***Mycobacterium tuberculosis* multidrug resistant strain M induces IL-17+IFNγ- CD4+ T cell expansion through an IL-23 and TGF-β-dependent mechanism in patients with MDR-TB tuberculosis**

Running title: Mechanisms of Th17 cell expansion in tuberculosis

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Abbreviations: AFB, acid fast bacilli; APC, antigen presenting cells; BCG, Bacillus Calmette-Guerin; DC, dendritic cells; HD, healthy donors; IFNγ, Interferon-gamma; IL, Interleukin; LAM: Latin-American-Mediterranean; LAP, latency-associated protein; MDR-TB: multidrug-resistant tuberculosis; MR, mannose receptor; PBMC, peripheral blood mononuclear cells; PPD, purified protein derivative; TB, tuberculosis; Th, T helper; TLR, toll-like receptor; TGF-β, Tumor growth factor-beta.

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ABSTRACT

We have previously reported that T cells from patients with multidrug-resistant tuberculosis (MDR-TB) express high levels of IL-17 in response to the MDR strain M (Haarlem family) of *Mycobacterium tuberculosis* (*M.tuberculosis*). Herein, we explore the pathways involved in the induction of h17 cells in MDR-TB patients and healthy tuberculin reactors (PPD$^+$HD) by the M strain and the laboratory strain H37Rv. Our results show that IL-1β and IL-6 are crucial for the H37Rv and M-induced expansion of IL-17$^+$IFNγ^- and IL-17$^+$IFNγ^+ in CD4$^+$ T cells from MDR-TB and PPD$^+$HD. IL-23 plays an ambiguous role in Th1 and Th17 profiles: alone, IL-23 is responsible for *M.tuberculosis*-induced IL-17 and IFNγ expression in CD4$^+$ T cells from PPD$^+$HD whereas, together with TGF-β, it promotes IL-17$^+$IFNγ^- expansion in MDR-TB. In fact, spontaneous and *M.tuberculosis*-induced TGF-β secretion is increased in cells from MDR-TB being the M strain the highest inducer. Interestingly, TLR-2 signaling mediates the expansion of IL-17$^+$IFNγ^- cells and the enhancement of latency-associated protein (LAP) expression in CD14$^+$ and CD4$^+$ T cells from MDR-TB, which suggests that M strain promotes IL-17$^+$IFNγ^- T cells through a strong TLR-2-dependent TGF-β production by antigen-presenting cells and CD4$^+$ T cells. Finally, CD4$^+$ T cells from MDR-TB patients infected with MDR Haarlem strains show higher IL-17$^+$IFNγ^- and lower IL-17$^+$IFNγ^+ levels than LAM-infected patients. The present findings deepen our understanding on the role of IL-17 in MDR-TB and highlight the influence of the genetic background of the infecting *M.tuberculosis* strain on the *ex vivo* Th17 response.

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INTRODUCTION

Interleukin-17 (IL-17) is a proinflammatory cytokine secreted by hematopoietic cells including representatives of the adaptive (e.g. CD4$^+$ and CD8$^+$ αβ T cells) and the innate (e.g. NK, NKT and γδT cells) immune responses [1, 2]. IL-17 plays an important role in chronic inflammatory disorders as well as in host immune responses against extracellular and intracellular pathogens [2-4]. Th17 cells were described to be protective by accelerating the recruitment of Th1 cells to the site of infection in *M.tuberculosis*-vaccinated mice [5], contributing to the formation of the granuloma, and reducing the bacterial burden in BCG-infected mice [6]. Nevertheless, excessive production of IL-17 in response to repeated exposure to mycobacterial antigens has been associated with extensive lung pathology [7].

Human IL-17-secreting T cells can produce not only the hallmark interleukins IL-17A and IL-17F but also TNF-α, IL-22, IL-21, IL-4 and IL-10, according to the cytokine environment [8]. Human Th17 cells produce IFNγ in IL-12-enriched micro-environments. IL-17/IFNγ double-positive CD4$^+$ T cells, named Th17-Th1 cells, were found to be expanded in human inflamed tissues [9], peripheral blood T cells, and T-cell clones expanded from healthy donors (HD) [10, 11]. On the other hand, in patients with chronic bronchial asthma and other allergic disorders, cells derived from memory Th17 cells express IL-4 in response to an IL-4 enriched environment [12]. Also, Th17 cells can produce IL-10 in response to *Staphylococcus aureus* [13], supporting the concept of Th17 heterogeneity and plasticity under different microenvironments. Regarding *M.tuberculosis*–induced Th17 cells, an increase in IL-17$^+$IFNγ$^+$ CD4$^+$ memory T cells was observed in response to H37Rv strain in non-BCG vaccinated HD [14]. Besides, patients with active pulmonary TB showed to promote a high Th17 response to cell lysates of H37Rv strain, which was mainly dependent on IFNγ$^+$IL-17$^+$ CD4$^+$ T lymphocytes [15].

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In the early 1990s, two major MDR-TB outbreaks were detected in two overpopulated urban areas of Argentina (Buenos Aires and Rosario cities). Epidemiological, bacteriological, and genotyping data allowed the identification of the respective outbreak strains named M and Ra which belong to the Haarlem (H2) and the Latin American-Mediterranean (LAM3) families of *M. tuberculosis*, respectively. Although these strains have been isolated from human immunodeficiency virus (HIV)-infected patients [16], they soon it disseminated to immunocompetent individuals and managed to perpetuate in the community. In a systematic countrywide survey performed in 2003-2009, M and Ra strains still accounted for 29% and 11% of all MDR-TB cases in the period [17] .

We have previously demonstrated that M strain induced an altered T-helper 1/T-helper 2 (Th1/Th2) profile in T cells from healthy tuberculin reactors (PPD$^+$HD) and from TB patients, being M strain a weaker inducer of IFNγ and CD8-dependent cytotoxic activity [18]. In contrast, an expansion of CD4$^+$ and CD8$^+$ IL-17-producing T cells was observed in MDR-TB patients in response to *M.tuberculosis* strains. This increase was associated to a differential expansion of IL-17$^+$IFNγ^- within the CD4$^+$ T cell subset and this effect was more evident when M strain was used as antigen [19]. In the present work we explore the underlying mechanisms involved in IL-17$^+$IFNγ^- and IL-17$^+$IFNγ^+ memory T cell expansion taking into account the genotype of the infecting strains.

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METHODS

Ethics Statement: This work was carried out in accordance with the revised version of the Declaration of Helsinki (2013) of the World Medical Association, and was reviewed and approved by the following Bioethics Committees, Academia Nacional de Medicina (Decision Number 23-03-2010), Hospital Muñiz (DN 131-07, Project Number 145) and the Teaching and Research Committee of the Buenos Aires City government (DN 1217 2010).

Patients: Blood samples were obtained from MDR-TB patients hospitalized in the Phthisiopneumonology Institute University of Buenos Aires, placed in grounds of F. J. Muñiz Hospital, Buenos Aires, Argentina. Patient informed consent was obtained according to the guidelines of the ethics committee of the F. J. Muñiz Hospital. All patients were diagnosed by the presence of recent clinical respiratory symptoms, abnormal chest radiography, a sputum smear test positive for acid-fast bacilli (AFB), and the identification of *M.tuberculosis* in culture. Exclusion criteria included a positive test for HIV and the presence of concurrent infectious diseases or noninfectious conditions (cancer, diabetes, or steroid therapy). Sputum smear examination and mycobacterial culture were performed in agreement with standard procedures. Susceptibility to isoniazid, rifampicin, ethambutol, and streptomycin was determined conforming to World Health Organization standards. Susceptibility to kanamycin, *p*-aminosalicylic acid, and cycloserine was tested following Canetti, Rist, and Grosset method, whereas the pyrazinamidase test was used to infer pyrazinamide susceptibility [20]. Available MDR *M.tuberculosis* isolates were genotyped by IS*6110* DNA fingerprinting and spoligotyping, using standardized protocols [21, 22]. A total of 31 MDR-TB patients were included (17 men and 14 women; median age [25th to 75th percentiles] 32 [23 to 55] years). Percentages of different *M.tuberculosis* lineages

among MDR-TB patients in this study were as follows: LAM, 43%; Haarlem, 50% (80% of whom were infected with M strain); T, 4%; and other 3%. All MDR-TB patients showed a radiologically advanced pulmonary disease and were under specific treatment according to the infecting strain's drug susceptibility at the time of the study (Supplementary Table 1). Twenty one patients were sputum smear positive: median number of AFB/field [25th to 75th percentiles] for patients infected with Haarlem strains: 3 (0.4-10), and for those infected with LAM strains: 2.5 [0.3 to 10]. Sixteen BGC vaccinated healthy volunteers donors were recruited as controls from laboratory personnel of the Academia Nacional de Medicina upon written informed consent as approved by the research ethics board of the institution. They were classified according to their reactivity to purified protein derivative (PPD) and interferon-γ release assays (QuantiFERON-TB Gold In-Tube assay, Qiagen, Valencia, CA, USA) in latently TB infected (PPD$^+$HD, PPD$^+$IGRA$^+$, 4 men and 4 women, median age [25th-75th percentiles] 30 [27-56] years) and in healthy donors (PPD$^-$HD, PPD$^-$IGRA$^-$, 4 men and 4 women, 32 [25-55] years.

Peripheral blood mononuclear cell: PBMC from heparinized blood were isolated by Ficoll-Triyosom gradient centrifugation and suspended in RPMI 1640 (HyClone; Thermo Scientific, Waltham, MA USA) containing 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% heat-inactivated fetal calf serum (Gibco® , Thermo Scientific), hereafter identified as complete medium.

