Comparative Analysis of Induced CD4⁺ Tregs (iTreg) In Response to CD8⁺ CTL Induction From PBL By MHC-I Bound Mart-1 Peptide Pulsed DC or Autologous Tumor Cells with Freshly Isolated TIL from Human Melanoma.

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Abstract

CD4⁺CD25⁺thymus derived Tregs (tTregs) and peripherally induced Tregs (pTreg) are known to control auto-destruction that needs stimulation through major histocompatibility complex-II (MHC-II). For cellular immunotherapy of cancer, tumor associated antigen (TAA, these are also self-antigens) specific *in vivo* induced CD8⁺ Cytotoxic T Lymphocytes (CTL) are rapidly eliminated. Under *ex vivo* condition TAA specific CTL generated with purified CD8⁺ cells continued to grow and remain active for months. *Ex vivo* induced CD8⁺ CTL with un-fractionated Peripheral Blood Lymphocytes (PBL) stimulated with Mart-1₂₇₋₃₅ A2 peptide pulsed autologous dendritic cells (DC), died within three weeks. Whereas Influenza (Flu₅₈₋₆₆) peptide antigen specific CTL induced identically, continued to expand and remain functional beyond four weeks. No significant difference between binding affinity of Mart-1₂₇₋₃₅ peptide and Flu₅₈₋₆₆ peptide to HLA A2 was observed. Upon activation CD8⁺ cells from the cultures expressed MHC-II molecules along with other activation and homing receptors. Expanding CD4⁺ cells were isolated from *In vitro* Co-cultures (IVC) with PBL for Mart-1 peptide and Flu peptide. CD4⁺ cells isolated from Mart-1 IVC secreted significantly higher amount of IL-10 and IL-4 upon re-stimulation with autologous DC and/or anti-CD3 antibody, than CD4⁺ cells isolated from Flu PBL IVC. In a similar fashion, CD4⁺ cells isolated from PBL IVC with autologous Tumor or from freshly obtained tumor tissues were found to secrete significant amount of IL-10, subsequently CD8⁺ CTLs in such cultures did not survive for long. This finding suggests that CD4⁺CD25⁺ iTreg (or pTreg for *in vivo*) cells induced *in vivo* / *in vitro*, negatively regulate effect or immune response via CD8⁺ CTL against tumor by elaborating IL-10. In this process activated CD8⁺ CTL against different antigens might provide differential additional signals via newly expressed MHC-II molecules to the CD4⁺ cells as needed for different antigens.

Keywords: tTreg; pTreg; iTreg; Immune regulation; Tumor immunotherapy

Introduction

Thymus is not full proof eliminator of self- antigen reactive T cells hence spontaneously arising TAA specific CTLs could be detected *in vivo* as well as could be activated for CTL mediated

tumor immunotherapy. Unfortunately most of the time such CTLs are short lived. Sakaguchi [1,2] showed that a set of T cells emerging from the thymus naturally as CD4⁺CD25⁺ regulatory T cells (nTreg; now termed as tTreg) has a critical role in the control of autoimmune pathology. Tregs cells are also generated in the periphery and are referred to as peripherally inducible Treg (pTreg) cells. Both tTreg and pTregs show regulatory roles in the periphery [2, 3]. It is not possible to differentiate between tTreg and pTreg when CD4⁺CD25⁺ T cells are isolated from peripheral blood³. For the sake of argument it could be said that unless there is a need to stop newly induced self-reactive T cells (or CTL) *in vivo*, there is no need for pTreg. These CD4⁺ pTreg might recognize some ligand (epitope) associated with MHC-II molecules for their activation and expansion. In humans also pTregs are induced from CD4⁺CD25⁺ cells in response to activation of self reactive cells⁴. Unfortunately the ligand associated with locally available MHC-II molecules are either not known or too difficult to identify such specific ligand *in vivo* [3]. Previously in *ex vivo* experiments we have demonstrated iTreg mediated down regulation of CTL response in a relevant human tumor system [5]. We compared freshly isolated Treg cells as natural tTregs with *in vitro* induced (CD4⁺CD25⁺FoxP3⁺) iTreg cells. Using Mart-1₂₇₋₃₅ epitope as a prototypic self-but-melanoma-associated epitope, we tested the effect of these two types of Treg cells on the activation and expansion of the epitope specific CD8⁺ CTL in cultures. We found that tTreg cells were not suppressive towards generating Mart-1₂₇₋₃₅ epitope specific CTL when the CTL precursors are stimulated by the relevant peptide-loaded fully activated DC. On the other hand iTregs emerging from CD4⁺CD25⁺ precursors, in the cultures were found to be significantly suppressive [5]. We further found that while the CTL responses in the Mart-1 model are exquisitely sensitive to suppression by the iTreg, CTL responses against a foreign and dangerous antigen such as influenza Flu₅₈₋₆₆ are not. These results indicated that when CTL precursors, with specificity for a self but tumor-associated epitope, are optimally stimulated, tTreg are

