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1. The aim of this study was to understand the role of lung epithelial cells in development of natural as well as adaptive immunity in lungs against pathogens and the modulation of this process by air borne particles.

2. Human as well as mouse lung epithelial cell lines had negligible levels of surface TLR2, TLR4 and TLR9 expression. A significant proportion (20.45±1.21% and 22.31±1.62% respectively) of MH-S macrophage cell line however expressed TLR2 and TLR4 markers. Approximately 10% of MH-S cells had a basal expression of TLR9 molecule.

3. Both lung epithelial cell lines A549 and LA4 as well as MH-S cells showed high intracellular TLR4 expression [A549 (65.33±7.18%), LA4 (54.02.72 ± 3.14%), MH-S (35.2±6%)].

4. Surface expression of TLR2, 4, 9 on LA4 cell line were substantially upregulated in response to a mixture of TNFa and IFNy.

5. Expression of TLR2 and TLR4 on LA4 cells could be marginally augmented by LPS in a dose dependent manner. On MH-S cells, LPS had less effect on TLR4 receptors, whereas a marked increase was seen in the expression of TLR2 receptor on LPS treated MH-S cells.

6. TLR2 and TLR4 expressions on LA4 and MH-S cells could be modulated in response to BCG infection. At MOI (100:1) expression of TLR2 and TLR4 was significantly increased in LA4 and MH-S cells exposed to BCG. The effects seem to be higher than that caused by LPS. However like LPS, BCG also did not induce a substantial change in surface expression of TLR4 receptors on MH-S cells.

7. Silica by itself induced a small yet significant decline in basal expression of TLR2 and TLR4 receptors on MH-S cells. Silica particles could also significantly reduce BCG induced augmented TLR2 expression on MH-S cells. High level of TLR2 expression in response to LPS was however not...
changed significantly, by silica particles. While silica particles significantly decreased basal expression of TLR4 receptors, no effect of silica particles was noticed in LPS treated MH-S cells. A small yet significant decreased was noticed on TLR4 expression in BCG treated MH-S cells.

8. Treatment with LPS resulted in marked boosting of intracellular TLR4 molecules on LA4 cells. No effect of BCG on intracellular TLR4 expression was noticed. Treatment with silica particles resulted in a small yet significant decrease (about 30%) in TLR expression.

9. In presence of BCG (MOI 1:1 or 10:1) LA4 cells had a significantly higher growth rate than the control untreated LA4 cells. At higher MOI 100:1, the recovery of LA4 was not altered. In contrast, at all test concentrations of BCG, growth of MH-S cells was significantly inhibited at all time point. The epithelial cell line actually seems to proliferate faster in presence of BCG at MOI (1:1 and 10:1). The effect of BCG sonicates on cell numbers of LA4 and MH-S cell lines in culture, was similar to what was seen with intact BCG cells.

10. To study cellular uptake, BCG cells were labelled with a fluorescent dye (DiI C18), so that uptake of the fluorescenated bacteria by epithelial cells and macrophages could be examined flow cytometrically. Unstained BCG cells have negligible fluorescence, but after treatment with DiI C18 dye, 82% of BCG cells were labelled with fluorescence.

11. In order to assess the uptake of BCG by epithelial and macrophage cell lines, DiI C18 tagged BCG was added to A549, TC-1, LA4 and MH-S cell cultures for different time points. Results indicated that with the progression of time, increased number of culture cells became associated with fluorescence coupled BCG. At all time points a significantly larger proportion of MH-S macrophages were found to be associated with BCG as compared to various lung epithelial cell lines. Thus there is a time dependent uptake/association of BCG with all types of cells.
12. Effect of microfine crystalline silica particles at different doses (10, 50, 100 μg/ml) on cell recovery was examined using LA4 and MH-S cells in culture. Silica particles inhibited significantly the growth of LA4 as well as MH-S cells. The inhibition was almost complete at 50 and 100 μg/ml concentration of silica particles. At 100 μg/ml of silica, recovery of LA4 and MH-S cells fell by approximately 52% and 65% respectively at 24 hrs, time point. These results indicate that microfine crystalline silica particles are toxic to LA4 and MH-S cell lines.