Antigens: Clinical isolates representative of the MDR outbreak *M.tuberculosis* M strain and the laboratory *M.tuberculosis* strain H37Rv were grown in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI, USA) at 37°C in 5% CO_2. Mycobacteria were harvested in the log phase, washed three times, and suspended in pyrogen-free PBS.

Bacteria were inactivated by gamma-irradiation, suspended in PBS at a 600 nm optical density of 1.0 ($\approx 10^8$ bacteria/ml), and stored at -20°C until use.

PBMC cultures: PBMC (2×10^6 cells/ml) were cultured during 48 h in polystyrene tubes (Falcon, BD Bioscience, Franklin Lakes, NJ, USA) at 37°C in a humidified 5% CO_2 atmosphere in complete medium alone or in the presence of gamma-irradiated bacilli of M or H37Rv strains at a 2:1 *M.tuberculosis* to PBMC ratio. For blocking experiments, monoclonal antibodies directed against a) cytokines: IL-23p19 (10 µg/ml, affinity purified polyclonal antibody; immunogen: *E.coli*-derived rhIL-23p19, goat IgG, R&D Systems, Minneapolis, MN, USA), IL-1β (10 µg/ml, clone AS10, IgG1,k, mouse IgG1), and IL-6 (10 µg/ml, clone MQ2-13A5, rat IgG1,κ) (both from BD Bioscience) or TGF-β (10 µg/ml, clone 500-M66, Mouse IgG1,κ, Peprotech Inc. Rocky Hill, NJ, USA), b) pattern recognition receptors: TLR-2 (10 µg/ml, clone TL2.1, Mouse IgG2a,κ κ), TLR-4 (10 µg/ml, clone HTA, mouse IgG2a,κ) and mannose receptor (10 µg/ml, clone 15-2, Mouse IgG1, κ) (all three from Biolegend Inc, San Diego, CA, USA), Dectin-1 (10 µg/ml, clone 259931, mouse IgG2B, R&D Systems) and CD14 (10 µg/ml, clone M5E2, Mouse IgG2a, κ, Biolegend) or c) the corresponding isotype-matched antibodies (10µg/ml) were added at the onset of the PBMC cultures in order to determine the role of each cytokine in *M.tuberculosis*-induced Th17 immune responses. In some assays, PBMC from PPD[+]HD were cultured for 48 h alone or with *M.tuberculosis* strains in the presence or not of recombinant IL-23 (1 ng/ml, Biolegend), recombinant TGF-β (0.5-5 ng/ml, Prepotech, Rocky Hill, NJ, USA) or IL-23 plus TGF-β while PBMC from MDR-TB patients were cultured for 48 h with the strains in the presence or not of anti-IL-23p19, anti-TGF-β or anti-IL-23 plus anti-TGFβ neutralizing antibodies (10 µg/ml for both antibodies). Afterwards, cells were tested for active-caspase-3, IFNγ and IL-17

expression by flow cytometry and supernatants were collected and stored at -70ºC for subsequent IL-17 and TGF-β detection.

Immunofluorescence analysis:

Surface expression: Surface CD25 and latency associated protein (LAP) expression was determined on 48 h-cultured PBMC by staining cells with FITC or APC conjugated anti-CD4 and PE- or PerCP/Cy5- anti-CD25 (all from BD Bioscience) and PerCP/Cy5- anti-LAP (TGF-β1) monoclonal antibodies (Biolegend Inc).

Intracellular cytokine and active-caspase-3 expression: Intracellular IL-17, IL-1β, IL-23 and IFNγ expression was determined in PBMC cultures stimulated with *M.tuberculosis* strains after 24h or in 48 h cultured PBMC (for monocytes and lymphocyte staining respectively). Briefly, Brefeldin A (5 µg/ml; Sigma Chemical Co., St. Louis, MO, USA) was added 4h before finishing the culture to block cytokine secretion, and cells were surface stained with the following anti-human antibodies: PE-Cy5- or FITC anti-CD4 (BD Bioscience) or PerCP/Cy5.5 anti-human CD14 (Biolegend Inc); then fixed with 0.5% paraformaldehyde and permeabilized with fluorescence-activated cell sorter permeabilizing solution 2 (BD Bioscience) before PE- anti-IL-17 (RD), PE- anti-IL-1β (eBioscience Inc., San Diego, CA, USA), PE- anti-IL-23 p19 (R&D Systems), FITC- or APC-anti-IFN-γ (Biolegend Inc.), FITC-anti- active-caspase-3 (BD Bioscience) or the corresponding isotype control antibody was added. In other experiments, surface stained CD4$^+$CD25$^+$LAP$^+$ cells were tested for IL-17 and Foxp3 expression by employing a FITC-anti-human Foxp3 staining set (eBioscience Inc.) according to the manufacturer's instructions. Stained cells were analyzed by flow cytometry. Eighty thousand events were acquired for each cell preparation, using a FACSCan flow cytometer (BD Bioscience) with CellQuest software acquisition. FCS Express software (De Novo Software, Los Angeles, CA, USA) was used for analysis.

Lymphocyte or monocyte/macrophage gates were set according to forward and side-scatter parameters, excluding cell debris and apoptotic ones. The percentage of positive cells in lymphocytes and macrophages gates was determined and then the number of positive cells within 1×10^6 PBMC (n) was calculated for each individual on the basis of the percentage and absolute number of $CD4^+$ and $CD14^+$ cells. As previously shown [18] , MDR-TB patients showed lower levels of $CD4^+$ cells than PPD^+HD.

Results were expressed as a) number of positive cells/1×10^6 PBMC (n) or b) % of caspase-3 cells within the $IL-17^+IFN\gamma^-$, $IL-17^+IFN\gamma^+$ and $IL-17^-IFN\gamma^+$ $CD4^+$ cells subsets.

Cytokine assays: IL-17 and TGF-β production in 48h-PBMC cultures supernatants was assessed using commercial enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions: IL-17 (sensitivity 4 pg/ml, range 4–500 pg/ml), TGF-β (sensitivity 8 pg/ml, range 8-1000 pg/ml) (both from eBioscience Inc.).

Statistical analysis: Data in tables were expressed as medians and 25th to 75th percentiles while in graphics were depicted as boxes representing median values (line) and 25th to 75th percentiles and error bars indicating maximum and minimum values. Data analysis was performed using the following One way Anova tests: a) nonparametric Kruskal-Wallis test followed by Dunn's Multiple Comparison Test to compare MDR-TB patients and PPD^+HD responses; b) non-parametric Friedman test followed by Dunn's Multiple Comparison Test to compare responses to different treatments within each group. All statistical analyses were two-sided, and the significance level adopted for *p* values was <0.05. The analysis was performed using the statistical software SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA) and Graphpad Prism 5.0 (Graphpad Software Inc., San Diego, CA, USA).

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RESULTS

IL-23, TGF-β, IL-6 and IL-1β participate in *M.tuberculosis*-induced Th17 response in MDR-TB patients

It has been shown that IL-23, IL-1β and IL-6 are essential for human Th17 differentiation while the role of TGF-β remains controversial [8]. Thus, we first evaluated whether these cytokines could modulate Th17 response by adding specific neutralizing antibodies at the onset of PBMC culture. Cells from MDR-TB were stimulated with both strains, H37Rv and M, while in PPD$^+$HD and PPD$^-$HD the neutralization assays were performed only in M-stimulated PBMC cultures due to the low IL-17 levels induced by H37Rv [19]. As observed in Table 1, M and H37Rv-induced IL-17 production by PBMC from MDR-TB and PPD$^+$HD was markedly reduced due to IL-1β, IL-6 and IL-23p19 neutralization, while anti-TGF-β reduced IL-17 amounts only in MDR-TB. In the same line, relative (Supplementary Figure 1A) and absolute numbers of CD4$^+$IL-17$^+$ cells were reduced upon neutralization of IL-1β, IL-6 and IL-23p19 in both MDR-TB (Supplemental Figure 1B) and PPD$^+$HD (Supplemental Figure 1C) while anti-TGF-β reduced their numbers only in MDR-TB. The same trend was observed in PPD$^-$HD but number of CD4$^+$IL-17$^+$ cells was negligible (data not shown) and thereafter cells from PPD$^-$HD were withdrawn from further analysis. Our results suggest that IL-1β, IL-23 IL-6 and TGF-β participate in *M.tuberculosis*-specific Th17 responses in MDR-TB patients.

TGF-β promotes expansion of IL-17$^+$IFNγ$^-$ cells in MDR-TB patients

We have previously demonstrated that the strong Th17 response observed in MDR patients was the consequence of an increased proportion of IL-17$^+$IFNγ$^-$ CD4$^+$ cells [19]. Herein, we evaluated whether IL-1β, IL-6, IL-23p19, and TGF-β modified the

proportion of IL-17⁺IFNγ⁻ or IL-17⁺IFNγ⁺ cells within the CD4⁺ cells. As observed in Figure 1, neutralization of IL-1β, IL-6 and IL-23p19 reduced strongly the number of IL-17⁺IFNγ⁻ and IL-17⁺IFNγ⁺ cells in H37Rv- and M-stimulated PBMC from MDR-TB. Interestingly, the neutralization of TGF-β decreased the number of IL-17⁺IFNγ⁻ cells induced by both strains while it only increased the number of IL-17⁺IFNγ⁺ induced by M strain in cultures from MDR-TB patients. In PPD⁺HD, neutralization of IL-1β and IL-6 reduced the numbers of both Th17 cells subsets while anti-IL-23p19 and anti-TGF-β had not effect. These results indicate that IL-1β, IL-6 and IL-23 are crucial for the expansion of both IL-17⁺ T cells subsets independently of the strain, whereas TGF-β shifts Th17 cells towards an IL-17⁺IFNγ⁻ phenotype in M-stimulated CD4⁺ T cells from MDR-TB patients.