not much of an impediment but iTregs are [5]. Growing body of evidence suggests that Treg cells consist of a heterogeneous group of CD4⁺ T cells [6]. Evidence for the negative role of Treg cells on anti-tumor immune response underscores the need for blocking Tregs in tumor immunotherapeutic schema. It is also necessary to find out the possible reason for the induction of pTreg and the epitope (if any) they react to for their expansion to suppress tumor antigen specific CTL. Keeping in mind the difficulties to understand *in vivo* induction of tumor antigen specific pTreg, in this present work we wanted to induce CD8⁺ CTL response against tumor associated but self-antigen

Mart-1₂₇₋₃₅ and Flu derived antigen Flu₅₈₋₆₅ as foreign antigen using un-fractionated PBL in order to explore the functional characteristics of the CD4⁺CD25⁺ Treg cells induced in identical culture conditions with the two different antigens. We also worked with CD4⁺CD25⁺ cells isolated from PBL + autologous Tumor IVC or from number of freshly obtained tumor tissues. In all these three conditions we found remarkable similarity of IL-10 production by induced CD4⁺CD25⁺, a common mechanism of stopping self-antigen or tumor reactive CTL expansion.

Materials And Methods

Study Subjects

Healthy individuals or melanoma patients were included in the study with appropriate approval from the institute's regulatory board. Blood samples from HLA A2 positive donors were taken for the study with informed consent. The study population consisted of subjects diagnosed with primary cutaneous melanoma with or without systemic metastasis. Informed consent was obtained from each participant who volunteered to donate 50 milliliters peripheral blood, when possible surgically removed tumor tissue. The details of the collection of samples and cell preparation have been described [5, 7].

Tissue culture

Tissue cultures were performed in IMDM medium (HyClone Laboratories Inc., USA) supplemented with 10% Fetal Bovine Serum (FBS) (GIBCO Inc., USA), henceforth described as complete medium.

T cell purification

CD4⁺ and CD8⁺T cells were purified using Dynal magnetic beads (Invitrogen, USA), as described earlier [5]. Purity of the isolated cells was verified and > 98% pure cells were used for the study.

DC culture

The method of the generation of myeloid DC from Peripheral Blood Mononuclear Cells (PBMC), used in this study has been described previously [5, 8]. Briefly, monocytes/macrophages were isolated as adherent cells from Ficoll-Hypaque gradient derived PBMC. The adherent cells were cultured in complete medium containing 1000 U/ml of GM-CSF (R & D Systems, Minneapolis, MN) 1000 U/ml of IL-4 (R & D Systems, Minneapolis, MN) for 7 days. The non-adherent and loosely adherent dendritic cells were harvested by vigorous washing.

Expansion of epitope specific T cells in *In vitro* co culture (IVC)

CD8⁺ T (purity > 98%) cells were co cultured with peptide pulsed DC as described earlier [5, 8]. Briefly, human peripheral blood derived CD8⁺ T cells were co-cultured with autologous DCs pulsed with 100µg/ml of either Mart-1₂₇₋₃₅ or Flu MP₅₈₋₆₆ peptide (T cells: DC=10:1). These cultures were maintained in complete medium supplemented with 100U/ml IL-2. Expansion of antigen specific CTL was quantified 7-10 days post co-culture by staining with respective epitope specific tetramers (1st Stimulation). These CTL were further re-stimulated under similar conditions for 3 more times at 7-10 days interval and the expansion of antigen specific CTL were quantified by tetramer staining before each re-stimulation.

