

Immune Response to mycobacteria

Differential B-cell responses are induced by *Mycobacterium tuberculosis* ESAT-6 and Ag85A synthetic peptides

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This study aimed at assessing the usefulness of antibody reactivity in the diagnosis of pulmonary tuberculosis when evaluating the B-cell responses to the ESAT-6 and Ag85A synthetic peptides, the recombinant antigen ESAT-6 (rESAT-6) and antigen 85 (rAg85) and the non-recombinant PPD antigen. A total of 60 creole individuals were included in this study. Patient group was conformed of 20 individuals, whilst control group was composed by 40 healthy individuals. The B-cell responses of pulmonary tuberculosis patients and healthy individuals were evaluated by an IgG-ELISA. The diagnostic accuracy of these methods was assessed by using receiver operating characteristic (ROC) curve analysis. The results showed that the method using the ESAT-6 peptides, rESAT-6 and PPD antigens revealed low specificities, although these remained limited between 40.0 % and 77.5 %.2; whilst the two remaining methods, anti-12033 IgG and anti-12034 IgG, showed the highest sensitivities of 100.0 % (Negative Predictive Value (NPV)=100.0). Regarding the Ag85A peptides, anti-10998 IgG was found to be the most specific (100.0 %, Positive Predictive Value (PPV) =100.0). Whilst anti-11006 IgG showed the highest sensitivity of 95.0 % (NPV=90.0) but was the lowest in specificity (22.5 %, PPV=38.0). Conclusion, the use of highly sensitive and specific peptides may obviate the need for a *M tuberculosis* culture in the initial diagnostic approach to pulmonary tuberculosis.

Key Words: Synthetic peptides, recombinant antigen ESAT-6 (rESAT-6), recombinant antigen 85 (rAg85).

Identification of *Mycobacterium tuberculosis* genes that inhibits MHC class II presentation on dendritic cells

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CD4 T cells are essential for the formation and maintenance of adaptive immune responses against *Mycobacterium tuberculosis* (*Mtb*). It has been shown that *Mtb* uses a variety of mechanisms for inhibiting antigen presentation to CD4 T cells in macrophages and dendritic cells especially to non-secreted proteins. We hypoth-

esize that *Mtb* inhibits MHC class II antigen presentation on infected dendritic cells by reducing the amount of peptide-MHC complexes without a substantial reduction of the total MHC on the surface of the cells. Through the screening of an H37Rv cosmid library expressed in *M. smegmatis* and Y-Ae antibody detection of the I-Ea peptide in the context of a non secreted form of ESAT-6 we have been able to identify six cosmids which contain genes responsible for the diminished class II presentation. A subsequent study of one of the cosmids (M2D10) suggests that a member of the PE_PGRS family could be responsible for the significant decrease in class II presentation showed in multiple independent experiments. Some PE_PGRS family members have been implicated in the intracellular replication capability of *Mtb* and are up-regulated under low iron conditions. This PE_PGRS gene has been showed to be non-essential for in-vitro growth but its deletion from BCG has a very clear phenotype. We will attempt to prove, using T cell transgenic mice in vaccination experiments, that the deletion of this gene from the parental *Mtb* or BCG strain should enhance class II presentation and improved its efficacy as a vaccine for control and prevention of tuberculosis.

Key Words: MHC Class II, immune response, PE_PGRS.

TLR2 and TLR4 are both involved in *Mycobacterium tuberculosis* induced macrophage death