IL-23 and TGF-β promote the expansion of CD4⁺IL-17⁺IFNγ⁻ cells by inducing IFNγ⁺ cells death in MDR-TB.

To confirm the modulatory role of IL-23 and TGF-β on the *M.tuberculosis*-induced expansion of Th17 memory cells observed in MDR-TB, PBMC from 6 PPD⁺HD were cultured for 48h alone or with M strain with or without the addition of IL-23 and/or TGF-β and then IL-17 and IFNγ expression were tested within CD4⁺ T cells. As shown in Figure 2A, IL-23 alone enlarged the number of IL-17⁺ cells subsets in M-stimulated cells and TGF-β alone inhibited IL-17 and IFNγ expression while co-addition of IL-23 and TGF-β enhanced IL-17⁺ T cells numbers through the expansion of IL-17⁺IFNγ⁻ cells.

Considering that human Th17 clones are less susceptible to TGF-β-induced cell death than Th1 and Th2 clones [23], we evaluated whether the *M.tuberculosis*-induced expansion of IL-17⁺IFNγ⁻ cells observed in MDR-TB was due to TGF-β-induced cell death of IFNγ⁺ cells. For this purpose, the expression of active caspase-3 (a-caspase-

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3) which is associated with cell death signaling pathway [24], was evaluated in a) PBMC from PPD[+]HD cultured for 48 h in the presence or not of IL-23 and/or TGF-β and b) PBMC from MDR-TB patients cultured with or without anti-TGF-β and/or anti-IL-23p19. As it is shown in Figure 2B, H37Rv and M strains enhanced the expression of a-caspase-3 in IL-17^{+} cells from PPD[+]HD and co-addition of IL-23 and TGF-β increased the percentages of a-caspase-3^{+} cells in IL-17^{+}IFNγ^{+} subsets but markedly reduced their percentage in IL-17^{+}IFNγ^{−} CD4^{+} cells (Figure 2B). Interestingly, a low proportion of a-caspase-3^{+} cells was observed within the IL-17^{+}IFNγ^{−} subset from MDR-TB patients and this proportion increased when IL-23 and TGF-β were simultaneously neutralized (Figure 2C). In contrast, IL-17^{+}IFNγ^{+} cells showed a high percentage of a-caspase-3^{+} cells that diminished by IL-23 and TGF-β neutralization. A similar tendency was observed in IL-17^{−}IFNγ^{+} cells from MDR-TB patients (data not shown). These results suggest that IL-23 and TGF-β could act in concert to promote the expansion of Th17 cells with an IFNγ[−] phenotype in response to *M.tuberculosis* strains by inducing cell death in IFNγ^{+} cells from MDR-TB patients.

TGF-β secretion and LAP^{+} cells are enhanced in CD4^{+} and CD14^{+} cells from MDR-TB.

It was shown that spontaneous and *M.tuberculosis*-induced TGF-β production is enhanced in PBMC from patients with active TB [25]. Thus, we evaluated the production of TGF-β in supernatants of cultured PBMC. Spontaneous TGF-β production was markedly increased in MDR-TB when compared to PPD[+]HD and the strains enhanced TGF-β levels only in MDR-TB (Figure 3) being strain M the highest inducer.

Mature TGF-β is associated to a latency-associated protein (LAP), which provides a disulfide-linked shell hindering interaction of TGF-β with its cellular receptors

and to a latent TGF-β–binding protein, which anchors the complex to the cell surface [26]. This latent TGF-β complex has been detected in the cell surface of macrophages and T cells that exert their inhibitory activity through secretion of the bound latent complex upon activation. So, we determined the numbers of CD14$^+$ and CD4$^+$ cells that express latent TGF-β by measuring surface membrane LAP expression in 48 h-cultured PBMC. As observed in Figure 3, the number of CD14$^+$LAP$^+$ and CD4$^+$LAP$^+$ cells increased in no-stimulated PBMC from MDR-TB compared to PPD$^+$HD. Besides, both strains enhanced LAP$^+$ cells values being M a higher inducer when comparing with H37Rv in MDR-TB. Considering that LAP has been found in activated human CD4$^+$Foxp3$^+$ regulatory T cells (Tregs) [27] as well as circulating Foxp3$^-$CD4$^+$ T cells with suppressor activity [28] and taking into account that MDR-TB patients show enhanced proportion of circulating CD25^{high}CD4$^+$ (Tregs) [18], we wondered if the enhanced LAP expression in CD4$^+$T cells was ascribed to this subset. We found that not only Tregs but also conventional activated CD25^{low}CD4$^+$ T cells showed enhanced LAP expression and *M.tuberculosis* strains enhanced its expression in both subsets (Supplementary Figure 2A). Furthermore, we observed that 90-95% of IL-17 expression was detected in *M.tuberculosis*-stimulated Foxp3$^-$LAP$^-$CD4$^+$ cells subsets (data not shown), suggesting that LAP$^+$ cells participate as regulatory cells and not as direct Th17 effector cells in MDR-TB. These findings, in combination with the results of blocking experiments, suggest that the high amounts of free TGF-β and cells binding latent TGF-β could be involved in the expansion of IL-17$^+$IFNγ$^-$ cells via IFNγ down-regulation.

Dectin-1 and TLR-2 induce expansion of IL-17$^+$IFNγ$^-$ CD4$^+$ T cells in MDR-TB patients

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As the recognition of *M.tuberculosis* is mediated by a complex network of pattern recognition receptors [29], we also wondered if TLR-2, TLR-4, MR and Dectin-1 participated in Th17 response induced by MDR clinical isolates of *M.tuberculosis*. As shown in Table 2, blockade of TLR-2, TLR-4 and Dectin-1 diminished IL-17 amounts in PBMC supernatants from MDR-TB and PPD$^+$HD while no differences were observed by blocking MR. We also evaluated if these receptors were involved in the expansion of both Th17 subsets in MDR-TB and in PPD$^+$HD. As observed in Figure 4 A and B, blockade of Dectin-1 markedly diminished the number of antigen-stimulated CD4$^+$ IL-17$^+$IFNγ^- and IL-17$^+$IFNγ^+ cells from MDR-TB and PPD$^+$HD, making no differences among strains. Blockade of TLR-2 inhibited *M.tuberculosis*-induced IL-17$^+$IFNγ^- cells numbers while enhanced numbers of M-induced IL-17$^+$IFNγ^+ cells only in MDR-TB. Besides, anti-TLR-4 reduced the number of *M.tuberculosis*-induced IL-17$^+$IFNγ^+ in PPD$^+$HD. These results propose that while Dectin-1 is essential for a whole Th17 response, TLR-4 sustains IL-17$^+$IFNγ^+ cells in PPD$^+$HD and TLR-2 shifts IL-17$^+$ cells towards an IFNγ^- phenotype in MDR-TB.

The expansion of LAP$^+$ cells in MDR-TB patients is dependent on TLR-2 signaling

Given that interactions between microbial ligands and TLRs in APCs and/or T cells will shape the T cell profiles evoked by modulating their expansion and functionality [30], we assessed whether Dectin-1, TLR-2 and TLR-4 were involved in the expansion of CD4$^+$LAP$^+$ T cells induced by *M. tuberculosis* strains in MDR-TB. As observed in Figure 4C, anti-TLR-2 reduced the number of *M. tuberculosis*-stimulated CD14$^+$ and CD4$^+$ LAP$^+$ cells from MDR-TB, while anti-TLR-4 did not. In line with this, anti-TLR-2 decreased the number of Tregs and conventional activated CD4$^+$ T cells in antigen-stimulated PBMC from MDR-TB (Supplementary Figure 2B). In PPD$^+$HD, the differences were negligible due to low LAP$^+$ cell levels (data not shown). Altogether,

these results suggest that TLR-2 would be involved in the induction of LAP expression in CD4$^+$ T and CD14$^+$ cells from MDR-TB.

TGF-β secretion and LAP$^+$ cells levels correlate with bacillary load.

We have previously showed that the strong Th17 response observed in MDR-TB patients correlates with the presence of acid fast bacilli in sputum smear (AFB) [19]. Herein, we evaluated whether TGF-β secretion and the number of LAP$^+$ cells in cultured PBMC was associated to presence/absence of AFB in sputum using Ziehl–Neelsen stained smears and bacillary load (bacilli/field). As it is shown in Figure 5, not only LAP$^+$CD4$^+$ cell values but also TGF-β levels correlated with the bacillary load in *M. tuberculosis*-stimulated PBMC of all the AFB$^+$ patients studied

MDR-TB patients infected with Haarlem strains show high proportion of IL-17$^+$IFNγ$^-$ CD4$^+$ cells.

We finally evaluated whether MDR-TB infected with strains belonging to Haarlem or LAM families showed differential *in vitro* expansion of IL-17$^+$IFNγ$^-$ and IL-17$^+$IFNγ$^+$ cells. For this purpose, MDR-TB patients were divided in four groups according to the infecting strain and the presence of AFB in sputum smears at the time of the study. Besides, as some patients were infected with MDR *Mtb* strains belonging to the LAM family, PBMC from all patients were also stimulated with the MDR Ra strain which belongs to this family. Haarlem- and LAM-infected AFB$^+$ patients showed higher IL-17$^+$ cells numbers than those AFB$^-$ (Figure 6). However, higher numbers of IL-17$^+$CD4$^+$ cells were observed in PBMC from Haarlem-infected patients when compared to LAM-infected patients in spite of the fact that both groups showed similar sputum smear bacillary loads [bacilli/field: Haarlem= 7 (0.6-10); LAM= 7 (0.8-10)]. M-stimulated CD4$^+$ T cells from AFB$^+$ patients produced higher numbers of IL-17$^+$IFNγ$^-$ cells compared with cells from AFB$^-$ patients. Also, IL-17$^+$IFNγ$^-$ cells numbers were higher in

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cells from Haarlem-infected patients than in cells from LAM-infected patients. In contrast, in AFB$^+$ patients, the numbers of IL-17$^+$IFNγ^+ CD4$^+$ T cells were lower than in AFB$^-$ patients, and AFB$^+$ Haarlem-infected patients showed the lowest levels. Remarkably, the M strain was the highest IL-17$^+$IFNγ^- and the lowest IL-17$^+$IFNγ^+ cell inducer.

