TREGS SUPPRESS MACROPHAGE TUMORICIDAL FUNCTIONS

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Macrophages are well-known for their tumoricidal functions. In order to ascertain the effect of Regulatory T cells (Tregs) on macrophage tumoricidal functions, macrophages purified from healthy individuals were co-cultured with Tregs for 48 hours and cytotoxicity assay was performed with macrophage sensitive lymphoma cells, U937. Results clearly suggested that Tregs significantly inhibit (p<) macrophage mediated tumor cell killing, as observed in cultures using different E:T ratios.

Increasing evidence supports the existence of elevated numbers of $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells (Treg cells) in solid tumours and hematologic malignancies (Ha, 2009) as well as in murine models. Treg cells protect the host from autoimmune disease by suppressing self-reactive cells (Zou, 2006). In cancer, Treg cells may also block anti-tumour immune responses (Yang and Ansell, 2009). Particularly in the context of cancer, Treg-cell frequencies and function are important because increased numbers might favour tumour development or growth and influence the course of the disease. The suppressive effect of naturally occurring Treg cells against tumour-specific $CD8^+$ T cells was established in a poorly immunogenic B16 melanoma model (Côté *et al*, 2008). Macrophages, on the other hand, play a key role in immune system (Galani *et al*, 2010). The functions of macrophages within tissues are homeostatic, regulating the local and systemic milieu through diverse plasma membrane receptors and varied secretory products. They react to, and themselves generate, signals that influence growth, differentiation, and death of other cells, recognizing and engulfing senescent and abnormal cells, especially tumour cells. These activities contribute substantially to recognition and defence functions against invading microorganisms, foreign particulates, and other immunogens. Innate immune functions of macrophages complement their contributions to acquired humoral and cellular immunity, in which they regulate activation of T and B lymphocytes; this is achieved in part through their specialized derivatives, dendritic cells (DCs) of myeloid origin. Macrophages, with or without DCs, process and present antigen, produce chemokines and cytokines such as interleukin-1 (IL-1), IL-6, IL-12, IL-18, IL-23, tumour necrosis factor-α (TNFα), and IL-10, and phagocytose apoptotic and necrotic cells. Here, effects of Tregs on macrophage functions were evaluated.

MATERIALS AND METHODS

Subjects and Experimental DesignPurification of Treg cells

$CD4^+$ $CD25^+$ Treg cells were purified by MACS, according to manufacturer's instruction (MiltenyiBiotec, GmbH). In brief, PBMC were labelled with a CD4 free antibody cocktail, conjugated with magnetic beads and passed through a magnetic column. $CD4^+$ cell enriched flow through were then labelled with CD25 microbeads. Using another magnetic column, flow through containing $CD4^+$ $CD25^-$ population and column-bound $CD4^+$ $CD25^+$ Treg cells were collected separately.

Preparation of Macrophage

Venous blood was collected from patients and healthy individuals in heparinized tubes and separated on lymphocyte separation medium (MP Biomedicals, Irvine, CA, USA) at 2000 rpm for 30 min. Leucocytes were recovered from interface, washed, counted and adhered on plastic surface for 2 hrs. Adherent fractions were checked for its CD14 positivity and >90% $CDI4^+$ macrophages were used in different in vitro assays.

Cytotoxicity assay

The cytotoxicity of Macrophages against U937 cells were tested by LDH release assay. U937 cells (1 X 104) were plated as target and PBMC were added as effector in different E:T ratios (1:10, 1:50, 1:100) and were co-cultured for 4 hours. Cell free supernatant was used to measure the level of released LDH.

Flow cytometric analysis for cell surface markers

Flow cytometric analysis for surface phenotypic markers (CD40, CD80 and CD86) of macrophage was performed after purification and culture of macrophage. Macrophages were washed with FACS buffer and labeled with 20μl of different anti-human fluorescence labelled antibodies for 30 mins at 4°C in dark as per manufacturer's recommendation. After labelling, cells were washed in FACS buffer, fixed in 1% paraformaldehyde in PBS and cytometry was performed by using Cell Quest software on a FACScan flow cytometer (Becton Dickinson, Mountainview, CA). Suitable negative isotype controls were used to rule out the background fluorescence. The data was generated by cytofluorometric analyses of 10,000 events. Percentage of each positive population and mean fluorescence intensity (MFI) were determined by using quadrant statistics.

