## Mycobacterium tuberculosis Promotes Regulatory T-Cell Expansion via Induction of Programmed Death-1 Ligand 1 (PD-L1, CD274) on Dendritic Cells

To the Editor—$CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs) are critical for the maintenance of immune tolerance by suppressing immune cell functions. In the context of infection such as *Mycobacterium tuberculosis*, Tregs can also suppress efficient protective immune responses against the pathogen and its effective clearance [1]. Results from studies of patients with tuberculosis and experimental models have shown that Tregs are expanded and accumulated at the site of infection. These Tregs efficiently inhibit the arrival of effector T cells in the lungs, production of interferon (IFN)–$\gamma$ and $\gamma\delta$ T-cell responses to *M. tuberculosis* [2–4]. However, mechanisms underlying the expansion of Tregs are not clear.

Periasamy et al [5] identified that Treg expansion by *M. tuberculosis* requires expression of the protein programmed death-1 (PD-1, CD279). Inhibition of PD-1 by blocking antibodies or small interfering RNA prevented the Treg expansion. However, the questions that remain unanswered are as follows: does *M. tuberculosis* trigger dynamic changes in the expression of signaling molecules on innate immune cells that, in turn, facilitate Treg expansion, and if so, which innate pattern recognition receptors are implicated in this process? Because Treg expansion in the periphery requires costimulatory signals and antigen presentation by antigen-presenting cells such as dendritic cells (DCs), we surmised that the Treg expansion observed by the Periasamy et al [5] might reflect the modulation of DCs by *M. tuberculosis*. After education by *M. tuberculosis*, these DCs then might provide signals for expansion of Tregs.

To address our hypothesis, immature DCs were derived by culturing peripheral blood monocytes (isolated by using CD14 microbeads; Miltenyi Biotec) from healthy blood donors after ethical committee permission, in the presence of granulocyte microphage colony-stimulating factor (GM-CSF; 1000 IU/$10^6$ cells) and IL-4 (500 IU/$10^6$ cells). The 5-day-old immature DCs (0.5x$10^6$/ml) were either cultured in the presence of cytokines alone or cytokines plus whole cell lysates (WCLs) of pathogenic M. tuberculosis H37Rv (5 microgram/mL) for 48 h. The DCs were washed thoroughly and were cocultured with 0.2 × $10_6$ $CD4^+$ T cells at 1:10 ratio for 96 h. $CD4^+$ T cells were isolated by using CD4 microbeads. The DCs and $CD4^+$ T cells were from unrelated donors, and hence CD3 and CD28 stimulation was not provided. The frequency of $CD4^+CD25^+FoxP3^+$ Tregs was analyzed using flow cytometry (LSR II, BD Biosciences). Surface staining was performed with fluorochrome-conjugated monoclonal antibodies (MAbs) to CD4 and CD25 (BD Biosciences), and intracellular staining for FoxP3 was performed using fluorochrome-conjugated MAb to FoxP3 and kit from eBioscience. We found that *M. tuberculosis*–treated DCs significantly expand Tregs (3.5% ± 0.5% in control vs 6.6% ± 0.8% in *M. tuberculosis*–treated DCs; n = 5; Figure 1A). The results suggest that the Treg expansion observed by Periasamy et al [5] was indeed attributable to modulation of DC (or innate immune cells in general) functions by *M. tuberculosis*.

We then attempted to identify the mechanisms by which *M. tuberculosis*–treated DCs expand Tregs. Periasamy et al [5] identified PD-1 on Tregs as a key molecule involved in Treg expansion, indicating that molecules of PD-1 axis are implicated in Treg expansion by *M. tuberculosis*–treated DCs. Therefore, we analyzed the expression of PD-1 and its ligands PD-L1 (CD274) and PD-L2 (CD273) on DCs after stimulation with WCLs. The expression of these molecules were analyzed by flow cytometry by using the fluorochrome-conjugated MAbs (BD Biosciences). As shown in Figure 1*B*, *M. tuberculosis* selectively induced PD-L1 on DCs (12.4% ± 1.2% in controls vs 78.86% ± 12.8% in *M. tuberculosis*–treated DCs; n = 6), although the expression of PD-L2 and PD-1 were not altered. In fact, the expression of these molecules was either absent or negligible.