DISCUSSION

It is well known that triggering naïve CD4 αβ T cells with MHC/peptide antigens in the presence of IL-6 and/or IL-21, IL-1β, and TGF-β directs their differentiation to the Th17 lineage while IL-23 tends to strengthen and maintain this lineage commitment [8]. As we previously reported that MDR-TB patients show a high proportion of IL-17 producing cells [19], in the present study we explored the involvement of the above-mentioned cytokines in Th17 responses of MDR-TB patients and PPD+HD. Our results showed that IL-1β and IL-6 are critically involved in the Th17 response induced by *M.tuberculosis* strains by promoting the expansion of IL-17+IFNγ− and IL-17+IFNγ+ CD4+ T cells in MDR-TB as well as in PPD+HD. We also found that the neutralization of both cytokines inhibits the expansion of IL-17−IFNγ+ cells in PPD+HD (data not shown), suggesting that IL-1β and IL-6 are also implicated in the *M.tuberculosis*-induced IFNγ response, what is in agreement with Saunders et al [31].

Cytokines promoting the differentiation of human naïve T cells into IL-17-secreting cells are also committed to the regulation of IL-17 production by memory T cells [32]. Likewise, human Th17 cells can be generated from effector memory CD4+ T cells [32-34] and Treg cells [35]. The role of IL-1β and IL-6 that we found for the overall Th17 response in MDR-TB is in agreement with results demonstrating that IL-1β, alone or in association with IL-23 and IL-6, increases IL-17 production in CCR6+ memory T cells from healthy donors, while IL-1β and IL-12 promote the differentiation of memory CCR6+CXCR3+ Th17/Th1 cells specific for pathogenic and commensal microbes [36]. Regarding IL-23, it was previously found to have certain overlapping functions with IL-12 by inducing IFNγ secretion in human CD4+ T cells [37]. However, we found an ambiguous role for IL-23 in *M.tuberculosis*-induced IFNγ expression in CD4+ cells. On one hand, IL-23 amplifies IFNγ expression in T cells from PPD+HD and, on the other hand, it strongly enhances the IL-17+IFNγ− profile in MDR-TB, suggesting a direct role

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of IL-23 on Th1 shift towards IL-17$^+$IFNγ$^-$ cells in these patients. This dual behavior was confirmed in cells from PPD$^+$HD in which the addition of IL-23 increases IFNγ expression in CD4$^+$ T cells and the co-addition of TGF-β lead to a marked reduction of IFNγ expression together with a significant expansion of the IL-17$^+$IFNγ$^-$ population, what is in agreement with Santarlasci et al [23]. The involvement of TGF-β in Th17 cell differentiation is ambiguous, having positive [38] or negative effects [32, 39, 40] according to its concentration along the T cell differentiation/activation processes. As herein shown, and in accordance to the literature [41], high amounts of TGF-β are present in control and *M.tuberculosis*-stimulated cells from MDR-TB. In fact, the high TGF-β secretion and the enhanced percentage of CD14$^+$ and CD4$^+$ T cells binding latent TGF-β might suppress Th1 response and, together with IL-23, increase the proportion of IL-17$^+$IFNγ$^-$ cells in these patients, as supported by our TGF-β neutralization assay results. It has been demonstrated that a-CD3/a-CD28 stimulated Th17 clones are less prone to TGF-β-induced apoptosis than Th1 cells and it has been proposed that TGF-β favors Th17 expansion by directly inhibiting Th1 cells survival [23]. Herein, we show that a-caspase 3$^+$ cells levels induced by the strains were lower in IL-17$^+$IFNγ$^-$ cells and higher in IL-17$^+$IFNγ$^+$ and in IL-17$^-$IFNγ$^+$ cells from MDR-TB patients than in PPD$^+$HD. Furthermore, the assays carried out with neutralization of TGF-β and IL-23 in MDR-TB cells and their addition to PPD$^+$HD cells suggest that both cytokines would sustain the expansion of IL-17$^+$IFNγ$^+$ cells by inducing a subtle cell death of IFNγ expressing CD4$^+$ T cells.

The balance between Th1 and Th17 responses has been associated to the expression of C-lectin like and Toll-like receptors on APC within the TB context [14, 33, 34, 42, 43]. In our hands, the blockade of Dectin-1 decreased the *M.tuberculosis*-induced Th17 response in MDR-TB and in PPD$^+$HD by inhibiting the expansion of IL-17$^+$IFNγ$^-$ and IL-17$^+$IFNγ$^+$ cells but not of IL-17$^-$IFNγ$^+$ cells, confirming that IL-17$^+$IFNγ$^+$

cells are derived from the Th17 and not from the Th1 subsets [10, 44]. Besides, TLR-2 participated in the *M.tuberculosis* induced expansion of IL-17$^+$IFNγ^+ cells and the inhibition of IL-17$^-$IFNγ^+ cells in MDR-TB patients. However, we did not find a participation of TLR-2 in the production of IL-17 from PPD$^+$HD cells, as has been reported in HD without previous exposure to *M.tuberculosis* or BCG vaccination [14]. We consider that our results could be ascribed to an enhanced TLR-2-mediated production of TGF-β by APCs [45] or cell-cell contact suppression by CD25$^+$CD4$^+$ T cells binding TGF-β/LAP complex which are known to express TLR-2 [46, 47] and up-regulate TGF-β expression upon TLR-2 signaling [48]. Indeed, TLR-2 blockade inhibited the *M.tuberculosis*-induced LAP expression in CD14$^+$ cells as well as in Tregs and conventional activated CD4$^+$ T cells from MDR-TB patients.

On the other hand, we showed that TLR-4 signaling is involved in *M.tuberculosis*-induced expansion of IL-17$^+$IFNγ^+ cells from MDR-TB and from PPD$^+$HD but it does not affect the Th1 response, suggesting a differential contribution of TLR-4 and TLR-2 in the modulation of Th17 subsets and Th1 responses in MDR-TB and in PPD$^+$HD, in agreement with Van der Weerdonk [14]. Thus, our results indicate that M strain promotes a high Th17 response in MDR-TB by the expansion of IL-17$^+$IFNγ^- cells as a consequence of a strong TLR-2 dependent TGF-β production by APC and Tregs and conventional activated CD4$^+$T cells (summarized schematically in Supplementary Figure 3).

Interestingly, we found that free TGF-β and LAP$^+$CD4$^+$ T cells levels correlated with bacillary loads in sputum from MDR-TB patients at the time of the study, which is in agreement with experimental models showing that TGF-β suppresses Th1 responses and lung inflammation and enhances lung bacillary load during the chronic phase in the murine model of infection [49]. Thus, it is tempting to speculate that the

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strong TGF-β elicited by the host in an attempt to diminish the severe inflammatory process could favor the persistence of high number of bacilli in MDR-TB.

Finally, we demonstrated that patients that are infected with Haarlem strains and have a positive AFB sputum show the highest numbers of IL-17$^+$IFNγ$^-$ cells and the lowest numbers of IL-17$^+$IFNγ$^+$ cells, underlining the strong ability of the M strain, which belongs to the Haarlem family, to switch the Th17 profile to an IL-17$^+$IFNγ$^-$ phenotype. Thus, our present work extends our previous results [19] and show that the genetic background of the infecting *M. tuberculosis* strain and its bacterial burden can modulate *in vitro* Th17 profiles through an expansion of IL-17$^+$IFNγ$^-$ cells.

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Conflict of interest: None of the authors have any competing financial interests in relation to this work.

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Table 1: IL-23, TGF-β, IL-1-β and IL-6 are involved in IL-17 secretion by MDR-TB patients' PBMC

			No antibodies	a-IL-23p19	a-TGF-β	a-IL-1β	a-IL6
IL-17 (pg/ml)	MDR-TB	C	87 (41-117)	64 (60-104)	91 (95-106)	76 (70-96)	65 (59-70)
		H37Rv	165 (94-171)	90 (48-125)*	92 (53-133)*	78 (39-101)*	99 (53-131)*
		M	207 (82-242)	83 (47-120)*	97 (81-116)*	76 (27-114)*	73 (29-98)*
	PPD$^+$HD	C	5 (4.0-6.0)	< 4	6 (4-10)	< 4	< 4
		M	17 (10-25)	7 (4-22)*	18 (10-29)	5 (4-15)*	7 (4-18)*

Table 1: PBMC from 10 MDR-TB patients and 8 PPD$^+$HD were stimulated for 48 h alone or with the strains H37Rv and M, in the presence or not of monoclonal antibodies against IL-23p19, TGF-β, IL-1β and IL-6. Then, IL-17 amounts (pg/ml) were determined in PBMC supernatants by ELISA. Results are expressed as median and 25-75 percentiles; statistical differences: *: $p < 0.05$ for antibody-treated vs. non-treated PBMC (Friedman test followed by Dunn's test).

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Table 2: Dectin-1, TLR-2 and TLR-4 are involved in high Th17 response in MDR-TB patients.