Intracellular assessment of perforin and granzyme B

Macrophages from coculture with or without Treg were incubated in presence of Golgistop for 6 hours. Adherent cells were then stained with perforin-FITC or granzyme B-FITC antibodies. Cells were analyzed on a FACSCalibur using CellQuest software.

ELISA for cytokine

IL-12 and IL-10 were measured from macrophage culture supernatant by ELISA using commercially available kits. IL-10, IL-12 were measured in PBMC culture supernatant. In brief, 96 well micro titre plate was coated with 50 or 100 ml of cell free supernatant and incubated over night at 4°C and blocked with 5% BSA for 2 hrs. After washing primary antibody in 1:1000 dilution was added into each well and incubated for overnight. Washing

was done by PBS with Tween 20. Bound cytokines were detected by peroxidase conjugated secondary antibody anti mouse IgG in 1:500 dilution to each well and incubated for 2 hrs. Colour was developed with TMB substrate solution. Reaction was stopped by 1N $H2SO^4$ and absorbance was measured at 450 nm using microplate reader (Tecan Spectra, Grodig, Austria).

RESULTS

Tregs mediate suppression on macrophage cell cytotoxicity to U937 cells

Macrophage cell cytotoxicity was measured towards U937 cells in presence of both autologous and allogenic Treg cells in culture. Cytotoxicity of macrophage was observed significantly less in presence of Treg cells. This extent of suppression is indifferent in case of autologous and allogenic Treg cells (Fig. 1).

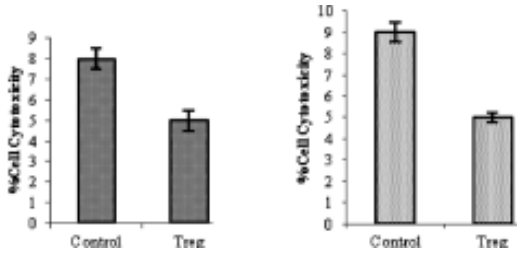

Fig.1. Cytotoxicity of Macrophages against U937: The cytotoxicity of macrophages against U937 cells were tested by LDH release assay. In brief, U937 cells were plated as target and macrophages were added as effector in different E:T ratios and were co-cultured for 4 hours. Cell free supernatant was used to measure the level of released LDH. (A) Autologous, (B) Allogenic Treg cells. Bar diagramme represents the mean value ± SD.

Treg suppress the expression of CD14, CD80 and CD86 macrophages

To study the activity status of macrophages in Treg induced condition, isolated macrophage and purified Treg cells were cocultured. Surface phenotypes for macrophage activity were studied by flow cytometry (Fig 2). CD14, which is expressed on monocytes and macrophages and required for macrophage activation is significantly downregulated in coculture with Tregs. Other two costimulatory molecules, CD80 and CD86, that are expressed on activated macrophages (and B cells) and act as costimulator for T cell stimulation, were also downregulated after Treg induction.