13. LA4 cells release very low level of NO (less than 1 μM) and that was not further augmented by LPS or BCG. Control MH-S cells also secreted very low levels of NO, but in this case both LPS and BCG markedly augment the secretion of NO by MH-S cells after 24 and 48 hrs. Addition of silica particles resulted in a marginal decline in NO secretion by BCG or LPS stimulated LA4 cells. No effect of silica was seen on LPS and BCG induced NO secretion by MH-S cells.

14. Primary lung epithelial (PLE) cells were isolated from mouse lungs using mouse epithelial cell enrichment kit. This technique efficiently removed leukocytes, endothelial cells and RBCs from single cell suspension. After magnetic separation, epithelial cell preparations were found to be devoid of leukocyte contamination.

15. PLE cells in culture were initially smaller in size but became larger and differentiated with cytoplasmic projections after 3-4 days in culture. To see the PLE cells morphology, cells were cultured in 48 wells culture-plate for few days and stained with hematoxylin. Morphology of PLE cells was very similar to LA4 lung epithelial cell line. Forward and side scatter of LA4 cells and primary lung epithelial cells were also comparable.

16. PLE cell were cultured for 3 days and processed for cell cycle analysis on flowcytometer (FACS). Relative numbers of cells in different phases of cell cycle were determined by using Modfit software. The results indicated that
82.97%, 7.70% and 9.33% PLE cells in culture were in G1/G0, G2/M and S phase respectively. These results indicate that PLE cells divide under culture conditions, at least till third day of culture.

17. Basal expression of TLR2 and TLR4 was examined on PLE and BAL cells. Our results indicate that 6-9% of PLE cells expressed TLR2 and TLR4 markers. On BAL cells, which comprise of essential alveolar macrophages, relatively higher expression of TLRs was seen.

18. BCG (1x10^6 cfu/mouse) and silica (100 μg/mouse) were intratracheally instilled into C57BL/6 mice. After 5 weeks, lung epithelial and BAL cells were isolated from control (instilled with PBS), BCG and silica instilled mice. Silica significantly decreased TLR2 and TLR4 expression by 30% and 42% respectively on BAL cells. The expression of TLR2 and TLR4 on PLE cells decreased by 55% and 45% respectively. While BCG significantly increased TLR2 expression by 44% and 68% in BAL and epithelial cells respectively. There was no effect of BCG on TLR4 expression on either primary lung epithelial or BAL cells.

19. BCG sonicates had similar effects on LA4 and PLE cells. As was the case with LA4 cells, silica particles were significantly toxic to PLE cells also. A 26% decline was observed in PLE cells recovery after 24 hrs of culture with 100 μg/ml of silica particles.

20. The recovery of sBCG (MOI 100:1) treated PLE cells in presence of silica (100 μg/ml) declined by 14% at 24 hrs. Thus silica particles and BCG had additive toxic effect on PLE cells.

21. AF-SWCNTs inhibited the growth of PLE cells at all test doses (10, 50 μg/ml). Recovery of cells exposed to AF-SWCNTs (50 μg/ml) fell approximately by 15% as compared to the cell recovery in control cultures at 24 hrs. AF-SWCNTs induced an almost complete block of S phase of cell cycle in PLE cells.
22. Peritoneal cells were stained with F4/80 (marker of macrophage cells) antibody. FACS results show that approximately 61% of peritoneal cells were F4/80 +ve (before washing). F4/80 cells were further enriched to 85% when PCs were allowed to adhere and non-adherent cells were washed off.

23. Recoveries of peritoneal cells and peritoneal macrophages (F4/80 +ve peritoneal cells) cultured with BCG decline at all concentrations of BCG sonicates (sBCG). At highest concentration of sBCG, recovery of peritoneal cells and peritoneal macrophages fell approximately to 30% and 48% respectively at 24 hrs. At 72 hrs time point, the decline was 43% and 61% respectively. Recovery of control peritoneal cells and peritoneal macrophages also fell in culture.