How does *M. tuberculosis* induce PD-L1 expression on DCs? DCs express a wide range of pattern recognition receptors, including lectin receptors and Toll-like receptors (TLRs), through which *M. tuberculosis* can interact and deliver signals [4]. We found that TLR2 and C-type lectin receptor DC-SIGN, but not mannose receptor, have a major role in the induction of PD-L1 (Figure 1*C* and data not shown). Blocking of both TLR2 and DC-SIGN appears to have an additive effect on reduction of PD-L1 on DCs, suggesting that both these receptors participate in the induction of PD-L1 on DCs after stimulation with *M. tuberculosis*.
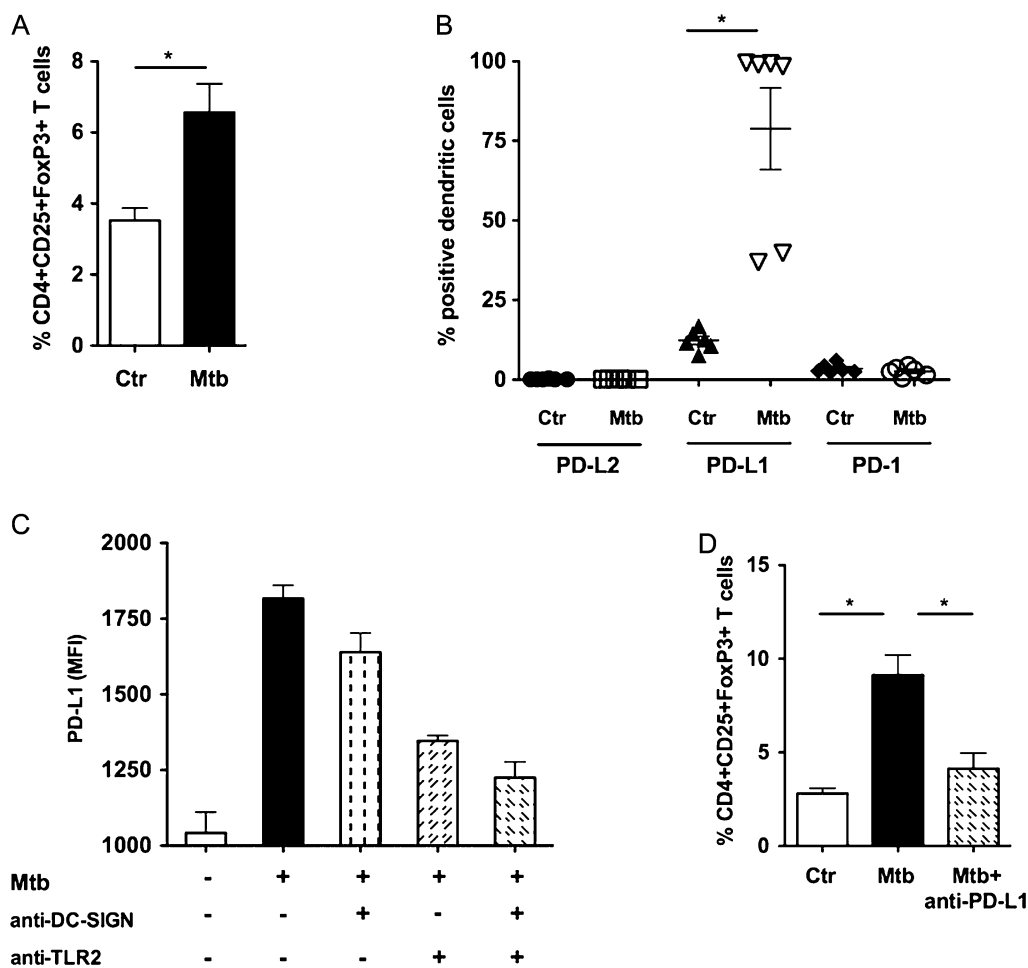


**Figure 1.** *Mycobacterium tuberculosis* promotes regulatory T-cell (Treg) expansion via TLR2- and DC-SIGN–dependent induction of PD-L1 on dendritic cells (DCs). Immature DCs were either cultured in the presence of cytokines alone (Ctr) or cytokines plus whole cell lysate (WCLs) of *M. tuberculosis* H37Rv. *A*, The DCs were cocultured with CD4$^+$ T cells and the frequency of CD4$^+$CD25$^+$FoxP3$^+$ Tregs was analyzed. Results are mean ± standard error of the mean of 5 independent experiments, and statistical significance as analyzed using Student *t* test (*$P < .01$). *B*, Expression of PD-1, PD-L1, and PD-L2 on DCs after stimulation with WCLs of *M. tuberculosis* (*open symbols*). Results are mean ± standard error of the mean of 6 independent experiments, and statistical significance as analyzed by Mann-Whitney test (*$P < .01$). *C*, Effect of TLR2 and DC-SIGN blockade on induction of PD-L1 on DCs after treatment with WCL of *M. tuberculosis*. DCs were preincubated with blocking antibodies to DC-SIGN or TLR2 (5 µg/mL; R&D systems) for 4–6 h, then treated with WCL of *M. tuberculosis* for 48 h. Results are mean ± standard error of the mean of 2 independent experiments. *D*, PD-L1 blockade on DC inhibits Treg expansion mediated by *M. tuberculosis*. Results are mean ± standard error of the mean of 3 independent experiments, and statistical significance was analyzed using Student *t* test (*$P < .05$).