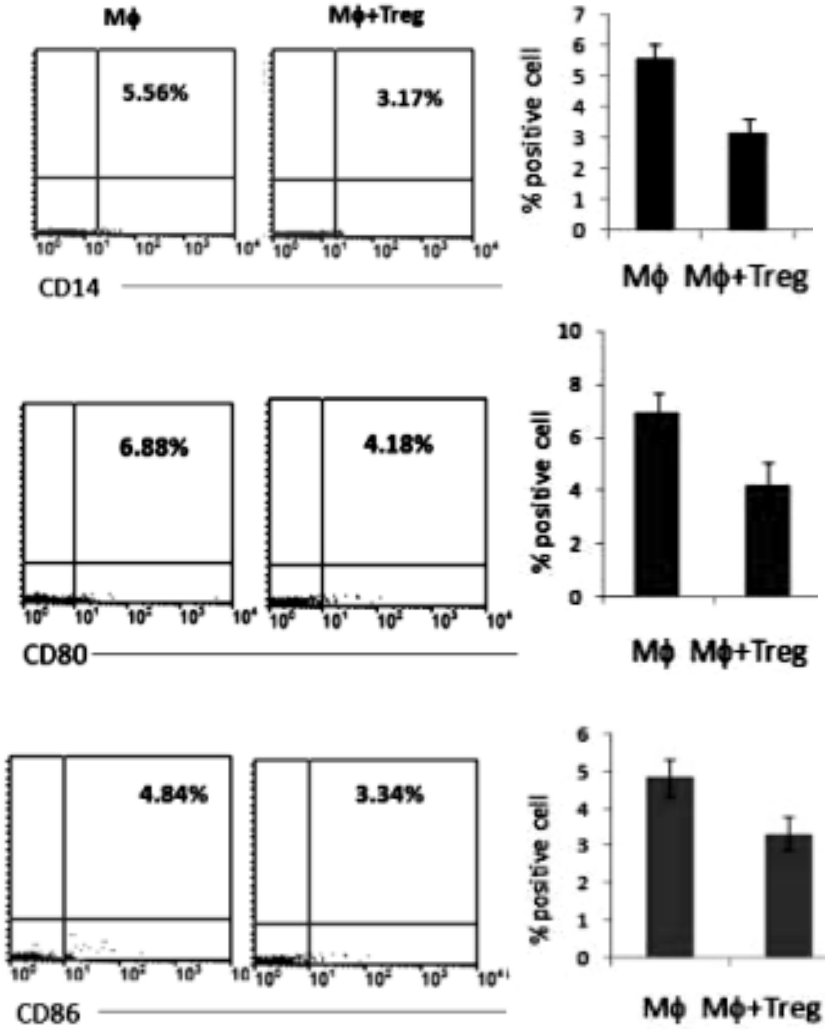

Fig.2. **Tregs downregulate expression of CD14, CD80 and CD86 on macrophages**: Macrophages were cocultured with MACS purified Treg cells for 24 hr and cells were subjected to FACS analysis for (A) CD14, (B) CD80 and (C) CD86. Bar diagram represent the mean value ± SD. *p< 0.01 in comparison to MΦ+Trg.

Tregs disturb type 1/type 2 cytokine balance in macrophages

Macrophages are the chief source of IL-12 and IL-10. Addition of Tregs with macrophages seriously inhibit the release of type 1 cytokine, IL-12, whereas, enhance the secretion of type 2 cytokine, IL-10 (Figure 3).

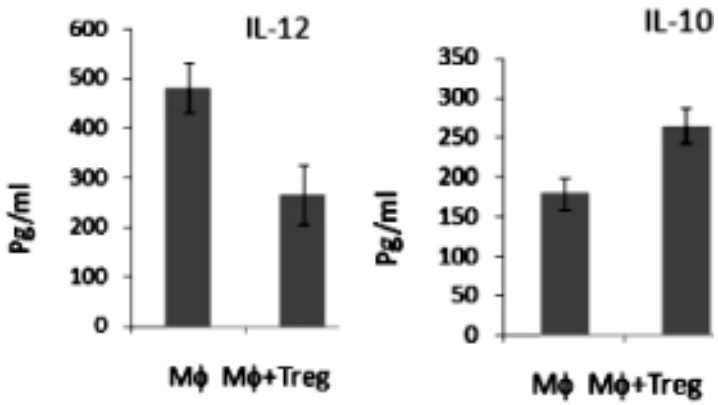

Fig.3. **Tregs disturb type 1/type 2 cytokine balances in macrophages**: Macrophages were cocultured with MACS purified Treg cells for 24 hr and supernatants from each culture were subjected to ELISA to detect the level of extracellular (A) IL-12 and (B) IL-10 secretion. Bar diagram represent the mean value ± SD.*p< 0.01 in comparison to MΦ+Trg.

Tregs downregulates perforin/granzyme B expression in macrophages

The fact, that tumoricidal function of macrophages largely depends on perforin/grazyme B expression in macrophages which prompted us to know the status of cytotoxic molecules, perforin and granzymeB within these macrophages. Tregs significantly downregulate the Perforin/Granzyme B content of macrophages (Fig 4).