			No MAb	α-TLR-2	α-TLR-4	a-MR	α-Dectin-1
IL-17 (pg/ml)	MDR-TB	C	94 (66-114)	66 (59-109)	90 (62-110)	103 (94-112)	87 (71-112)
		Rv	171 (144-270)	127 (55-171)*	133 (76-190)*	159 (105-225)	101 (54-168)*
		M	254 (177-423)	120 (38-149)*	141 (108-220)*	214 (159-353)	121 (28-149)*
		C	5 (4.2-6.5)	5 (4.8-5.7)	4 (4.0-4.5)	4.5 (4.1-6.5)	4 (4.1-6.3)
	PPD⁺HD	M	20 (10.1-25.7)	18 (9-29)	7 (4.5-22.5)*	17 (9.2-23.3)	5 (4.0-15.2)*

Table 2: PBMC from 10 MDR-TB patients and 6 PPD^{+}HD controls were stimulated for 48 h alone or with *Mtb* strains, in the presence or not of monoclonal antibodies against TLR-2, TLR-4, mannose receptor (MR) and Dectin-1. Then, IL-17 amounts (pg/ml) were determined in PBMC supernatants by ELISA. Results are expressed as median and 25-75 percentiles; statistical differences: *: $p<0.05$ for antibody-treated vs. non-treated PBMC (Friedman test followed by Dunn's test).

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Supplementary Table 1: Additional clinical feature of tuberculosis (TB) patients

MDR-TB patients	Chest X-ray results	Lineage	Treatment	Days of treatment	Time to acquire a negative sputum (days)
1	UC	Haarlem*	CS/OFL/KM/PAS	7	153
2	UC	LAM	CS/OFL/KM/PAS	168	232
3	UC	Haarlem*	CS/PAS/E/LZD	6	60
4	BC	LAM	CS/OFL/KM/PAS	39	62
5	BC	Haarlem*	CS/PAS/KM/OFL	112	245
6	BC	Haarlem*	CS/PAS/OFL/LZD	166	399
7	BC	Haarlem	CS/OFL/LZD	19	49
8	BC	Haarlem*	CS/OFL/KM/PAS	8	35
9	BC	Haarlem	CS/OFL/E/AMK/PZA	11	25
10	UC	Haarlem*	CS/OFL/KM/PAS	197	142
11	BC	T	OFL/PAS/KM/E	20	31
12	BC	T	OFL/E/S/PZA	88	63
13	UC	LAM	CS/OFL/E/LZD	22	56
14	BC	LAM	PAS/E/LZD/D/KM	68	55
15	BC	Haarlem	E/PAS/KM	76	69
16	UC	LAM	PAS/OFL/LZD/AMK	24	103
17	BC	LAM	PAS/OFL/ CS	34	74
18	BC	Haarlem*	PAS/OFL/E/KM	134	90
19	UC	LAM	PAS/KM/OFL/E/LZD	1	91
20	BC	Haarlem	PAS/OFL/E/S/PZA	76	135
21	BC	Haarlem	CS/OFL/KM/PAS	22	27
22	UC	LAM	PAS/E/OFL/S	70	67
23	B	LAM	PAS/ETH/KM/CS/OFL	21	131
24	BC	LAM	PAS/OFL/KM/E	87	84
25	BC	Haarlem*	PAS/CS/ETH/OFL/KM	60	59
26	UC	LAM	CS/OFL/PZA/E/S	35	29
27	BC	LAM	CS/ETH/KM	36	30
28	BC	Beijing	CS/KM/E/MOX	97	91
29	BC	LAM	CS/PAS/PZA/AMK	70	151
30	U	Haarlem*	LZD/LEV/CS/ETH/PAS	61	123
31	B	Haarlem*	PAS/OFL/S/PZA/E	32	31

*=Patients infected with M strain; Chest X-ray results: UC=unilateral with cavity; BC= bilateral with cavity; U= unilateral without cavity; B= bilateral without cavity; Drug abbreviations: AMK=amikacine; CS =cycloserine; D= deofloxacine; ETH=ethambutol;E=ethionamide; KM= kanamycin; LEV=levofloxacine; LZD= linezolid; MOX=amoxicillin;OFL=ofloxacine; PAS=Para-Aminosalicylate Sodium; PZA=pyrazinamide; S=streptomycin.

Legends to Figures:

Figure 1: IL-23, TGF-β, IL-6 and IL-1β differentially participate in *M.tuberculosis*-induced Th17 response. PBMC from 31 MDR-TB and 8 PPD$^+$HD were stimulated for 48 h alone (Control) or with H37Rv and M strains (2:1 bacteria to PBMC ratio), in the absence (--) or presence of antibodies against IL-23p19, TGF-β, IL-1β and IL-6 (a-IL-23p19, a-TGF-β, a-IL-1β and a-IL-6 respectively). IL-17 and IFNγ expression was determined in CD4$^+$ T cells by flow cytometry (FACS) and the number (n) of **(A)** CD4$^+$IL-17+IFNγ$^-$ and **(B)** CD4$^+$IL-17$^+$IFNγ$^+$ cells present in 1x10^6 cultured PBMC was calculated for each individual. Results are expressed as median and 25th-75th percentiles (boxes) with maximum and minimum values (error bars). Statistical differences: *: p<0.05 for treated vs. non-treated PBMC (Friedman test followed by Dunn's test).

Figure 2: IL-23 and TGF-β promoted the expansion of IL-17$^+$IFNγ$^-$ cells and modulated IL-17$^+$IFNγ$^+$ cell death. (A and B). PBMC from 6 PPD$^+$HD were cultured for 48 h alone (Control) or with H37Rv and M strains in the presence or absence of IL-23 (1 ng/ml) and/or TGF-β (5 µg/ml), and then IL-17, IFNγ and active-caspase-3 (a-caspase-3) expression was tested within CD4$^+$ T cells by FACS. Results are expressed as number (n) of CD4$^+$IL-17$^+$ cells, CD4$^+$IL-17$^+$IFNγ$^-$ and CD4$^+$IL-17$^+$IFNγ$^+$ cells in 1x10^6 cultured PBMC **(A)** and percentage of a-caspase-3$^+$ cells within IL-17$^+$IFNγ$^-$ and IL-17$^+$IFNγ$^+$ CD4$^+$ cells (% a-caspase-3) **(B). (C)** PBMC from 6 MDR-TB patients were cultured for 48 h alone (Control) or with the strains H37Rv and M in the presence or absence of anti-IL-23p19 (a-IL-23, 10 µg/ml) and/or anti-TGF-β (a-TGF-β, 10 µg/ml) monoclonal antibodies. Then % of a-caspase-3$^+$ cells was determined within CD4$^+$IL-17$^+$IFNγ$^-$ and CD4$^+$IL-17$^+$IFNγ$^+$ cells by FACS. Box plots indicating medians and 25th to 75th percentiles with maximum and minimum values are shown. Statistical differences: * p<0.05 for treated vs non-treated PBMC (Friedman test followed by Dunn's test).

Figure 3: Enhanced TGF-β secretion and absolute number of LAP$^+$ cells in PBMC from MDR-TB patients. PBMC from 10 MDR-TB and 6 PPD$^+$HD were cultured for 48 h alone (Control, C) or with *M.tuberculosis* strains and then TGF-β secretion was determined in PBMC supernatants by ELISA as well as LAP expression was determined in CD14$^+$ and CD4$^+$ cells by FACS. **(A).** TGF-β secretion. Results are expressed as pg/ml and medians and 25th to 75th percentiles are shown. Statistical differences: *: $p < 0.05$ for control vs. *M.tuberculosis*-stimulated PBMC or differences among strains (Friedman test followed by Dunn's test); a: $p < 0.05$ for M or Ra-stimulated PBMC from MDR-TB vs the corresponding data from PPD$^+$HD (Kruskal-Wallis statistics followed by Dunn's test). **(B and C).** Number (n) of CD14$^+$LAP$^+$ cells **(B)** and of CD4$^+$LAP$^+$ cells **(C)** present in 1×10^6 cultured PBMC; medians and 25th to 75th percentiles with maximum and minimum values are shown. Statistical differences: *: $p < 0.05$ for treated vs. non-treated PBMC or differences between strains (Friedman test followed by Dunn's test); a: $p < 0.05$ for MDR-TB patients vs PPD$^+$HD (Kruskal-Wallis statistics followed by Dunn's test).

Figure 4: Dectin-1, TLR-2 and TLR-4 are differentially involved in high Th17 response and LAP$^+$ cells expansion in MDR-TB patients. (A and B). PBMC from 10 MDR-TB patients and 6 PPD$^+$HD controls were stimulated for 48 h alone (Control, C) or with *Mtb* strains, in the absence (--) or presence of antibodies against TLR-2, TLR-4, and Dectin-1 (a-TLR-2, a-TLR-4 and a-Dectin-1). Then, the IL-17 and IFNγ expression was determined within the CD4$^+$ subset by FACS and the number (n) of CD4$^+$IL-17$^+$IFNγ$^-$ and of CD4$^+$IL-17$^+$IFNγ$^+$ cells in 1×10^6 cultured PBMC was calculated. Box plots show median and 25th-75th percentiles with maximum and minimum values; statistical differences: *: $p < 0.05$ for treated vs. non-treated PBMC (Friedman test followed by Dunn's test). **(C).** PBMC from 6 MDR-TB patients were stimulated for 48 h alone or with Mtb strains, in the absence (--) or presence anti-TLR-2 or anti-TLR-4

antibodies. Then LAP and CD25 expression was determined in CD4$^+$ and CD14$^+$ cells by FACS. Results are expressed as number (n) of CD4$^+$LAP$^+$ and CD14$^+$LAP+ cells in 1x10^6 PBMC (median and 25-75 percentiles with maximum and minimum values). Statistical differences: *=p<0.05 for antibody-treated vs. non-treated PBMC (Friedman test followed by Dunn's test).