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INTRODUCTION: Infection of macrophages with *Mycobacterium tuberculosis* (*Mtb*) leads to apoptotic or necrotic death. TLR2 and TLR4 have been involved in *Mtb* recognition. It is not known whether cell death induction depends on both TLR2 and TLR4. **OBJECTIVE:** To investigate the importance of TLR2 and TLR4 for *Mtb*-induced macrophage death. **METHODS:** Cell death (apoptosis and necrosis), and intracellular TNF- α and IL-10, in response to *Mtb* infection were determined in murine macrophage lines either expressing (wt) or deficient for TLR2, TLR4 or MyD88. Anti-TLR2 and anti-TLR4 Abs, and MAPK inhibitors were used to examine the role of TLRs and of p38 and ERK1/2 in macrophage death, respectively. **RESULTS:** Infection of wt macrophages with *Mtb* induced apoptosis. Deficiency in TLR2, TLR4 or MyD88 resulted in a reduction of apoptosis whereas TLR4 deficiency resulted in a significant increase in necrosis upon infection. Treatment of wt macrophages with PGN or LPS did not induced macrophage death; however, simultaneous treatment with both PGN and LPS induced apoptosis. Pre-treatment of wt macrophages with anti-TLR2 and anti-TLR4 Abs completely abolished *Mtb*-induced apoptosis and increased necrosis when pre-treated with the anti-TLR4 Ab. Pre-treatment of wt macrophages with the MAPK inhibitors before infection resulted in significant inhibition of apoptosis. TLR2- and TLR4-deficient macrophages displayed lower levels of iTNF- α and higher levels of iIL-10 compared to wt macrophages upon infection with *Mtb*. **CONCLUSIONS.** *Mtb*-induced

macrophage death is dependent on both TLR2 and TLR4 and correlates with MAPK activation and the production of TNF- α and IL-10.

Key Words: *M. tuberculosis*, macrophage death, TLRs.

Study of memory T cells specific for *M. tuberculosis*

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INTRODUCCION: The phenotyping of memory immune cells that mediates the cellular immune response against *M. tuberculosis* may lead to new diagnostic methods and more effective vaccines. **Objectives:** The purpose of this work was to determine differences in memory T cell populations specific for PPD, ESAT-6, peptide pools from ESAT-6 and CFP10 and sonicate of *M. tuberculosis* between patients with TB and healthy controls. **Methods:** Ex vivo phenotyping of four different T cell subpopulations of memory T cells populations: central memory (T_{CM}) [CD62L⁺/CD45RO⁺], effector memory (T_{EM}) [CD62L⁻/CD45RO⁺], effector (T_{EF}) [CD62L⁻/CD45RO⁻] and naïve T cells (T_N) [CD62L⁺/CD45RO⁻] in PBMCs from patients and control individuals (PPD+ and PPD-) upon stimulation with antigens (10µg/ml each) was performed by Flow Cytometry (FC). **Results:** Using an antibody panel to analyze memory markers (CD45RO; CD62-L) on CD3⁺CD4⁻ and CD3⁺CD4⁻IFN- γ ⁺ T-cells specific for *M. tuberculosis* we detected a different expansion capacity of the four populations of T cells studied in the two groups of individuals examined. Notably it was found a significant higher percentage of CD4⁺ T_{CM} and lower percentage of T_{EF} in TB patients compared to controls. On the other hand, compared to PPD- individuals, PPD+ healthy donors showed lower percentage of T_{CM} and higher percentage of T_{EF} in both CD4⁺ and CD8⁺ T cells. **Conclusions:** further studies regarding differences in memory T cells sub-populations between TB patients and healthy individuals may lead to understand individual responses that protects or favors the development of TB.

Key Words: memory T cells, IFN- γ ⁺, EAT-6.

Study of the immunological profile towards *M. bovis* antigens in naturally infected cattle

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A number of studies have determined the contribution of TH1 and TH2 responses to protective immunity and pathology of *Mycobacterium bovis* infection. However, much of that information is derived from experimentally infecting cattle with *M.*

bovis and few data from naturally infected animals is available. **OBJECTIVES AND METHODS:** The aim of this study was to characterize the immunological profile towards *M. bovis* antigens of naturally infected cattle by measurement of cytokine mRNA expression in peripheral blood mononuclear cells (PBMCs) and to determine which lymphocyte subsets are involved in recall proliferation of PBMCs from *M. bovis* infected cattle in response to *M. bovis* antigens. **RESULTS AND CONCLUSIONS:** Consistent with data from experimentally *M. bovis* infected cattle, naturally infected animal displayed a Th1 cytokine profile in response to PPDB stimulation. Production of INF- γ mRNA by PBMCs after PPDB stimulation statistically distinguishes between infected and healthy herds, suggesting this molecule as an *M. bovis*-infection marker. As it happens in experimental infected cows, CD4 and $\gamma\delta$ TCR cells from a naturally *M. bovis*-infected herd are the predominant T cell responding in recall responses to PPDB.

