

Research article

Flowcytometry – A rapid tool to correlate functional activities of human peripheral blood lymphocytes with their corresponding phenotypes after *in vitro* stimulation.

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Published: 7 August 2002

Received: 15 April 2002

BMC Immunology 2002, 3:9

Accepted: 7 August 2002

This article is available from: <http://www.biomedcentral.com/1471-2172/3/9>

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Abstract

Background: While dealing with mixed *in vitro* lymphocyte cultures one is faced with the problem of relative contributions of different populations to the activity being studied. This is especially true in the controversy relating to the contributions of lymphocyte sub-populations to the Lymphokine Activated Killer (LAK) phenomenon. Flowcytometry can be used to highlight relative contributions of lymphocyte subpopulations towards LAK activity without resorting to difficult purification strategies. We set up long-term *in vitro* lymphocyte cultures, stimulated them with cytokines IL-2/IL-12, recorded their phenotypic changes and cytotoxic activity against U-937 tumor targets.

Results: The results indicated that natural killer cells (NK) constituted the predominant proliferating cell population in the cytokine stimulated cultures. Flowcytometric evidence revealed that CD56+ T cells contributed little to LAK activity against U937 target cells as compared to cells with NK phenotype which were predominantly responsible for spontaneous killing of the tumor targets. The two cytokines, IL-2 and IL-12, had an additive effect on cell proliferation and spontaneous cytotoxicity.

Conclusion: Flowcytometry can be used to rapidly delineate phenotypic changes in immune cells after stimulation and simultaneously correlate them with corresponding functional activity. This approach may find application as a initial screening tool for studying different types of cells in mixed cultures and their respective activities under stimulatory / inhibitory conditions.

Background

The *in vitro* culture of human peripheral blood lymphocytes in IL-2 results in the generation of cytotoxic cells that lyse tumor targets. Natural Killer (NK) cells are the main lymphocytic population constitutively expressing p75 β chain of the IL-2 receptor and exhibiting up-regulation of LFA-1 (Lymphocyte Function associated Antigen-1) molecule upon IL-2 stimulation. Consequently, incubation of peripheral blood lymphocytes with IL-2 induces

selective activation of NK cells, rapid increase of NK activity followed by generation of LAK activity and proliferation. This has been referred to as the LAK phenomenon[1]. Similarly IL-12 treatment of peripheral blood mononuclear cells (PBMC) or pure NK cells within a few hours, induces, an enhancement of cytotoxic ability. It was observed that the maximum enhancement obtained with IL-12 is less than that obtained with IL-2 and comparable to that observed with IFN- γ . However, IL-12

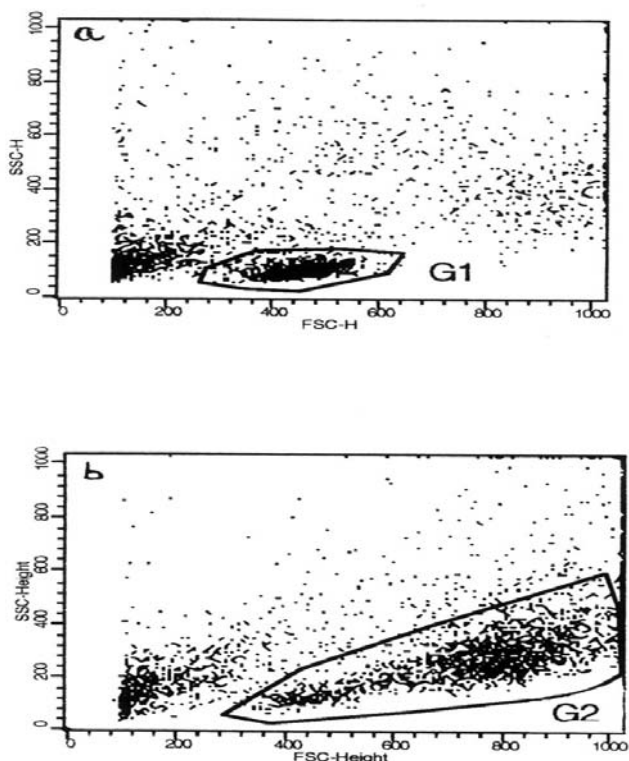


Figure 1
Representative scatter plot of basal and stimulated lymphocytes by flowcytometry : The gate G1 in figure 1a represents the basal lymphocytes while gate G2 in figure 1b represents cells after *in vitro* stimulation with cytokines.

