by *Mtb*. Therefore, we first blocked the C5a receptors of Mo/MΦ isolated from HD and TB patients, then stimulated these cells with H37Rv or the M strain, and measured IL-12p40, IL-10 and TNF-α secretion.

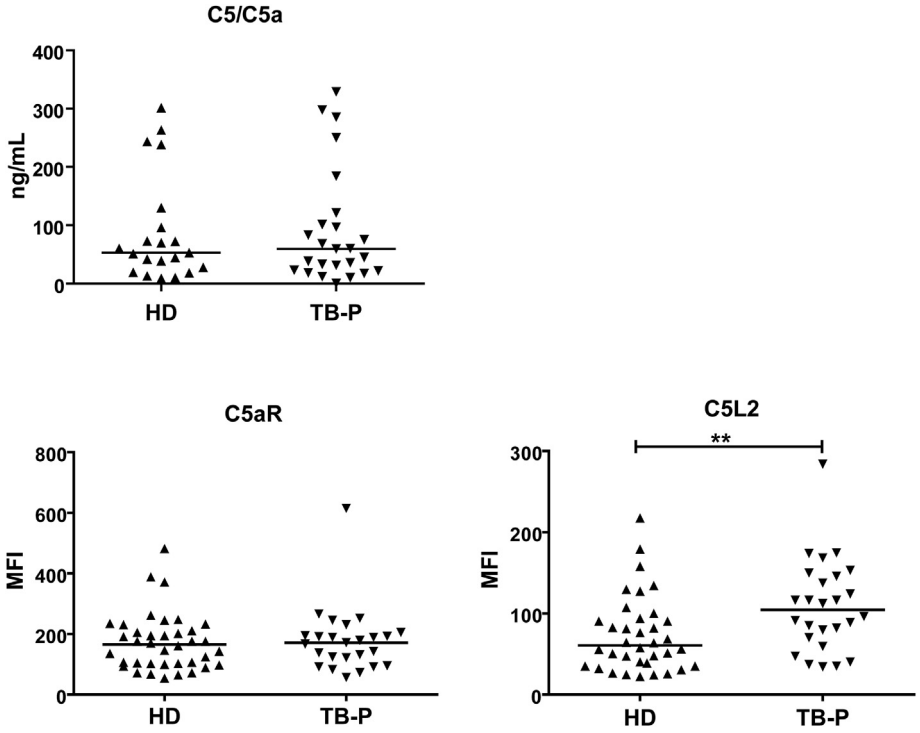

Fig. 1. C5/C5a levels and C5aR and C5L2 expression ex vivo. C5/C5a levels were measured in sera from TB patients (TB-P; n = 25) and healthy donors (HD; n = 21) by ELISA (upper panel). Median fluorescence intensity (MFI) of C5aR (lower left panel) and C5L2 (lower right panel) was determined on $CD14^+$ cells of peripheral blood mononuclear cells from TB patients (n = 26) and HD (n = 36) by FACS. The horizontal line in each scatter plot represents the median of the analyzed parameters. Statistical significance: **p < 0.01 for HD vs. TB patients.