Key Words: *Mycobacterium bovis*, cattle, immune responses.

Role of cathelicidin in the immunopathogenesis of pulmonary experimental Tuberculosis

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OBJETIVE: To establish the role of cathelicidin in the immunopathogenesis of pulmonary tuberculosis as well as to determinate the cell types that produce cathelicidin in response to infection. **MATERIALS AND METHODOS:** In a murine model of progressive disease and latent infection previously described it was evaluated by immunohistochemistry, transmission electron microscopy (TEM) and real-time PCR the induction and expression of cathelicidin in pneumocytes, bronchial epithelium and alveolar macrophages in response to *M. tuberculosis* infection. **RESULTS:** Cathelicidin is over expressed in day 1 and 21 in the progressive disease model and in day 3 in latent infection model as well. This data correlates with mRNA genetic expression. Using double staining, we observed that cathelicidin is mainly produced in those cells infected with *M. tuberculosis* such as macrophages and type II pneumocytes. Same phenomena were confirmed using ultrastructural analysis by TEM. The main cathelicidin producing cells are bronchial epithelial cells and alveolar macrophages which actively participate in the control of infection. TEM analysis also shows cathelicidin- *M. tuberculosis* colocalization. **CONCLUSION:** Expression of cathelicidin CRAMP is highly increased in response to *Mtb* infection. In the days of high inflammation rates a substantial increase in cathelicidin production is shown, which might suggest an immunomodulatory role for this peptide. Colocalization of the cathelicidin and *M. tuberculosis* in phagocytic vesicles indicates an antimicrobial role for cathelicidin. Previous observations could sup-

port immunomodulatory and antimicrobial therapies for the control of active pulmonary tuberculosis.

Key Words: Cathelicidin, tuberculosis, antimicrobial peptides.

***Mycobacterium* induce defined patterns of response in myeloid derived antigen presenting cells (APCs) from PPD+ and PPD- individuals**

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INTRODUCTION: The successful handling of *Mycobacterium* by APCs may be determinant for defeating the infection by these bacilli. **Objectives:** in this work it was explored apoptotic cell death of monocytes and expression of surface markers important for activation of T cells on monocyte derived DCs after their exposure to different species of *Mycobacteria* (*M. avium*, *M. bovis* BCG, *M. colombienses*, *M. tuberculosis* H37Rv). **METHODS:** Monocytes and DCs were obtained from PPD+ and PPD- healthy individuals. Expression of calreticulin, PDL1 and Annexin-V on monocytes and DCs, together with the expression of DC-SIGN, CD83, CD80, CD11c and CD14 on DCs were monitored by Flow Cytometry (FC). **RESULTS:** it was found that after 48 exposure to different species of *Mycobacteria*, a high proportion of monocytes from both PPD+ and PPD- individuals become Annexin-V⁺/PI⁻ cells and expressed high levels of PDL1. Interestingly, whereas in response to *mycobacteria*, monocytes from PPD- individuals expressed calreticulin this was not observed on monocyte from PPD+ individuals. In contrast to what was observed with monocytes, DCs did express neither calreticulin nor PDL1 in response to same stimuli. It was observed that *M. avium* and *M. colombienses* compared to *M. bovis* BCG or *M. tuberculosis* H37Rv, efficiently induced in all volunteer's DCs higher levels of CD80 and CD11c. Only on DCs from PPD+ individuals DC-SIGN was down modulated after exposure to all *mycobacteria*. **CONCLUSIONS:** Altogether our results suggest that depending upon their differentiation program myeloid derived APCs from PPD+ and PPD- individuals may exhibit defined patterns of response to *mycobacteria*.