was found to be effective at concentrations 2–3 order of magnitude lower than IL-2/IFN- γ . Maximum enhancement of NK mediated cytotoxicity was observed with a combination of IL-12 and IL-2, the effect of the two cytokines together being additive[2]. Another study revealed that cytokine stimulated NK cells use both granzyme/Fas ligand pathways of apoptotic induction to mediate cytotoxicity[3] and it was found that IL-2 and IL-12 both increase induction in mRNA coding for perforin/ granzymes A and B[4]. Hence the importance of both these cytokines in enhancing cytotoxicity cannot be overlooked.

However before this picture about the involvement of NK cells in LAK activity emerged there existed considerable controversy regarding the type of effector cells mediating this activity, particularly concerning the relative contributions by the two lymphocyte populations, NK Vs cytotoxic T cells [5–9]. Experiments in which, depletion of Asialo-GM (a glycolipid present on the surface of NK cells) was carried out, demonstrated, that the generation of LAK cells followed by IL-2 stimulation was considerably reduced,

thereby confirming that the NK cells are the major contributors of LAK activity[10]. Further, studies with sorted pure populations of CD3/NK cells also showed that the LAK phenomenon is predominantly mediated by IL-2 activated NK cells. Both precursor and effector cells of this activity were typically NK cells[11]. Direct evidence that NK cells are the mediators of LAK activity came from Immunotransmission Electron microscopic studies using colloidal gold labeled antibodies in which CD16 positive cells were shown to extend their protrusions deep into the target cells and it was found that the cytoplasmic granules and vacuoles of CD16 positive LAK cells were concentrated in the area of the binding site[12]. With the advent of flowcytometry and immunophenotyping we thought we could approach this dilemma in a different but simple and straightforward manner. Therefore we generated LAK cells by the method of Anita and Hersh[13] and carried out cytotoxicity experiments with the stimulated cells. For our cytotoxicity experiments we used the U-937 cell line which are derived from human histiocytic lymphoma as tumor targets. U937 cells share many surface markers and receptors with normal human monocytes and therefore express many monocyte like characteristics [14–17]. Concurrently we also assessed the changes in the phenotypic characteristics by flowcytometry. Flowcytometry is a powerful tool that allows one to study not only freshly isolated lymphocytes but also cells that have been cultured *in Vitro* and allowed to proliferate in the presence of stimulating factors. This helps to qualitatively as well as quantitatively assess the phenotypic nature of the proliferating cells *in Vitro*. In the present study our aim was to find out the relative contributions of cells of different phenotypes to spontaneous cytotoxicity and study the effect of long term *in vitro* stimulation of cytokines on mixed lymphocyte cultures.

Results

A study was undertaken to see the effect of IL-2 and/or IL-12 stimulation on a long term basis and correlate the phenotypic changes of the proliferating population corresponding to their cytotoxic abilities after *in vitro* culture of the peripheral blood lymphocytes. LAK cells were generated as described. At regular time points the cells were harvested. The chromium release cytotoxic assay was performed by the standard procedure and compared with the corresponding phenotypic characteristics of the cells, by flowcytometry. A dot plot was generated to gate for the cells after stimulation. A representative dot plot of unstimulated and stimulated cells is shown in Figure 1.

In the Figures 2 to 7, the line graph represents percentage ^{51}Cr release values indicating the percentage NK cytotoxicity at different time points in response to different stimuli. The bar diagrams indicate the corresponding phenotypic characteristics of the cells as assessed by flow-