24. There was a significant decline in recovery of peritoneal cells and peritoneal macrophages cultured with different doses of silica as was the case with MH-S cells. At high dose of silica (100 μg/ml), recovery of peritoneal cells and peritoneal macrophages fell approximately by 49% and 67% respectively at 24 hrs. The recovery further fell to 43% and 65% respectively at 72 hr time point.

25. There was a significant decline in recovery of peritoneal cells as well as peritoneal macrophages at all test doses. At high dose of AF-SWCNTs (50 μg/ml) recovery of peritoneal cells and peritoneal macrophages decreased by approximately 45% and 66% respectively at 24 hrs and further to 68% and 78% by 72 hrs.

26. The recovery of sBCG treated peritoneal cells and peritoneal macrophages fell approximately by 44% and 67% respectively of the recovery of sBCG treated in presence of silica at 72 hrs. The recovery of sBCG treated peritoneal cells and peritoneal macrophages fell approximately by 48% and 55% respectively in presence of AF-SWCNTs at 72 hrs.

27. Apoptotic response of PLE cells and peritoneal macrophages to BCG infection was also examined. Control cells and sBCG treated cells were stained with
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7AAD and Annexin V and analyzed on flowcytometer. In case of sBCG (MOI 10:1) treated PLE cells, percentage of apoptotic cells declined from 1.09% to 0.41%. Interestingly the same dose of sBCG enhanced the percentage of apoptotic cells from 0.66% of control to 2.80% in case of peritoneal macrophages. When cells were treated with higher concentration of sBCG (MOI 100:1), both PLE cells and macrophages showed enhanced apoptotic response.

28. Treatment with sBCG (MOI 10:1) induced no change in the percentage of necrotic cells (7AAD+ cells) in PLE cells. At the same dose of sBCG, necrotic (dead) cells in peritoneal macrophages, increased from 7.74% of control to 12.36%. At higher concentration of BCG sonicates (MOI 100:1) both type of cells showed necrotic response. The percentage of necrotic cells increased marginally from 5.43% of control to 6.71% in case of sBCG treated PLE cells; while at same dose of sBCG to peritoneal cells, necrotic cells increased from 7.74% of control to 18.16 % sBCG treated peritoneal macrophages. These results indicate that macrophages may be more sensitive to BCG sonicates than primary lung epithelial cells, in terms of apoptotic and necrotic responses.

29. Treatment with silica, LPS and BCG, induced significant release of TNFα in both primary lung epithelial cells (PLE) and peritoneal macrophages. Peritoneal macrophages released about 10 fold more TNFα than the PLE cells. BCG was a significantly more potent inducer of TNFα than LPS in both PLE cells and macrophages. In all cases, silica particles inhibited the release of TNFα in response to LPS as well as BCG.

30. Basal expression of MHC class II molecule was negligible on LA4, lung epithelial cells (about 0.5% +ve cells) and was about 5% of MH-S, macrophage cells. In both cases the expression of MHC II could be marginally boosted by LPS. Exposure to live BCG also did not have a substantial boosting effect on MHC II molecules on these cells.
31. More than 90% of both LA4 and MH-S cells expressed MHC I molecules. LPS induced a dose dependent increase in MHC I expression (mean fluorescence intensity) in both cell types. Similar results were obtained with BCG as an activating agent.

32. B7.1 was expressed on about 31% of LA4 cells but only on 16% of MH-S cells. Treatment with LPS had little effect on B7.1 expression on LA4 cells. A marked boosting of B7.1 expression on MH-S cells in response to LPS was however seen. Similar results were obtained by using live BCG as an activating agent.

33. LA4 cells did not have significant expression of ICAM-1 molecules, whereas more than 45% of MH-S cells expressed ICAM-1. LPS as well as BCG induced ICAM-1 expression on 3-5% of LA4 cells. Treatment with BCG had little effect on ICAM-1 expression on MH-S cells. Expression of ICAM-1 on MH-S cells in response to BCG was significantly increased.

34. The expression of B7.1 on LA4 cells was markedly enhanced by exposure to cytokines mixture. ICAM-1 and MHC II molecules are normally not expressed on LA4 cells. Cytokines mixture however induced expression of these molecules by more than 20% on LA4 cells.