Key Words: Calreticulin, apoptosis, PPD+/- individuals.

Transcriptional profile of the early immune response to *Mycobacterium tuberculosis*: an "in vitro" model

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INTRODUCTION: Both phagocytes and T cell-mediated innate and adaptive immune responses are essential to effectively control *Mycobacterium tuberculosis* infection.

The interplay between the pathogen and host immune cells is central to both immunity to the bacterium and pathogenesis of the disease. **OBJECTIVES:** To monitor the early immune response to *Mycobacterium tuberculosis* using the *in vitro* granulomatous response and microarray technology. **METHODS:** Human peripheral blood mononuclear cells were cultured in presence of H37Ra strain at 37°C, 5 % CO₂. Cellular aggregation was followed daily with phase contrast microscopy and Wright staining. Granulomas were collected at 24h, and RNA extracted and hybridized to Affymetrix microarrays (HG-U133). Raw data from microarray experiments was analyzed with dCHIP and GenMAPP programs to determine the significance of changes at the biological context. **RESULTS:** Daily microscopic examination revealed the gradual formation of granulomas in cells cultured in the presence of mycobacteria. Granulomatous structures persisted for 96h, and then began to disappear. Microarray analysis revealed the strong activation of genes of the innate immune response and antigen presentation pathways in response to mycobacterial cells. Among activated genes we found TLR-2, TNF-R1, IL-1, IL6, CXCR4, and several chemokine ligands. **CONCLUSIONS:** We have followed the *in vitro* immune reaction to live mycobacterial cells. It involves recruitment of immature phagocytes and lymphocytes, which later became highly active cells with large cell-membrane interdigitations and high phagocytic activity. This was accompanied by strong transcriptional activation of pro-inflammatory genes detected by microarray analysis in this *in vitro* model.

Key Words: Microarrays, Tuberculosis, immune response.

Interaction between *Mycobacterium tuberculosis* (MTB) and mononuclear phagocytes: influence of differentiation and phagosomal maturation on macrophage effector functions and cell death

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INTRODUCTION: Mtb disturbs phagosome and lysosome fusion, modify signaling pathways to prevent the stimulation of bactericidal effects. Evidences indicate that differentiation of phagocytic cells may influence an efficient activation of mechanisms against Mtb. **OBJECTIVE:** To determine the effect of differentiation and phagosomal maturation of human mononuclear phagocytes on effectors functions and cell death of these cells during the infection with Mtb.

METHODS: CD68, CD16 and CD14⁺ are determined by flow cytometry (FC). Differentiation of Mtb-infected and non-infected monocytes into macrophages derived from monocytes (MDM) was studied by microscopy and FC. Adherence/phagocytosis of fluorescent latex beads and FDA-labeled Mtb by FC and CFU; Mtb replication inside mononuclear phagocytes by CFU. TNF α , IL6, IL10, and IL1 β by CBA. Acidification of intracellular compartments by "Lysotracker-Red^{MR}" under epifluorescence microscope. **RESULTS:** TB Patients have increased percentages of CD14^{hi}CD16⁺ monocytes with a low expression of CD68,

suggesting less differentiation stage. During *in vitro* differentiation of monocytes into MDM (120 h) there is increase in cell size and granularity, CD68 expression, HLA-II, CD40 and CD86, the expression of CD68, CD86 and HLA-II and morphological changes are prevented by *Mtb* infection. MDM acquired more latex beads and *Mtb* than monocytes. However, MDM prevented *Mtb* replication and produced more IL-1 β , TNF α and less IL-10. MDM display high numbers of acid compartments than monocytes, but both cells similarly acidified *Mtb* phagosomes.

CONCLUSION: *Mtb* infection prevents differentiation of mononuclear phagocytes, and less differentiated cells have low anti-mycobacterial activities than MDM.

Key Words: Mononuclear phagocytes, mycobacteria and differentiation.