PBL IVC

10 x 10⁶ PBMC after Ficoll-Hypaque gradient cut were kept separately in the complete medium without any cytokines or frozen at liquid N₂ for later use. Once DC maturation was done, these PBL were co-cultured with autologous DCs pulsed with 100µg/ml of either Mart-1₂₇₋₃₅ or MP₅₈₋₆₆ peptide (T cells: DC=10:1). These cultures were maintained in complete medium supplemented with 100 U/ml IL-2. Expansion of antigen specific CTL in PBL IVC was quantified 7-10 days post co-culture by staining with respective epitope specific tetramers (1st Stimulation). These PBL IVC were further re-stimulated under similar conditions for 3 more times at 7-10 days interval and the expansion of antigen specific CTL were quantified by tetramer staining before each re-stimulation.

Phenotypic Analysis by flow cytometry

The immune fluorescence procedure for phenotypic and functional analysis by flow cytometry has been described previously [5, 9]. For FACS staining, anti-hIFN γ , anti-hIL-4, anti-hIL-10, anti-hTNF α , anti-hFoxp3, anti-hCD25, anti-hCD62L, anti-h4-1BB, anti-hOX40 and anti-hHLA-DR, DP, DQ antibodies were purchased from BD Biosciences; anti-hIL-2 antibody was procured from Biolegend (San Diego, CA)

Cytokine ELISA

Cytokines IFN γ , TNF- α , IL-2, IL-4 and IL-10 were quantified by ELISA (Duo Set ELISA Development System, R&D System, USA) as per manufacturer's protocol.

Tetramer assay

Analysis for Mart-1₂₇₋₃₅ antigen specific and MP₅₈₋₆₆ antigen specific TCR bearing T cells has been described previously [5, 8, 9]. The cells from IVC were washed twice in PBS and then incubated with 1 µl of MP₅₈₋₆₆ or Mart-1₂₇₋₃₅ conjugated to Allophycocyanin HLA-A2 tetramer (Beckman Coulter Inc., USA), and CD4/CD8 conjugated to FITC or PE (BD Biosciences, USA), at room temperature for 30 minutes. The stained cells were washed twice, and re-suspended in FACS buffer. Thereafter, the number of tetramer positive cells was determined using FACSCalibur (Becton Dickinson, USA) or MACSQuant (Miltenyi Biotech, Bergisch, Gladbach, Germany), and the acquired

cytofluorographic data were analyzed using the FlowJo software (TreeStar, Ashland, OR).

Tumor Infiltrating Lymphocyte (TIL) isolation and culture

This has been described earlier [10-12] briefly tumor tissue surgically removed from a melanoma patient was washed in complete medium and cut into very fine pieces with a sterile scissor and then mechanically disrupted by repeated pipetting. A portion of cell suspension obtained from these tissue samples were characterized at day zero for the baseline. While another portion of the cell suspension was cultured in complete medium with or without IL-2 (100 U/ml) for the TILs to proliferate and melanoma tumor cells to adhere to the base of the culture dish. In the mean time peripheral blood was obtained from the same donor and PBL was isolated as described above. Once the autologous tumor line was ready PBL IVC was setup in presence of autologous tumor and 100 U/ml of IL-2. These cells were allowed to proliferate for 2-3 weeks before isolating CD8⁺ and CD4⁺ cells. These cells were cultured separately for 2-3 days in the presence of 100 U/ml IL-2 before characterization of these cells with or without re-stimulation with anti-CD3 antibody. ELISA was performed with the supernatant as described earlier.

Statistical Analysis

Statistical analysis was performed according to standard methods for *in vitro* experiments. Student's T-test was used to derive the statistical significance (p value 0.05) with figure 6a.