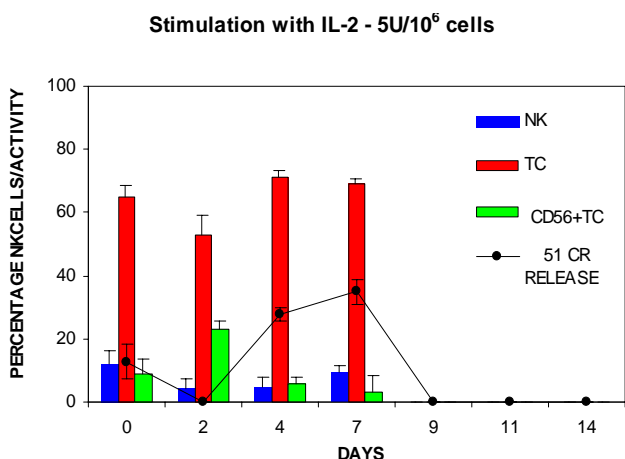


Figure 2
Stimulation with IL-2 (5 U/10⁶ cells): The line and bar graphs indicate the NK activity and the phenotypic characteristics respectively after *in vitro* stimulation with IL-2 for 0–14 days. The blue filled bar represents NK cells, the red bar represents T cells and the green bar represents the CD56 positive T cells. Results are expressed as Mean ± SD (n = 3).

cytometry. The total cells were analysed after appropriately applying the gate on the corresponding dot plots.

It can be seen that IL-2 at 50 U/10⁶ cells is very effective in inducing proliferation of NK cells & simultaneously augmenting cytotoxicity (Figure 3). The percentage of NK cells (blue bar) rises from a basal value of 12 ± 3 % of the total lymphocytes on day zero to 50 ± 5 % of the total lymphocytes on day 14. Simultaneously percentage cytotoxicity (line graph) also rises from a basal value of 12.7 ± 4.2 % on day zero to 86 ± 7.1 % on day 14 concurring with the increase in NK cells. When IL-2 at a concentration of 50 U/10⁶ cells was added along with IL-12 at a concentration of 125 U/10⁶ cells, there was an increase of NK phenotype up to 47 ± 5.2 % on day 11 and a corresponding maximum NK activity of 91 ± 6.5 % (Figure 5). Equally impressive augmentation of NK activity was seen even at lower concentrations of IL-2 and IL-12 in combination. At IL-2 concentration of 5 U/10⁶ cells along with IL-12 concentration of 25 U/10⁶ cells, there was an increase of NK phenotype up to 35 ± 3.1 % on day 9 and a corresponding maximum NK activity of 60 ± 9.4 % (Figure 4). But when IL-2 alone was added at the same low concentration, percentage of NK cells is only 9.4 ± 2.2 % on day 7 corresponding to maximum NK activity of only 35 ± 3.9 % (Figure 2). However IL-12 by itself at concentrations of 25 U or 125 U/10⁶ cells is unable to cause neither an increase in the NK phenotype nor in their cytotoxic ability (Figure 6 & 7).

From Figures 2,3,4,5,6,7 it is evident that initially on day 2 even though there is a transient increase in the CD56+ve T cells (green bars) up to 25–45% the corresponding increase in the chromium release values against U937 target cells is negligible even at a high dose of IL-2. In contrast, at later time points when the cytotoxicity goes up to 86–91%, there are very few CD56+ T cells as compared to the NK cell phenotype. The proportion of T cells (red bars) also has no bearing on the NK activity.

Discussion

Following low dose IL-2 treatment in leprosy patients, NK cell numbers were found to increase 6-fold and IL-2 therapy of HIV patients resulted in enhanced NK and LAK cell activity *in vitro*[18] IL-2 subcutaneously given resulted in, *in vivo* expansion of CD16 positive NK cells to very large numbers which showed LAK activity and ADCC (Antibody Dependent Cellular Cytotoxicity) *in vitro*[19]. In view of the above mentioned and other reports available in literature, a pilot study was undertaken to see the effect of IL-2 and/or IL-12 stimulation on a long term basis and study the phenotypic changes corresponding to the cytotoxic changes after *in vitro* culture of the peripheral blood lymphocytes.

The results obtained from these long term stimulation studies have thrown up interesting points for debate. The phenotypic analysis of the PBMC cultured in the presence of IL-2 and/or IL-12 from 0 to 14 days has brought to light that there is a gradual increase in the percentage of NK cells suggesting that these cells constitute the predomi-