35. Studies were also conducted on primary lung epithelial (PLE) cells as well as macrophages. For this purpose, PLE cells were derived from mouse lung by enzyme digestion to disperse cells followed by using magnetic bead separation technique to get rid of contaminating lymphoid cells and macrophages.

36. Bronchoalveolar lavage (BAL) cells and peritoneal macrophage preparations were used as primary macrophages.

37. Primary lung epithelial (PLE) cells and macrophages from lungs had substantially high expression of MHC class II molecules as compared to LA4 cells. Interestingly MHC I expression was found only in 32% of PLE cells.
whereas in LA4 epithelial cells MHC II expression was above 90%. Further while 31% of LA4 epithelial cells expressed B7.1, the expression of these molecules was negligible on PLE cells. Another interesting finding was the expression of ICAM-1 molecules that had negligible expression on LA4 cells whereas above 55% of PLE cells expressed these molecules.

38. Expression of B7.1, ICAM-1 and MHC molecules on per cell basis was compared between PLE and BAL cells. MFI of ICAM-1 was substantially higher on PLE cells as compared to BAL cells. Expression of B7.1 was relatively higher on BAL cells as compared to PLE cells. Under culture condition for three days, MHC II expression on PLE cells fell. The expression of ICAM-1 and B7.1 molecules was higher in cultured PLE cells as compared to freshly isolated cells.

39. MFI of MHC I as well as MHC II was significantly boosted in PLE cells isolated from BCG infected mice.

40. Similar results were also seen in BAL cells. No significant effect of BCG infection was observed on the expression of ICAM-1 molecules on both cell types. A small yet significant increase of B7.1 expression was observed in BAL cells from BCG infected mice.

41. Exposure to silica did not alter the expression level of any of these molecules on either epithelial or BAL cells. Interestingly when mice were exposed to BCG as well as silica, the expression of MHC molecules on PLE cells was boosted maximally. Similarly the expression of MHC II molecules on BAL cells was also highest in mice exposed to both BCG and silica.

42. BCG sensitized Th cells were isolated from BCG infected mice by positive selection using magnetic bead technology. Purified Th cells population comprised more than 95% of CD4+ T cells.
43. PLE as well as peritoneal macrophages were used for antigen presentation to BCG sensitized Th cells. PLE cells and PMs were incubated with BCG, washed and fixed with glutaraldehyde. Fixed APCs were co-cultured with Th cells and release of IL2 and IFNγ by Th cells was used as a parameter and T cell activation.

44. The time kinetics of release of IL-2 and IFN γ by Th cells as a result of activation by fixed antigen pulsed PLE cells in antigen presentation assay was studied. A peak of IL-2 secretion was observed, 24 hrs after the co-culture of Th cells and fixed PLE cells was initiated. The peak of IFN γ however came after 4 days of co-culture.

45. Cytokine secretion in antigen presentation assay by Th cells was directly proportional to the number of APCs and was dependent upon dose of BCG used for pulsing the APCs as well as on the duration of antigen pulse.

46. Cytokines were secreted only when sensitized Th cells were co-cultured with antigen pulsed macrophages or PLE cells. No cytokine secretion was observed if the Th cells were from control mice, not exposed to BCG or when antigen presenting cells were not pulsed with antigens. The amount of cytokines secreted was comparable, when macrophages or PLE cells were used for antigen presentation, suggesting there by efficacy of types of APCs in antigen presentation of Th cells, may be comparable.

47. Modulation of T-cell activation by micro-fine crystalline silica particles as well as acid functionalized single-walled carbon nanotubes (AF-SWCNTs) was also examined. Presence of silica particles at the time of antigen pulsing of APCs significantly boosted the T cell response when macrophages were used for antigen presentation. Exposure to silica had marginal or no effect on antigen presentation by PLE cells. AF-SWCNTs preparation did not boost T cell activation but had marginal inhibitory effect instead. Taken together our results clearly indicate that PLE cells have the ability to present mycobacterial antigens to Th cells and the process may be subject to modulation by airborne particles.