Figure 5: Correlation between TGF-β levels, percentage of LAP$^+$CD4$^+$ cells and bacillary load. Correlation between TGF-β levels, LAP$^+$CD4$^+$ cells and bacillary load in MDR-TB: TGF-β levels (pg/ml) **(A)** and number (n) of CD4$^+$LAP$^+$ cells **(B)** were compared with the number of bacilli per field detected in sputum from MDR-TB. Individual data and Spearman rho coefficients are shown.

Figure 6: MDR-TB patients infected with the M strain showed high proportion of IL-17+IFNγ$^-$ CD4$^+$ T cells. MDR-TB patients were grouped according to the presence or absence of AFB in the sputum (AFB$^+$ and AFB$^-$) and to the infecting strains (Haarlem family: AFB$^+$ n=10, AFB$^-$ n=5; LAM family: AFB$^+$ n=8, AFB$^-$ n=5). The numbers (n) of CD4$^+$ IL-17+ **(A)**, CD4$^+$ IL-17$^+$IFNγ$^-$ cells **(B)** and of CD4$^+$IL-17$^+$IFNγ$^+$ cells **(C)** present in unstimulated or H37Rv, M and Ra-stimulated PBMC cultures were calculated for each group (medians and 25th to 75th percentiles). Statistical differences: *: p<0.05 for AFB$^+$ vs AFB$^-$ individuals; a: Haarlem- vs. LAM-infected patients (Kruskal-Wallis statistics followed by Dunn's test).

Supplementary Figure 1: IL-23, TGF-β, IL-6 and IL-1β are involved in *M.tuberculosis*-induced Th17 response. PBMC from 31 MDR-TB patients **(A and B)** and 8 PPD$^+$ **(C)** healthy individuals were cultured for 48 h alone (Control) or with H37Rv and M strains in the absence (--) or presence of anti-IL-23p19, anti-TGF-β, anti-IL-1β or anti-IL-6 antibodies. Then the percentage of CD4$^+$ cells expressing intracellular IL17 was determined by FACS and number of CD4$^+$IL-17$^+$ cells present in 1x10^6 cultured PBMC (n) was calculated for each individual. **(A)**. Dot plots from one MDR-TB

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patient are shown and are representative of 31 patients studied. The numbers in the upper-right quadrant represent the percentage of CD4$^+$IL-17$^+$ cells within live lymphocyte gate. **(B and C).** Results are expressed as medians and 25th to 75th percentiles (boxes) with maximum and minimum values (error bars). *: p<0.05 for treated versus untreated PBMC (Friedman test followed by Dunn's test).

Supplementary Figure 2: High proportion of CD25$^+$ LAP$^+$ CD4$^+$ T cells in MDR-TB patients; role of TLR-2 on their expansion. (A). PBMC from 8 MDR-TB patients and 6 PPD$^+$HD were cultured for 48 h alone (Control) or with H37Rv, Ra or M strains. Then LAP and CD25 expression was determined in CD4$^+$ cells by flow cytometry. CD4$^+$ cells were classified in CD4$^+$CD25^{high} (T regulatory, Tregs) and CD4$^+$CD25^{low} (conventional activated) according to their CD25 fluorescence intensity. Results are expressed as number of CD4$^+$CD25^{high} or CD25^{low} expressing LAP$^+$ cells in 1x10^6 cultured PBMC (n) and are depicted as boxes showing median and 25-75 percentiles with maximum and minimum error bars. Statistical differences: *=p<0.05 for control vs. Mtb-stimulated cells (Friedman test followed by Dunn's test); a=p<0.05 for MDR-TB vs. PPD$^+$HD (Kruskal-Wallis statistics followed by Dunn's test). **(B).** PBMC from 6 MDR-TB patients were stimulated for 48 h alone or with *M.tuberculosis* strains, in the presence or not of anti-TLR-2 or anti-TLR-4 monoclonal antibodies. Then the number of CD4$^+$CD25$^{high/low}$ LAP$^+$ cells was determined. Box plots show median and 25-75 percentiles with maximum and minimum values. Statistical differences: *=p<0.05 for antibody-treated vs. non-treated PBMC (Friedman test followed by Dunn's test).

Supplementary Figure 3: Schematic model representing the mechanisms used by M strain to induce high levels of TGF-β secretion by APCs and CD4$^+$LAP$^+$ T cells leading to the IL-17+ IFN-γ$^-$ cell subset expansion in MDR-TB patients.

Upper panel: IL-17 secretion: Antigen presenting cells (APCs) from MDR-TB patients and PPD$^+$ healthy donors (PPD$^+$HD) recognize *Mycobacterium tuberculosis* (*Mtb*)