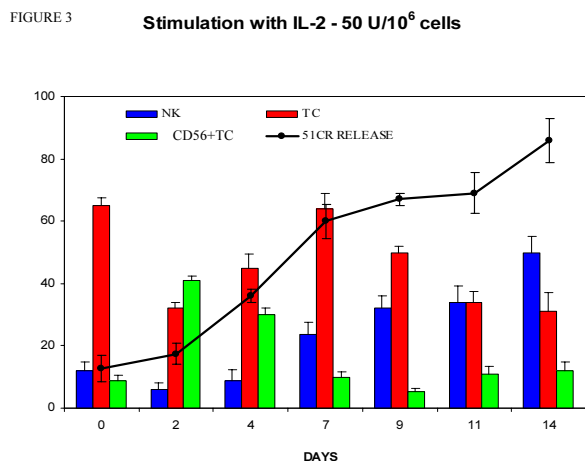


Figure 3
Stimulation with IL-2 (50 U/10⁶ cells): The line and bar graphs indicate the NK activity and the phenotypic characteristics respectively after *in vitro* stimulation with IL-2 for 0–14 days. The blue filled bar represents NK cells, the red bar represents T cells and the green bar represents the CD56 positive T cells. Results are expressed as Mean ± SD (n = 3).

nant cell population in these cultures. This observation is consistent with the results of others where they have shown an increase up to 73% of NK cells on day 9 in response to IL-2 and IL-12 [20]. Even at low concentrations when IL-2 is added along with IL-12, it is able to cause substantial increase in NK cell proliferation and activity (Figure 4) comparable to the effect seen when IL-2 is added alone at higher concentrations (Figure 3). Hence the two cytokines together appear to have an additive effect as reported by other workers [21].

The most striking observation from our experiments is that it provides clear evidence that the "CD56+ T cells" contribute little to cytotoxic activity against the U937 target cell line. It is seen from the figures 2,3,4,5,6,7, that when NK cell number increases, the NK activity also increases. Initially on day 2 even though there is a transient increase in the CD56+ T cell population a corresponding increase in the chromium release values is not observed at a low dose of IL-2 (figure 2) and only a very marginal increase is seen at a higher dose of IL-2 (figure 3) although the CD56 positive T cells are more than double the basal level. In contrast, at later time points when the cytotoxicity goes up to 86–91%, there are very few CD56 positive T cells as compared to the NK cell phenotype reaffirming that it is the NK phenotype that is mostly responsible for the spontaneous killing of U-937 tumor targets. Similar

FIGURE 4 Stimulation with IL-2 + IL-12 (5U +50 U/10⁶ cells)

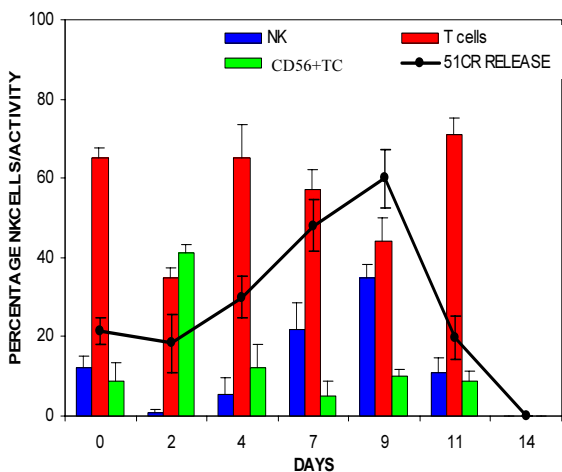


Figure 4
Stimulation with IL-2+IL-12 (5 U + 50 U/10⁶ cells): The line and bar graphs indicate the NK activity and the phenotypic characteristics respectively after *in vitro* stimulation with IL-2 and IL-12 for 0–14 days. The blue filled bar represents NK cells, the red bar represents T cells and the green bar represents the CD56 positive T cells. Results are expressed as Mean ± SD (n = 3).

FIGURE 5 Stimulation with IL-2 + IL-12 (50U+125U/10⁶ cells)