Arachidonic acid (AA)-induced cell death during *Mycobacterium tuberculosis* infection is Affected by the stage of Monocyte/Macrophage Differentiation

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In response to *Mycobacterium tuberculosis* (*Mtb*) infection, mononuclear phagocytes undergo apoptosis and necrosis depending on the stage of differentiation. Pharmacological blockade of phospholipase A (PLA-2) during the *Mtb* infection prevents necrosis, increased the number of TNF+ cells and decrease *Mtb* growth, suggesting that AA maybe determinants of the apoptosis/necrosis balance, thus playing a critical role during *Mtb* infection.

AIM: To determine the effects of AA on mononuclear phagocytes cell death and to validate a chromatographic method to quantify AA production.

METHODS: Human promonocytes (U937) were differentiated or not into macrophage-like cells using PMA (U937D), treated with AA, infected or not with *Mtb* and treated with inhibitors of PLA2 and cell death. Using flow cytometry the following parameters were determined: cell death by DIOC6/Propidium Iodide, TUNEL and Annexin V staining; intracellular TNF- α and IL-10; superoxide anion production using hydroethidine oxidation. Chloroform:Methanol extracts are esterified and lipid profiles were studied by gas chromatography.

RESULTS: AA induced apoptosis and necrosis of U937 cells but U937D cells underwent only apoptosis. In *Mtb* infected U937D, AA increased both types of death and increased the percentage of TNF- α + cells and reduced IL-10+ cells. Blockade experiments indicated that AA-mediated death is TNF- α - and IL-10-independent. AA mediated cell death in U937, but not in U937 D, seems to be influenced by PLA-2; it is noteworthy that calcium blockade prevented necrosis

and apoptosis, and U937 cells produce more superoxide anion in response to AA. Both types of cells produce the same fatty acids, and differences may be related with the amount of them.

Sensitivity of mononuclear cells to AA depends on the stage of differentiation and calcium, and probably oxidative stress.

Key Words: Arachidonic acid, apoptosis, necrosis.

Identification of IFNG-Producing cells and definition of their proliferative capacity in response to mycobacterial antigens in healthy controls, tuberculosis patients and house-hold contacts

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Pulmonary tuberculosis (TB) patients have reduced IFN γ production in response to specific mycobacterial antigens, in comparison with healthy individuals infected with *Mycobacterium tuberculosis*; however, it is not known whether the reduced responsiveness is restricted to a particular T cell subset.

AIM: To quantify the IFN γ -producing CD4⁺ and CD8⁺ cells T and their proliferative response to the non-specific mycobacterial (PPD), specific (CFP-10/ESAT-6) and the latency (HspX) antigens in 26 patients with active pulmonary TB (TBP), 30 household contacts (HHC) of TBP, and 30 tuberculin positive (TST+) healthy subjects not recently exposed to TBP. **METHODS:** Peripheral blood mononuclear cells from TBP, HHC, and TST+ were stained with CFSE and stimulated with PPD, HspX, CFP-10 and ESAT-6 during 144 h. The percentage of CD4⁺IFN γ ⁺ and CD8⁺IFN γ ⁺ cells and proliferating cells were analyzed by flow cytometry. **RESULTS:** TBP had decreased number of CD4⁺IFN γ -producing cells in response to all antigens used, whereas CD8⁺IFN γ -producing cells were decreased in response to PPD and ESAT-6, but not to CFP-10 and HspX. Decreased proliferation was observed in both CD4⁺ and CD8⁺ cells from TBP after stimulation with all antigens. HHC showed the higher IFN γ and proliferative responses irrespective of the antigen employed. The ratio of CD4⁺/CD8⁺ cells showed that CD4⁺ are the main IFN γ -producers and proliferative responders to PPD, also that the ratio of CD4⁺IFN γ ⁺/CD8⁺IFN γ ⁺, in response to ESAT-6 and PPD, was decreased in TBP. **CONCLUSION:** TBP patients had decreased CD4⁺IFN γ producing and CD4 and CD8 proliferating cells. HHC exhibited the higher frequency of CD4⁺IFN γ ⁺-producing CD4⁺ and CD8⁺ proliferating cells.

Key Words: IFN-gamma, RD1 antigens, latency.