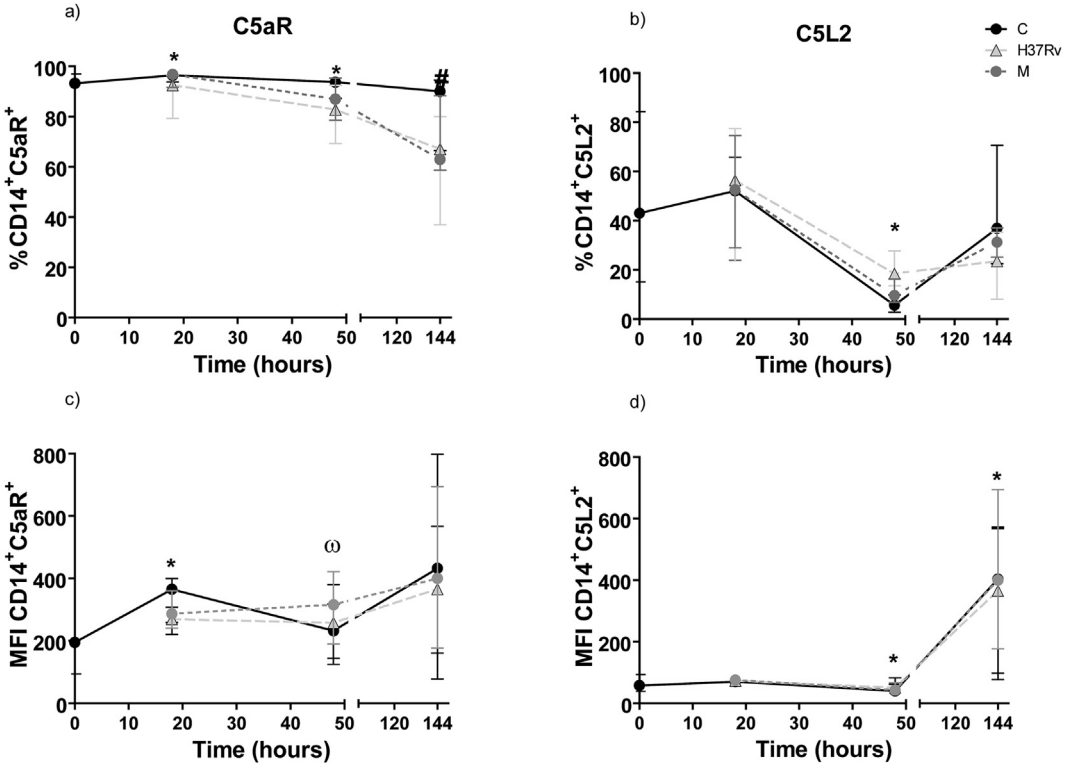

Fig. 2. Expression of C5a receptors along Mo differentiation to MΦ. The expression of C5aR (a,c) and C5L2 (b,d) was evaluated on $CD14^+$ Mo from buffy coats (HD; n = 10) stimulated or not with H37Rv and M strains at a ratio of 2 *Mtb*: 1 Mo for 18, 48 or 144 h. The data are expressed as the median ± interquartile ranges (%) (a,b) and the median fluorescence intensity (MFI, c,d) of positive cells. Statistical significance: *p < 0.05 for control vs. H37Rv; ω p < 0.05 for control vs. M; #p < 0.05 for control vs. both strains.