strains (H37Rv and MDR M strain) through Dectin-1, TLR-2 and TLR-4 pattern recognition receptors. Consequently, APCs secrete IL-1β, IL-6, IL-23 and TGF-β triggering the production of IL-17 by CD4$^+$ T cells (upper grey panel). **Lower panels: Differential expansion of IL-17$^+$ T cell subsets in MDR-TB and PPD$^+$HD:** IL-1β and IL-6 secreted by APCs are essential for IL-17$^+$IFNγ$^+$ and IL-17$^+$IFNγ$^-$ CD4+ T cells expansion in MDR-TB and PPD$^+$HD (lower grey panel). Particularly, in PPD+HD, APCs recognize Mtb strains through TLR-4 and together with IL-23 promote IL-17$^+$IFNγ$^+$ cells expansion (green panel). In the case of MDR-TB patients, APCs recognize M strain via TLR-2 secreting large amounts of TGF-β. Additionally, M strain can be further recognized by CD4$^+$CD25^{high}Foxp3$^+$ (T regulatory, Treg cells) and CD4$^+$CD25^{low}Foxp3$^-$ (conventional activated cells) through TLR-2, inducing the upregulation of LAP/TGF-β complex (LAP) expression and promoting the expansion of both subsets. TGF-β secreted by APCs and CD4$^+$LAP$^+$ T cells acts jointly with IL-23 to support the marked expansion of IL-17$^+$IFNγ$^-$CD4$^+$ T cells (pink panel) which are responsible for the enhanced Th17 response observed in MDR-TB patients.
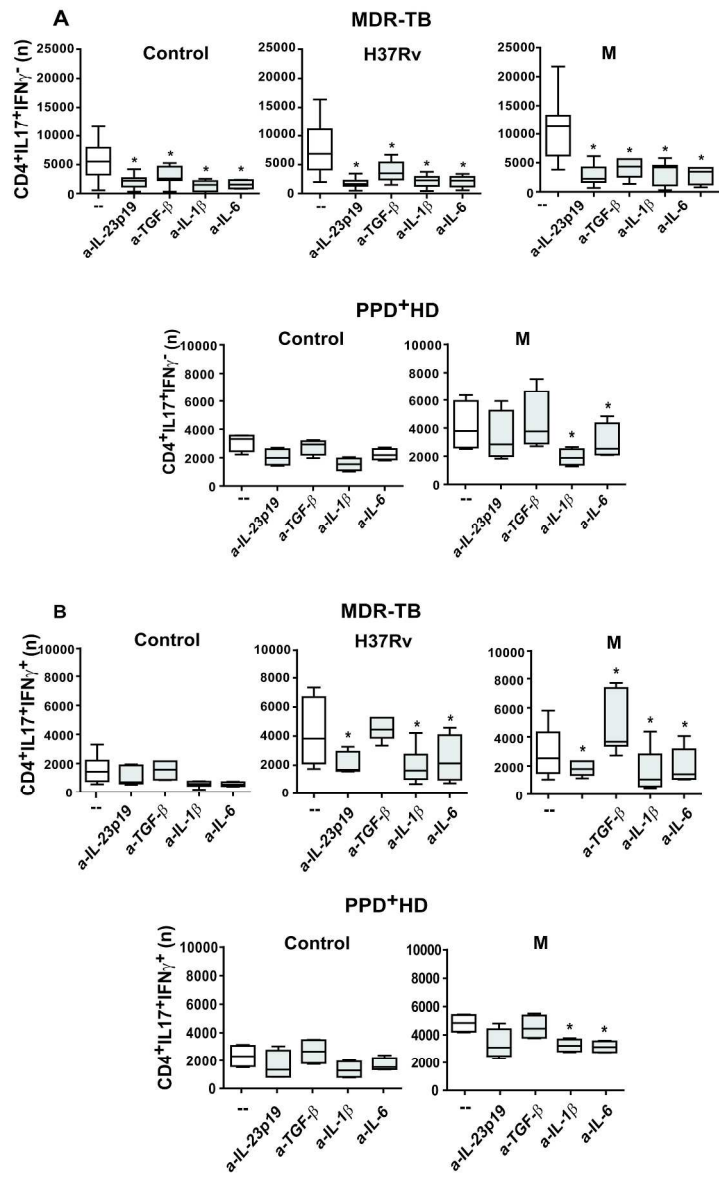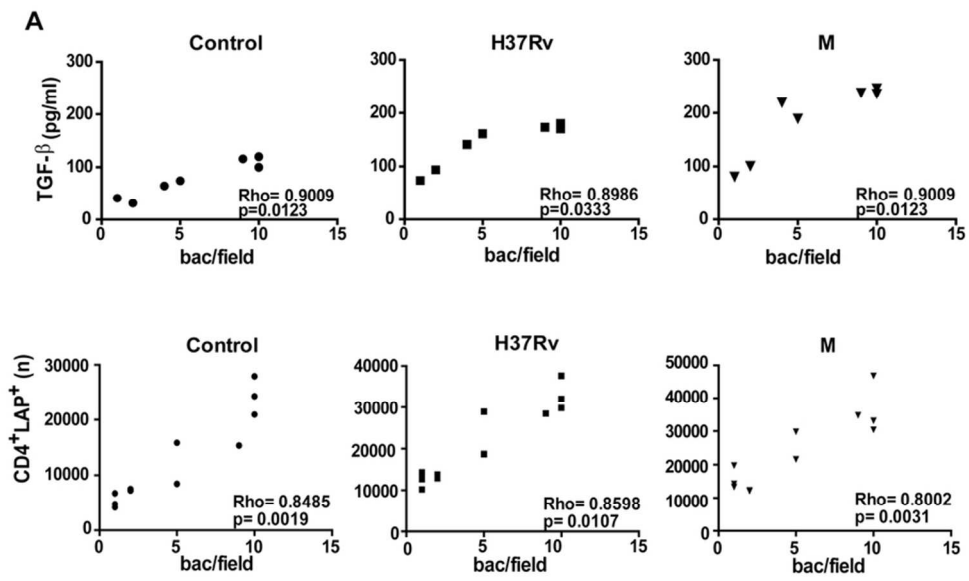
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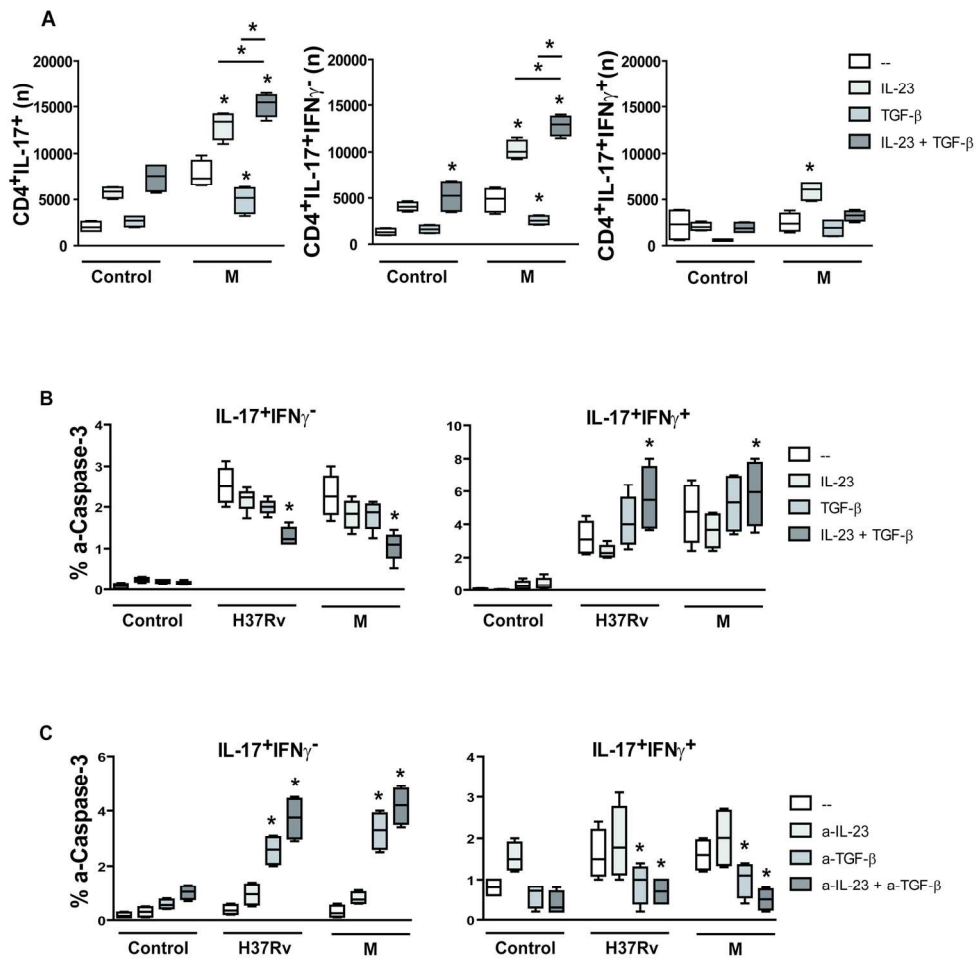
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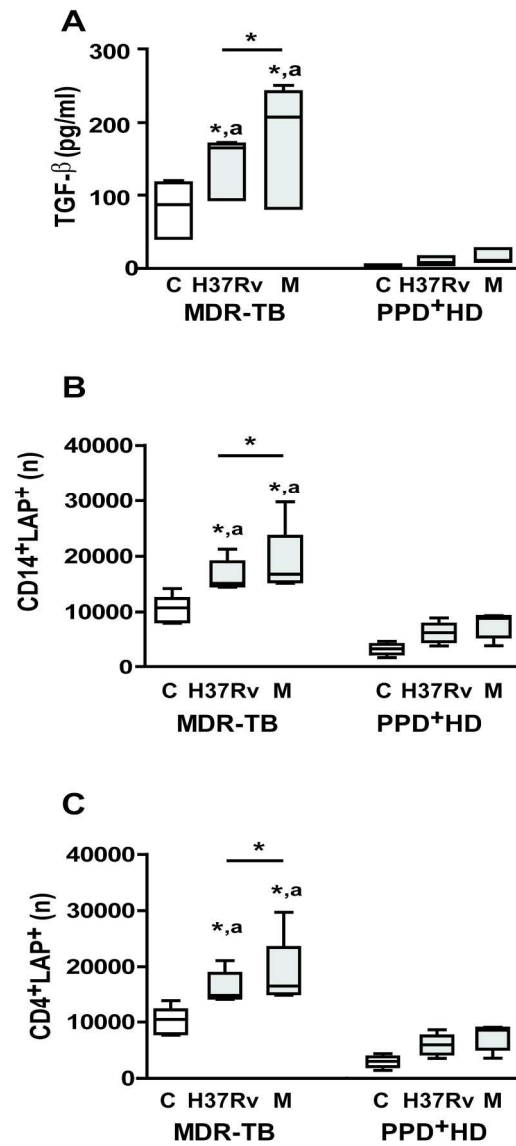
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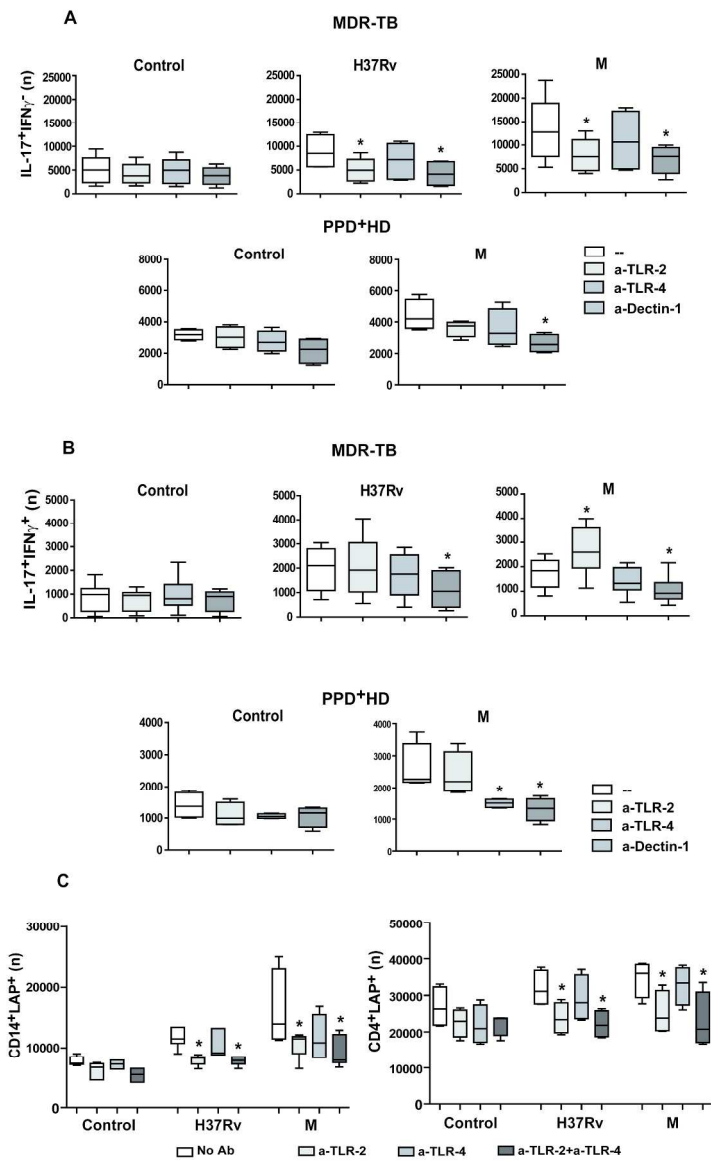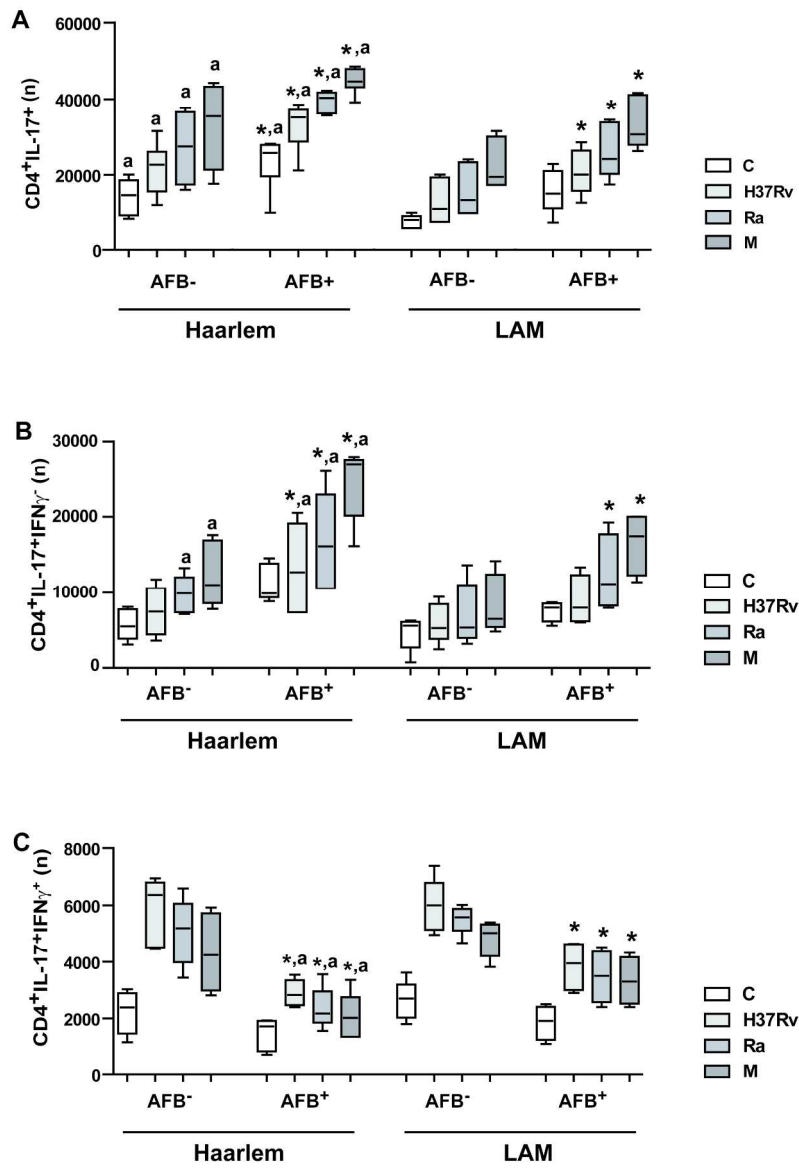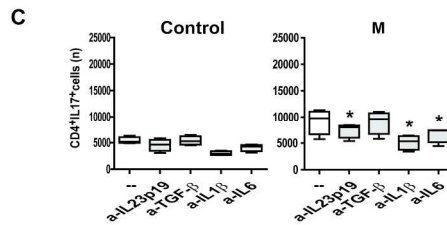
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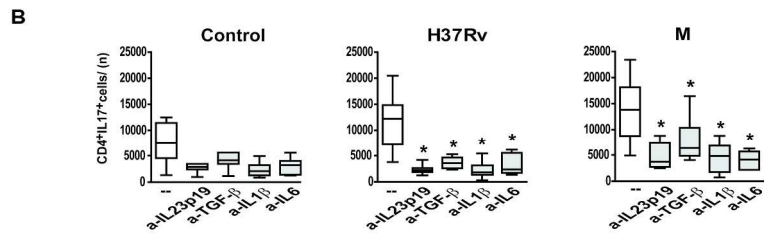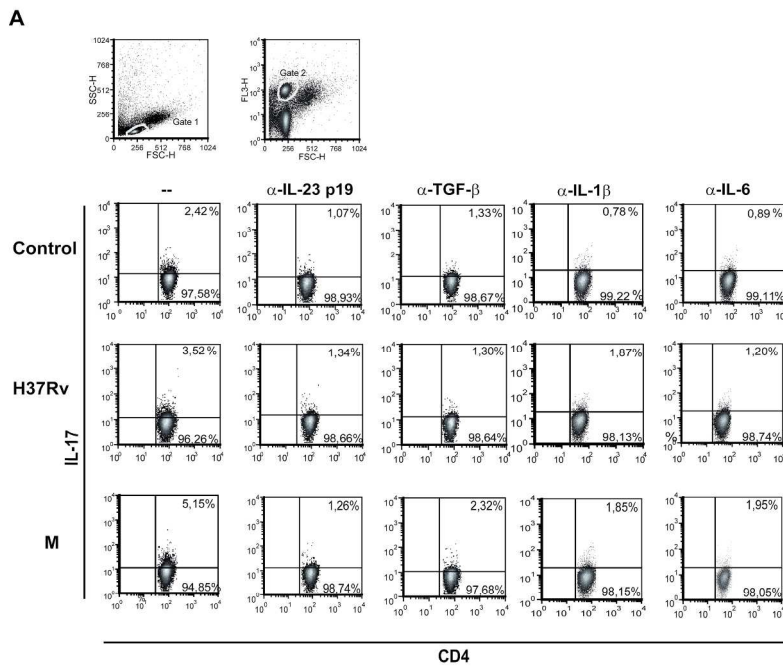79x48mm (300 x 300 DPI)