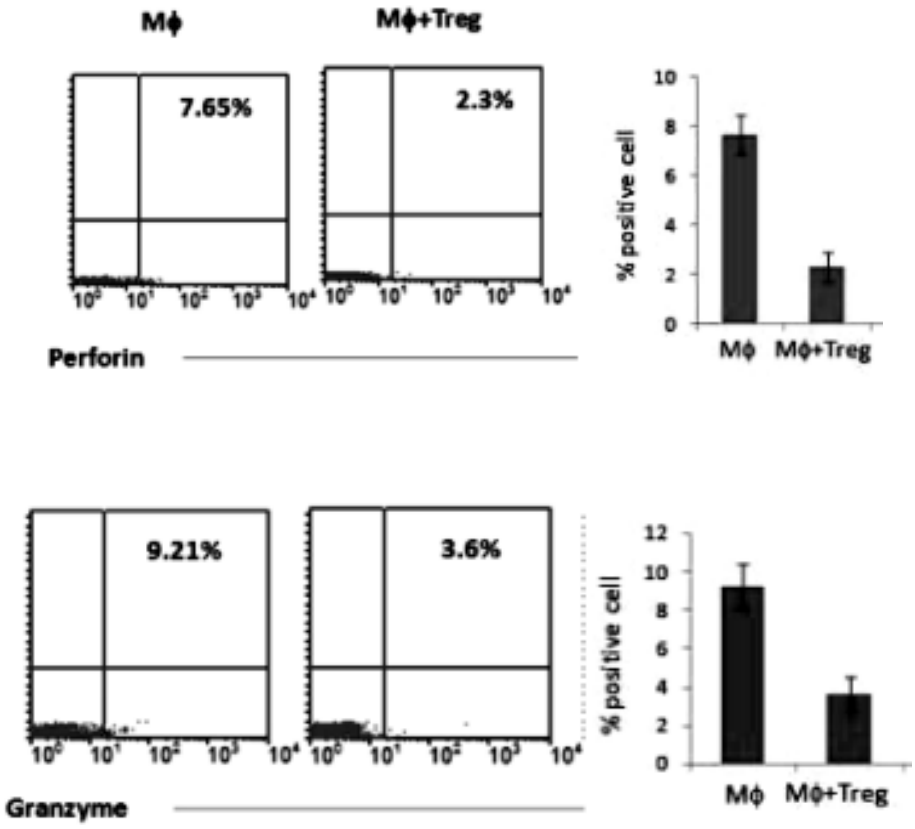

Fig.4. **Tregs downregulate perforin/granzyme B expression in macrophages**: Macrophages were cocultured with MACS purified Treg cells for 24 hr and cells were subjected to FACS analysis for perforin/Granzyme B expression. *p< 0.01 in comparison to MΦ+Treg.

DISCUSSION

Till date, little is known on Treg mediated effects on an antigen presenter as well as tumour killer cells, macrophages. To ask this basic question, we have isolated macrophages Treg cells from human peripheral blood and allowed them to crosstalk with each other. In experiments, we have seen the natural tumour killing efficacy of macrophages was significantly decreased in presence of Tregs to macrophage sensitive lymphoma (U937) cells. This inhibition of cytotoxic activities of macrophages may be due to downregulation of cytotoxic molecules, perforin/granzyme B.

In this cytotoxic machinery, type 1 cytokine skewness played a crucial role. Macrophages are the chief source of IL-12 (Ma X, 2001). Tregs significantly inhibit the release of IL-12 from macrophages and upregulates type 2 cytokine IL-10.

To manifest the above described effects, crosstalk between Tregs and macrophages is crucial. Macrophages talk with Tregs and effector T cells by means of co-stimulatory molecules, CD80/CD86 on macrophages and CTLA4/CD28 present on Tregs/Teffs. Higher CD80/CD86 on macrophages allow them to interact with effector T cells. Tregs significantly downregulate CD80/CD86 on macrophages. With these overall effects, Tregs significantly suppress the tumoricidal functions of macrophages.

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