The induction of PD-L1 on DCs by *M. tuberculosis* suggests that its interaction with PD-1 on T cells is responsible for Treg expansion. To confirm this, following treatment of DCs with WCLs of *M. tuberculosis*, cells were washed and incubated with blocking antibody to PD-L1 (10 microgram/mL; eBioscience) for 4 h. DCs were then cocultured with $0.2 \times 10_6$ CD4$^+$ T cells at 1:10 ratio for 96 h. The frequency of CD4$^+$CD25$^+$FoxP3$^+$ Tregs was analyzed by flow cytometry. Of interest, blockade of PD-L1 on DCs led to significant inhibition of Treg expansion (2.8% $\pm$ 0.3% Treg in control DCs, 9.1% $\pm$ 1% Treg in *M. tuberculosis*–treated DCs, and 4.1% $\pm$ 0.8% Treg in *M. tuberculosis*–treated but PD-L1–blocked DCs; Figure 1*D*). Together, our results indicate that *M. tuberculosis*–induced Treg expansion requires DCs and TLR2- and DC-SIGN–dependent induction of PD-L1 on these innate immune cells.

Several reports have demonstrated that Tregs are expanded after *M. tuberculosis* infection. Our results, along with those of Periasamy et al [5], provide insight on how the Tregs are expanded. Two possibilities, however, can account for the Treg expansion. First, *M. tuberculosis* modulates antigen-presenting cells, leading to secretion of chemokines, such as CCL22 and CCL17, which attract Tregs to the site of infection. Furthermore, interaction of PD-L1 on the modulated antigen-presenting cells with PD-1 leads to expansion of migrated Tregs. Second, Tregs can also be generated (induced Tregs) and expanded from the infiltrated CD4$^+$ T cells at the site of infection via PD-L1 and PD-1 interaction. Indeed, the PD-L1 and PD-1 signaling pathway has been shown to be important for the generation of induced Tregs by DCs [6]. Therefore, molecules that target migration of Tregs and PD-1–PD-L1 pathway might have use in vaccination and therapy of tuberculosis [7–10].

## Notes

***Potential conflicts of interest.*** All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

**Jamma Trinath,[1,2] Mohan S. Maddur,[1,3,4] Srini V. Kaveri,[1,3,4,5] Kithiganahalli N. Balaji,[2,a] and Jagadeesh Bayry[1,3,4,5,a]**

[1]Institut National de la Santé et de la Recherche Médicale, Unité 872, Paris, France; [2]Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore, India; [3]Centre de Recherche des Cordeliers, Equipe 16- Immunopathology and therapeutic immunointervention, Université Pierre et Marie Curie, Paris 6, UMR S 872; [4]Université Paris Descartes, UMR S 872, France; [5]International Associated Laboratory IMPACT (Institut National de la Santé et de la Recherche Médicale–Indian Council of Medical Research), National Institute of Immunohaemotology, Mumbai, India

## References

1. Belkaid Y. Regulatory T cells and infection: a dangerous necessity. Nat Rev Immunol **2007**; 7:875–88.

2. Shafiani S, Tucker-Heard G, Kariyone A, Takatsu K, Urdahl KB. Pathogen-specific regulatory T cells delay the arrival of effector T cells in the lung during early tuberculosis. J Exp Med **2010**; 207: 1409–20.

3. Kursar M, Koch M, Mittrucker HW, et al. Cutting edge: regulatory T cells prevent efficient clearance of *Mycobacterium tuberculosis*. J Immunol **2007**; 178:2661–5.

4. Harding CV, Boom WH. Regulation of antigen presentation by *Mycobacterium tuberculosis*: a role for Toll-like receptors. Nat Rev Microbiol **2010**; 8:296–307.

5. Periasamy S, Dhiman R, Barnes PF, et al. Programmed death 1 and cytokine inducible SH2-containing protein dependent expansion of regulatory T cells upon stimulation With *Mycobacterium tuberculosis*. J Infect Dis **2011**; 203:1256–63.

6. Wang L, Pino-Lagos K, de Vries VC, Guleria I, Sayegh MH, Noelle RJ. Programmed death 1 ligand signaling regulates the generation of adaptive Foxp3$^+$CD4$^+$ regulatory T cells. Proc Natl Acad Sci USA **2008**; 105:9331–6.

7. Bayry J, Tchilian EZ, Davies MN, et al. In silico identified CCR4 antagonists target regulatory T cells and exert adjuvant activity in vaccination. Proc Natl Acad Sci USA **2008**; 105:10221–6.

8. Ha SJ, Mueller SN, Wherry EJ, et al. Enhancing therapeutic vaccination by blocking PD-1-mediated inhibitory signals during chronic infection. J Exp Med **2008**; 205: 543–55.

9. Alvarez IB, Pasquinelli V, Jurado JO, et al. Role played by the programmed death-1-programmed death ligand pathway during innate immunity against *Mycobacterium tuberculosis*. J Infect Dis **2010**; 202: 524–32.

10. Kaufmann SH. Fact and fiction in tuberculosis vaccine research: 10 years later. Lancet Infect Dis **2011**; 11:633–40.

DOI: 10.1093/infdis/jir820