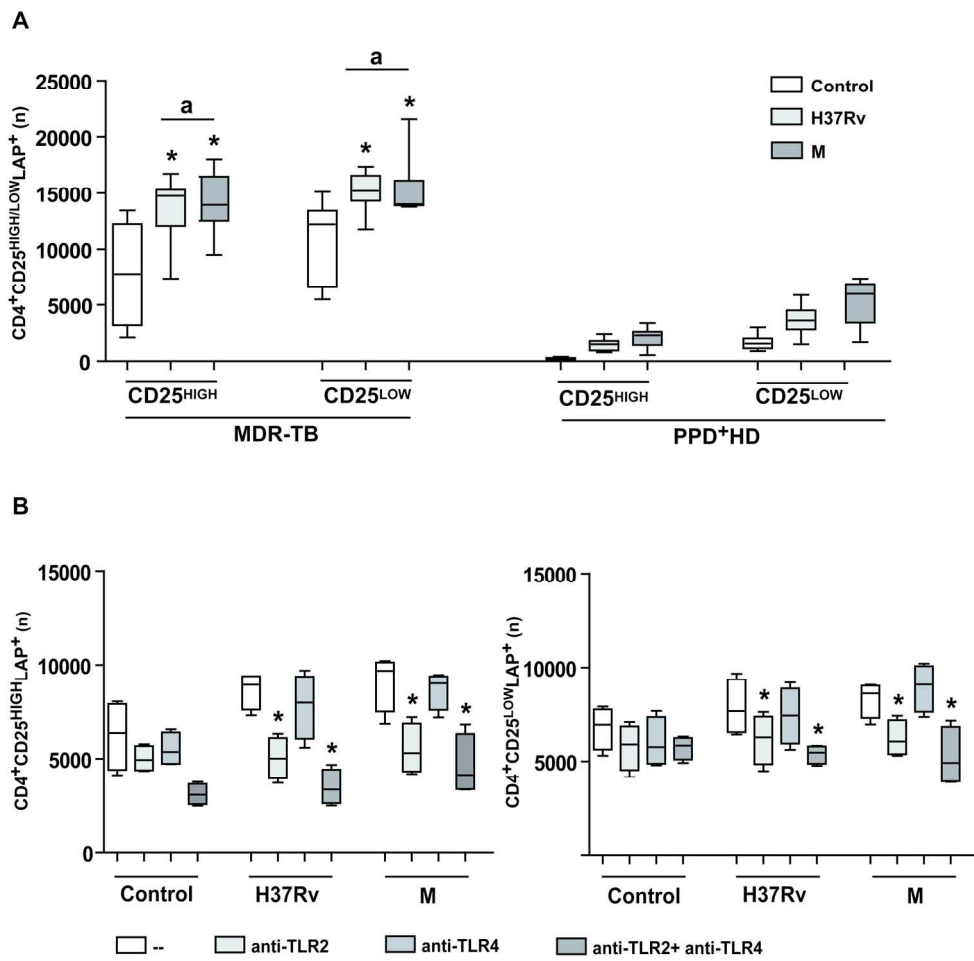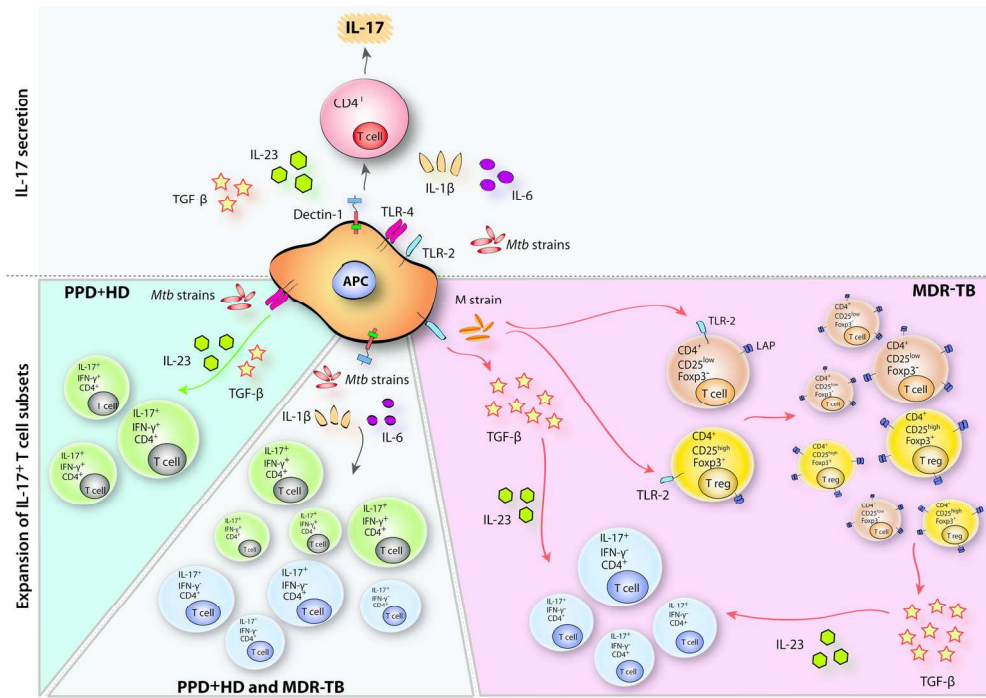199x285mm (300 x 300 DPI)

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