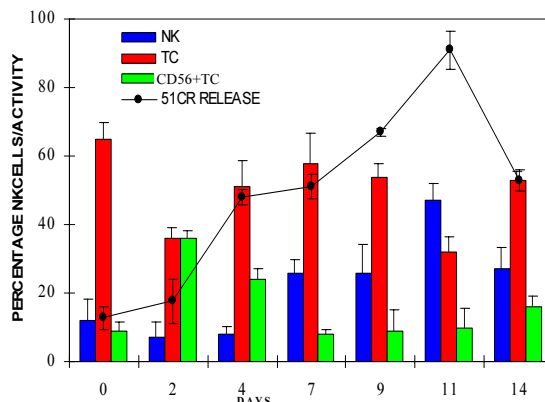


Figure 5
Stimulation with IL-2+IL-12 (50 U + 125 U/10⁶ cells): The line and bar graphs indicate the NK activity and the phenotypic characteristics respectively after *in vitro* stimulation with IL-2 and IL-12 for 0–14 days. The blue filled bar represents NK cells, the red bar represents T cells and the green bar represents the CD56 positive T cells. Results are expressed as Mean ± SD (n = 3).

studies have been carried out using other tumor targets such as K562, COLO, WILMS, MELANOMA and P815. Some researchers have reported the ability of CD56 positive T cells to exhibit spontaneous killing against some of the targets such as K562 or P815, upon stimulation with IL-2 [22–25]. However Lanier and Philips who carried out extensive studies on the LAK phenomenon have emphasised that although CD16+ T cells exhibit non-MHC-restricted cytotoxicity to some targets upon IL-2 stimulation, their contribution was quantitatively and qualitatively very minimal as compared to the NK subset [7,11]. The differences in the ability of lymphocytes of different phenotypes to respond to IL-2 stimulation and carry out spontaneous lysis of different targets could probably be attributed to the differential expression of inhibitory receptors present on the targets such as KIR and CD94:NKG2 [26].

Another important observation is that IL-12 by itself at concentrations of 25 U or 125 U/10⁶ cells is unable to cause neither an increase in the Natural Killer phenotype nor in their cytotoxic ability (Figure 6 &7). This is in agreement with the reported literature where it has been reported that IL-12 can act only on pre-stimulated cells and has little or no effect on resting lymphocytes [27]. IL-12 is a cytokine produced to some degree by B lymphocytes but mostly by phagocytic cells including monocytes, macrophages, neutrophils, epidermal cells, keratinocytes and dendritic cells which are among the first cells to encounter

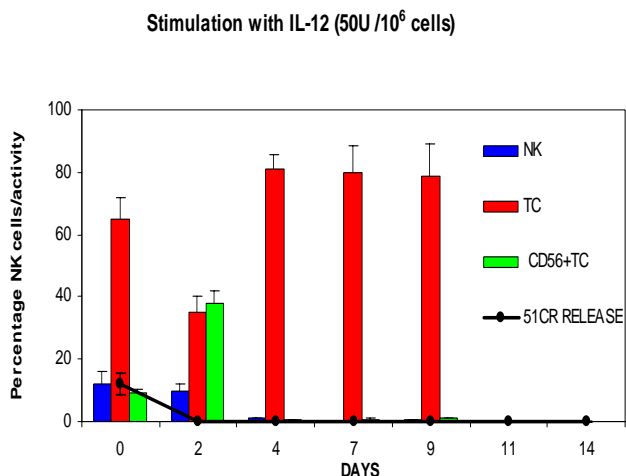


Figure 6
Stimulation with IL-12 (50 U/10⁶ cells): The line and bar graphs indicate the NK activity and the phenotypic characteristics respectively after *in vitro* stimulation with IL-12 for 0–14 days. The blue filled bar represents NK cells, the red bar represents T cells and the green bar represents the CD56 positive T cells. Results are expressed as Mean ± SD (n = 3).

a foreign antigen during infection in response to bacteria, bacterial products, intracellular parasites, etc [2]. It was found that IL-12 induced directional migration of highly enriched preparations of NK cells and CD3 activated cells but not resting T cells and monocytes. When NK cells were treated overnight with IL-12 they showed augmented binding to epithelial cells (EC) by the LFA /ICAM pathways. Thus by inducing migration and interaction with EC, IL-12 regulates crucial determinants of NK cell recruitment in tissues[20]. It is believed that IL-12 plays an instrumental role in tilting the immune response in favor of T helper type 1 by generation of Th1 cells and consequently initiates CMI[28]. Since IL-12 enhances cytotoxicity and augments proliferation by antigen specific T cells and NK cells, IL-12 may facilitate antigen specific and nonspecific cytotoxic immune defense against intracellular pathogens such as *M. tuberculosis*. An intriguing property of IL-12 is its capacity to induce proliferation by cytolytic T cell only upon co-stimulation with antigen. IL-12 may thus be a candidate to control the cytolytic arm of initial immune response to pathogens by inducing expansion of cytotoxic T cell only when antigen is encountered [29]. The biologic significance of the multiplicity of cytokines which can stimulate the proliferation of T and NK cells is unclear. It may be that the various lymphokine growth factors are differentially produced at different time points during the ontogeny of the immune system at different stages in the development of an immune response or at different sites within the body or lymphoid tissues [30]. Our studies have confirmed the importance of multiple cytokine stim-