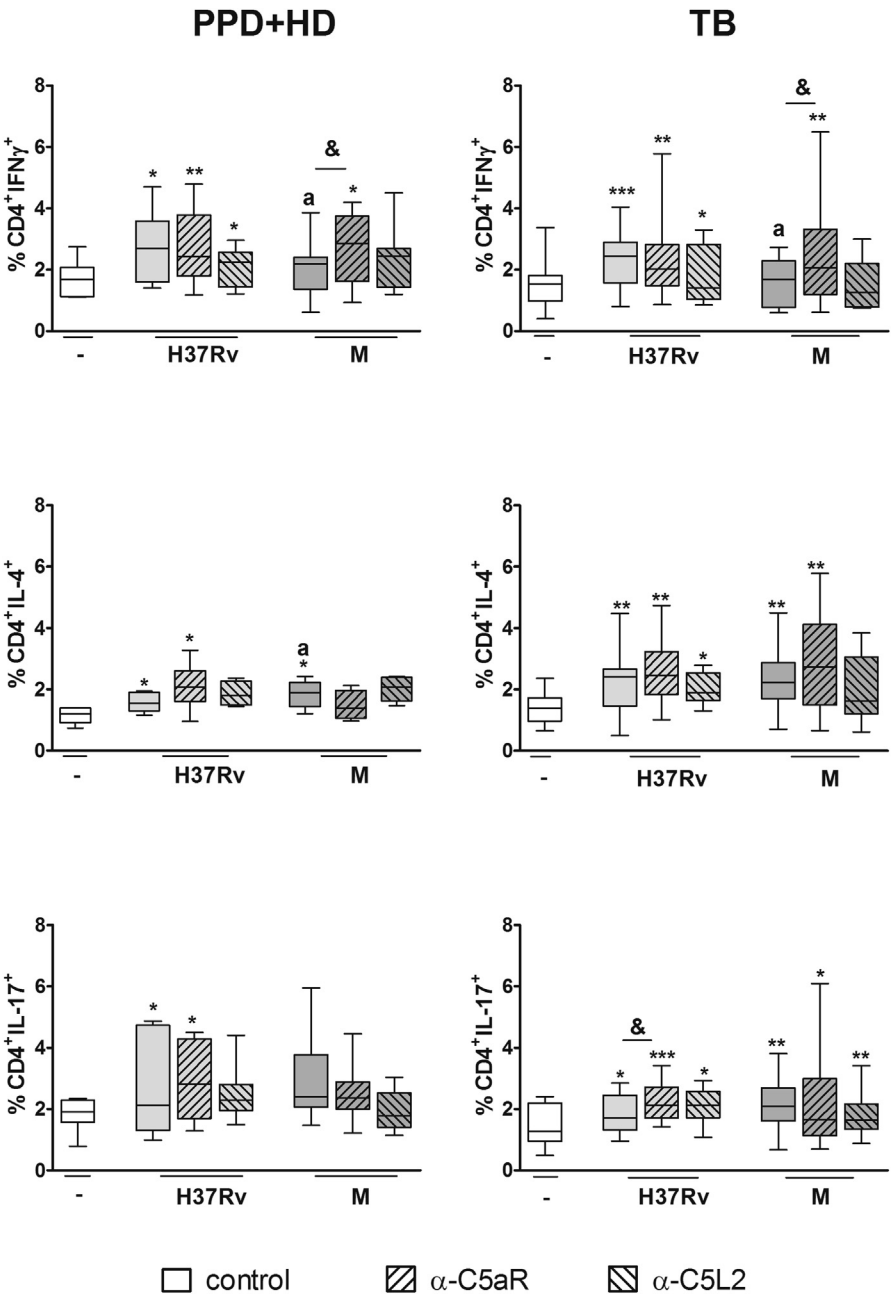

Fig. 3. C5a receptors modulate cytokine production by CD4$^+$ T-cells. Mo from healthy donors with positive PPD skin test (HD; n = 9–10) and TB patients (TB-P; n = 22) were treated or not with anti-C5aR (α-C5aR) or anti-C5L2 (α-C5L2) blocking antibodies prior to *Mtb* stimulation. As C5/C5a source, 20% of autologous serum was added to RPMI medium. Upon 18 h of culture, autologous lymphocytes were added for 5 days. The expression of intracellular IFN-γ, IL-4 and IL-17 (upper, middle and lower panel respectively) were measured in CD4$^+$T cells. As control groups, non-stimulated cells treated or not with α-C5aR or α-C5L2 blocking antibodies were used; as no differences between controls were found, only the untreated, non-stimulated cell groups are shown. The results are expressed as percentage (%, boxes represent median and 25–75 percentiles and whiskers data range). Statistical significance: *p < 0.05, **p < 0.01, ***p < 0.005 for control vs. strains; & p < 0.05 *Mtb* vs. *Mtb* + α-C5aR or α-C5L2; a p < 0.05 for H37Rv vs. M.

In general, H37Rv induction significantly increased the secretion of these cytokines in Mo/MΦ from TB patients and HD, whereas the M strain elicited a weaker response (Table 1). The blockage of C5aR in Mo/MΦ from HD enhanced IL-12p40 secretion irrespective of the *Mtb* strain used and the same effect was observed in cells from TB patients stimulated with H37Rv. Upon anti-C5L2 treatment, IL-12p40 secretion only increased in H37Rv-stimulated Mo/MΦ from TB patients. In the case of IL-10 secretion, the addition of anti-C5aR to Mo/MΦ from TB patients only increased H37Rv-induced production, whereas no effect was observed on cells from HD. When C5L2 was blocked, the M strain induced increased levels of IL-10 in Mo/MΦ from TB patients. Finally, neither anti-C5aR nor anti-C5L2 modified TNF-α production significantly.

Altogether, these results indicate that, in Mo/MΦ stimulated with *Mtb*, C5aR modulates the secretion of IL-10 and IL-12p40, but not the secretion of TNF-α, in a donor- and strain-dependent manner. In contrast, C5L2 only has some effect on Mo/MΦ from TB patients.

4. Discussion

In mice, the innate peptide C5a was found to play a key role in the generation of acquired immune response to *Mycobacterium*

Table 1
Modulation of Mo cytokine production by C5aR and C5L2.

HD (n = 10)		IL-12p40 (pg/ml)	IL-10 (pg/ml)	TNF-α (pg/ml)
Control				
	–	15.8 (12.9–28.8)	0.0 (0–1)	26.7 (4–161)
H37Rv				
	–	245.0 (167–607.7)*	3.8 (0–57)*	320.4 (122–829)*
	α-C5aR	480.6 (195–976) &	7.7 (0–59)	179.3 (37–264)
	α-C5L2	282.9 (157.7–698)	3.9 (2–61)	362.9 (64–948)
M				
	–	67.7 (47–168.7) [a]	0.0 (0–2) [a]	140.4 (17–473)
	α-C5aR	186.4 (98.2–297) &	0.7 (0–9)	75.9 (20–603)
	α-C5L2	151.3 (83.2–384.4)	1.1 (0–5)	59.2 (8–1074)
TB (n = 10)		IL-12p40 (pg/ml)	IL-10 (pg/ml)	TNF-α (pg/ml)
Control				
	–	12.7 (1–55)	0.0 (0–2)	37.3 (3–201)
H37Rv				
	–	144.5 (31–531)*	47.7 (22–105)*	737.5 (235–1167)*
	α-C5aR	209.1 (78–970) &	55.0 (33–163) &	1025.0 (207–1678)
	α-C5L2	370.4 (76–981) &	71.1 (31–195)	1117.0 (305–1738)
M				
	–	42.7 (7–147) [a]	4.3 (0–28) *[a]	390.9 (58–913) *[a]
	α-C5aR	31.7 (17–191)	6.1 (1–30)	191.1 (29–447)
	α-C5L2	37.8 (7–226)	13.5 (2–90) &	274.7 (2–1544)

–: no blocking antibody. α-C5aR: anti-C5aR blocking antibody. α-C5L2: anti-C5L2 blocking antibody. The data are expressed as median (25–75 percentiles).
Statistical significances: *p < 0.05 for control vs. strains; & p < 0.05 for *Mtb* vs. *Mtb* + α-C5aR or α-C5L2; [a] p < 0.05 for H37Rv vs M.

bovis BCG by regulating the Th1 response [26]. Herein we demonstrate that its receptor, C5aR, can modulate the adaptive immunity to *Mtb* in the human host and that this modulation is dependent on bacterial and host factors. To the best of our knowledge, this is the first report demonstrating that C5aR is directly involved in the human $CD4^+$ T cell polarization induced by *Mtb*.

Several lines of evidence suggest that the C5a/C5a receptor axis has an active role in human TB [2,3], including the finding of increased C5a serum levels in TB patients [27]. In this work, we detected unaltered C5/C5a levels in sera from TB patients, probably due to a technical limitation of the ELISA assay used, which does not discriminate between both components of the system. Indeed, we cannot rule out a C5a increase at the expense of a decrease in C5, as described for C3/C3a in atypical hemolytic uremic syndrome [28]. Our most conspicuous finding regarding the ex vivo expression of the C5a-C5a receptor axis is the increased percentage of Mo expressing C5L2 in TB patients. This increment could be related to the high levels of IFN-γ found in plasma from TB patients [29], since it has been shown that IFN-γ upregulates C5L2 in a macrophage cell line [30]. Regarding C5aR, its ex vivo expression in cells from TB patients was similar to that of HD, even though *Mtb* was able to reduce the percentage of $CD14^+C5aR^+$ cells throughout the in vitro differentiation to MΦ (Fig. 2). This suggests that local responses to *Mtb* are not directly translated into the peripheral expression of C5aR in Mo. Moreover, H37Rv and the M strain modulated somewhat dissimilarly both C5a receptors during the differentiation to MΦ. Considering that the expression of the receptors can also be regulated by their ligands, these differences could be partially ascribed to an indirect effect of the strains through the modulation of C5a production by Mo/MΦ. In any case, the observed differences were insufficient to explain the results of the receptor blockage assays that are discussed below.

To date, most experimental research on TB immunology used only the laboratory strain H37Rv and the influence of *Mtb* genotypic variability on the immune responses has been largely underestimated until recently [15,17–20]. In our study, the most striking result was not observed with strain H37Rv but with the outbreak M strain. In this sense, the poor Th1 polarization evoked by the M strain in HD cells was related to a C5aR modulatory effect on Mo/MΦ, as evidenced in our experimental approach. C5a exerts its effects on T cell polarization through APC modulation [25,31], being the regulation of cytokines of the IL-12 family the most extensively studied [32,33]. In fact, C5a or C5aR were previously found to modulate in the same way IL-12p70 and its subunit p40 [7,8], and even other IL-12 family subunits such as p35, p28 and p19 [34]. In this work we analyzed IL-12p40 subunit and found that C5aR blockage enhanced its secretion by Mo/MΦ stimulated with *Mtb*; which is in line with other authors' results [8,34,35]. Remarkably, both *Mtb* strains included in the study exerted similar C5aR inhibitory effect on IL-12p40 expression as did a third clinical strain analyzed in our lab (data not shown). Unfortunately, this almost universal effect of C5aR on IL-12p40 expression cannot explain the IFN-γ levels observed in some of the strain/donor combinations in our study. Thus, our findings need to be interpreted with caution. In HD stimulated with the M strain, the C5aR-dependent modulation of IL-12p40 correlated with an effect on Th1 polarization, as C5aR blockage increased IL-12p40 secretion by APC and expanded $CD4^+IFN\text{-}\gamma^+$ cells, while $CD4^+IL\text{-}4^+$ cells tended to diminish. We postulate that the outbreak M strain, which has *per se* a poor capacity to induce IL-12p40 expression, exploits C5a-C5aR signaling to further reduce its production which, in turn, results in a poor Th1 response. Conversely, in H37Rv stimulated cells, the IL-12p40 increment exerted upon C5aR blockage does not expand $CD4^+IFN\text{-}\gamma^+$ cells, probably because H37Rv had already boosted this cytokine to a level above the threshold needed for IFNγ induction in both HD and TB patients. Moreover, in cells of TB patients stimulated with the laboratory H37Rv strain, the IL-12p40 increment observed upon C5aR blockage correlated with an increased percentage of $CD4^+IL\text{-}17^+$ cells rather than $CD4^+IFN\text{-}\gamma^+$. Since TB patients have a higher proportion of $IL\text{-}23R^+CD4^+$ T cells than HD [20], it is reasonable to think that they are more susceptible to IL-23 and therefore prone to a subsequent shift towards Th17. Finally, in the case of M strain-stimulated cells from TB patients, it was surprising to find that the lack of C5aR signaling led to an expansion of $CD4^+IFN\text{-}\gamma^+$ cells with no increase in IL-12p40. This result evidences that C5aR modulates other factors contributing to T helper cell polarization. In fact, C5aR regulates CD40 and MHC-II