ulation of lymphocytes and how NK cells are crucial to the development of spontaneous cytotoxicity and are the major contributors to LAK activity *in vitro* when U-937 cells were used as targets. The use of flowcytometry to understand the *in vitro* effects of cytokine stimulation makes one's task easier as studies can be carried out on mixed lymphocyte populations.

Conclusions

The use of flowcytometry as a tool in correlating the functional capacity of various subpopulations of mixed lymphocyte cultures to their phenotype has been demonstrated. A study was undertaken to correlate the phenotypic changes corresponding to the cytotoxic potential of human peripheral blood lymphocytes after long term *in vitro* stimulation with IL-2 and IL-12 cytokines. As reported in literature flowcytometric evidence also pointed to the fact that double positive cells constituting the cytotoxic T cells contribute little to Lymphokine Activated Killer activity, reaffirming that in a mixed lymphocyte culture it is the NK phenotype that is responsible for the spontaneous killing of tumor targets. Thus flowcytometry can be easily applied to situations where one needs to quickly understand the phenotypic changes with respect to their corresponding functional capacity in response to various stimulants or inhibitory agents even on mixed cells populations.

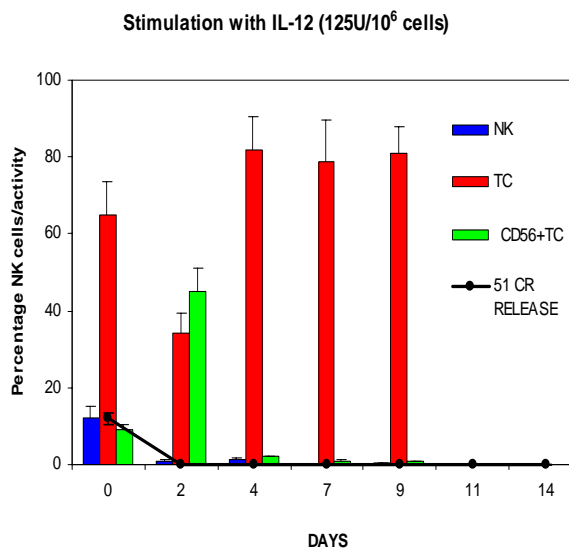


Figure 7
Stimulation with IL-12 (125 U/10⁶ cells): The line and bar graphs indicate the NK activity and the phenotypic characteristics respectively after *in vitro* stimulation with IL-12 for 0–14 days. The blue filled bar represents NK cells, the red bar represents T cells and the green bar represents the CD56 positive T cells. Results are expressed as Mean ± SD (n = 3).

Materials and Methods

Media and reagents

RPMI 1640 culture medium was supplemented with 5% heat inactivated FCS, 2 mM glutamine, 5 mM HEPES, 5×10^{-5} M 2-ME, 100 U/ml penicillin, 20 µg/ml gentamycin (pH 7.2–7.4) (Complete medium). Ficoll hypaque, RPMI-1640 and all other culture reagents were purchased from Sigma Chemical Co., St. Louis, U.S.A. $^{51}\text{Chromium}$ was obtained from BARC, Mumbai, India. Monoclonal antibodies were purchased from Becton and Dickinson, San Jose, CA. Some of the monoclonal antibodies were a kind gift from NIH, Bethesda, U.S.A. U937, tumor target cell-line was obtained from National facility for animal tissue and cell culture, DBT, Govt., of India, Pune, India. rIL-2 cytokine was purchased from Genzyme, USA and rIL-12 cytokine was a kind gift from Trinchieri G, Philadelphia, USA. All plastic ware were obtained from Costar, Cambridge, MA and Falcon, NJ, USA.