molecules in dendritic cells [20,26] and most probably this or other co-stimulatory molecules play a role in the results observed. Thus, these molecules should be evaluated in future research. Another factor to consider is that C5/C5a might have a direct impact on T cells. Even though we did not find ex vivo lymphocyte expression of its receptors, an upregulation of C5aR has been shown to occur in $CD3^+$ mice cells upon co-cultivation with BCG-infected MΦ [36]. Thus, we cannot rule out this possibility in the present work.

The role of C5a receptors in the modulation of adaptive immunity has been mainly focused on C5aR and much less is known about C5L2. In the present study we found that the absence of C5L2 signaling does not induce any change in the adaptive or the innate responses of HD. Remarkably, in Mo from TB patients, which had higher levels of C5L2 expression, this receptor repressed the IL-12p40 production induced by H37Rv. However, this effect did not lead to changes in the T helper cell profile and our experimental data failed to clarify the role of this ambiguous receptor. C5L2 has been postulated both as a positive and a negative modulator of C5aR and its role is still a contentious issue [37]. In any case, our results do not support an antagonistic or neutralizing role of this receptor on C5aR activity because the marginal participation of C5L2 in the parameters evaluated was in the same direction as C5aR.

It is worth to highlight that, despite the well-known pro-inflammatory activity of C5aR, our findings suggests that this receptor also has an important role in the modulation of T helper cell response in *Mtb*-stimulated Mo/MΦ.

Finally, it should be pointed out that the use of gamma irradiated *Mtb* is a limitation of the current work because of the abolishment of an active infectious system. Nonetheless, the results described herein could be ascribed to the interaction of host cell receptors with those *Mtb* surface antigens that withstand gamma inactivation and are subjected to modulation by signaling of C5a-C5a receptors. This is the case of *Mtb* antigens recognized by TLRs, such as glycolipids and glycoproteins. In fact in other models, the crosstalk between C5aR and TLRs has been shown to alter IL-12 family cytokines by manipulating these receptors [38]. In this line, variations in lipidic antigens among different *Mtb* lineages have been recently described [39,40] which might account for the differences described herein.

In conclusion, we demonstrate that C5aR modulates the T helper cell profile induced by *Mtb*. This modulation is carried out in a donor- and a strain-dependent manner, and, to a certain extent, is bound to partial inhibition of IL-12p40 secretion by Mo/MΦ. This effect is probably a homeostatic mechanism to limit hazardous consequences of host responses. We propose that, in the same manner as other pathogens found to manipulate C5a/C5aR to evade the host immune response [34,41], the highly successful M strain [21] takes advantage of the C5aR-mediated inhibition of Th1 polarization to survive in the human host and perpetuate in the community.

Author contributions

CSG was responsible for study design, data analyses and manuscript preparation. CSG, NY and JB performed the experiments. JB and LB contributed to laboratory work. AG was responsible for patient recruitment and selection and provided clinical data and sample collection. BL and VR were responsible for the characterization, selection and preparation of the *Mtb* clinical strain. NY, SB and MS contributed to the study design. MS conceived the experiments. NY, VR and MS contributed to manuscript preparation.

Disclosure

None of the authors or funding sources has either a commercial or other association that might pose a conflict of interest. No one involved in the publication process has a financial or other beneficial interest in the products or concepts mentioned in the submitted manuscript or in competing products that might bias his or her judgement.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tube.2016.12.005.

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