Preparation of human mononuclear cells (PBMC)

Blood was collected from 3 normal, healthy volunteers in heparinised containers. It was gently layered over equal quantities of Ficoll-Hypaque density gradient and centrifuged at 1800 rpm for 30 min. PBMC from the interface were washed twice in Hank's Balanced Salt Solution by centrifugation at 1500 rpm for 15 min. The washed PBMC were suspended in complete RPMI medium and adjusted to a volume of 10×10^6 cells/ml.

Long term stimulation studies

2.5×10^6 PBMCs were cultured in a total volume of 500 µl complete medium in 48-well tissue culture plates in the presence of (1) IL-2 (5 U/ 10^6 cells), (2) IL-2 (50 U/ 10^6 cells), (3) IL-2+IL-12 (5 U+25 U/ 10^6 cells), (4) IL-2+IL-12 (50 U+125 U/ 10^6 cells), (5) IL-12 (25 U/ 10^6 cells) and (6) IL-12 (125 U/ 10^6 cells). The cells were harvested and washed on days 2, 4, 7, 9, 11 & 14. Chromium release assay was performed and samples were also analyzed by flowcytometer at all time points including day 0 (fresh PBMC).

Phenotyping for flowcytometry

0.25×10^6 cells/250 µl medium were added to three tubes and 5 µl of isotype control, leucogate and monoclonal antibody anti-CD3PE + CD16/CD56 FITC conjugate was added to the respective tubes. The tubes were incubated for 15 min at 4°C in dark and washed with cold PBS at 1200 rpm for 5 min. The stained cells were fixed with 0.5 ml 1% paraformaldehyde and kept (covered with foil) at 4°C until further analysis on Flowcytometer (Becton and Dickinson, San Jose, CA) using Cell Quest software.

Statistical analysis

Statistical analysis (mean, standard deviation and graphics) was performed with Microsoft Excel.

$^{51}\text{Chromium}$ release assay

1×10^6 target U937 cells in 100 µl medium were labeled with 100 µci ^{51}Cr in a sterile screw-capped 4 ml falcon tube & incubated for 1 hr at 37°C in a CO_2 incubator. Target cells were then washed twice by centrifugation at 1200 rpm for 10 min and suspended in complete medium at a concentration of 0.1 million cells/ml. 0.01×10^6 radio-labeled target cells in 100 µl were mixed with 0.5×10^6 lymphocytes in 50 µl to give an E:T ratio of 50:1 in triplicates in a U-bottom 96-well tissue culture plate. Total volume was made up with complete medium and adjusted to 250 µl/well. Control wells containing only target cells were also processed simultaneously. For spontaneous cytotoxicity, one set of triplicates was set up in which volume was adjusted to 250 µl with medium alone. In another set, 100 µl of 1% Triton X and 150 µl medium was added and this was considered as maximum lysis. The 96-well plate was centrifuged at slow speed (1000 rpm) for 2–5 min to enable contact between effectors and targets. Incubation was carried out for 4 h at 37°C in a humidified CO_2 incubator. Plates were then centrifuged & 150 µl supernatant was carefully removed and transferred to a plastic vial. Counts were recorded in a Gamma counter and percentage cytotoxicity was calculated as follows:

$$\% \text{Cytotoxicity} = 100 \times \frac{\text{counts in test} - \text{counts in spontaneous lysis}}{\text{counts in maximum} - \text{counts in spontaneous lysis}}$$

List of abbreviations

LAK – Lymphokine Activated Killer

NK – Natural Killer cells.

LFA-1 – Lymphocyte Function associated Antigen.

PBMC – Peripheral Blood Mononuclear Cells.

ADCC – Antibody Dependent Cellular Cytotoxicity.

EC – Epithelial Cells.

ICAM – Ig superfamily Cell Adhesion Molecule.

CMI – Cell Mediated Immunity.

Authors' contributions

R Nirmala – Planned and executed the experiments.

P R Narayanan – Designed and guided the studies.

Both authors read and approved the final manuscript.

Acknowledgements

We wish to acknowledge the Indian Council of Medical Research (ICMR) for the support rendered. We also express our deep sense of gratitude to the scientific, clinical, technical, administrative and other staff of the Tuberculosis Research Centre (TRC), Chennai, INDIA, for all the help and co-operation rendered at all times.

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