Results

Expansion of CD8⁺ CTL for Mart-1₂₇₋₃₅ and FLU MP₅₈₋₆₆ epitopes with purified CD8⁺ cells and with unfractionated PBL in co-cultures

We tested the proliferation of Flu MP₅₈₋₆₆ and Mart-1₂₇₋₃₅ epitope specific naturally present CD8⁺ T cell precursors in co-cultures with respective peptide pulsed autologous DC. Figure 1a & 1b show an example of tetramer assay used for determining the expansion of MP₅₈₋₆₆ and Mart-1₂₇₋₃₅ epitope specific CD8⁺ T cells, respectively. As shown, specific precursors for both the epitopes exhibited substantial expansion following antigen presentation. MP₅₈₋₆₆ antigen specific CD8⁺ cells always gave better response than Mart-1₂₇₋₃₅ antigen specific CD8⁺. Figure 1c and 1d represent MP₅₈₋₆₆ and Mart-1₂₇₋₃₅ antigen specific intracellular IFN- γ production by *in vitro* expanded CD8⁺ T cells. MP₅₈₋₆₆ and Mart-1₂₇₋₃₅ antigen specific CTL showed specific intracellular IFN- γ production and it was further confirmed by ELISA. IFN- γ response by antigen specific CD8⁺ T cells upon *in vitro* are stimulation shown in (Figure 1e). There were variations in the precursor frequencies for the two epitopes MP₅₈₋₆₆ from 0.05-1.4 and Mart-1₂₇₋₃₅ from 0.01 to 0.50 (data not shown) amongst individual donors, but when compared for fold expansion of the respective epitope specific precursors following stimulation, both MP₅₈₋₆₆ and Mart-1₂₇₋₃₅ epitope specific precursors expanded remarkably well after primary stimulation (Figure 1b). Interestingly, while the fold expansion of Mart-1₂₇₋₃₅ and Flu MP₅₈₋₆₆ epitope specific T cells following 1st stimulation was comparable, the MP₅₈₋₆₆

epitope specific T cells were significantly higher following 2nd stimulation (Figure 1b). After four weeks of initiation of the cultures both Mart-1₂₇₋₃₅ and Flu₅₈₋₆₆ specific CTL were very active and secreted significant amount of IFN- γ upon stimulation with appropriate antigens (Table 1 and 2).

We then used the same *in vitro* Co-culture (IVC) method to expand antigen specific CD8⁺ cells using total PBL (un-fractionated). PBL from donors were stimulated with respective autologous matured DC pulsed with either Mart-1₂₇₋₃₅ peptide or Flu (MP₅₈₋₆₆) peptide. Cultures were kept in IL-2 (100U/ml) medium. Cultures were re-stimulated with DC pulsed with peptide every 7-10 days. Each time before re-stimulation some cells were taken out for tetramer analysis (Table 3 and 4). Table 3 shows that the expansion of Mart-1₂₇₋₃₅ antigen specific CD8⁺ CTL in the co-cultures using PBL from six donors. It is evident from the table that within 21 days Mart-1₂₇₋₃₅ specific CD8⁺ T cells disappeared from the co-cultures. Whereas Table 4 indicates that Flu₅₈₋₆₆ specific CD8⁺ CTL continued to expand and remained functional even after 35 days of culture. With all the donors we noticed Mart-1 specific CD8⁺ T cells in PBL IVC disappeared post third stimulation. These observations led us to three different thoughts; (a) The above phenomena might be due to significant difference between the binding affinity of Mart-1 and Flu peptide with A2 molecules on the DCs used for stimulation of the CD8⁺ cells in PBL IVC, that led to the faster elimination or loss of activity in responding CD8⁺ cells; or (b) Since DCs die after antigen presentation in 24 h the degree of expression of MHC-II molecule by activated T cells could make the difference. (c) There could be a significant difference between the phenotypic and functional properties of the expanding CD4⁺ cells, the induced Treg cells, in the co-cultures.

Difference in binding affinity to A2 for Mart-1 and Flu peptide

We used T2 cells, a gift from Dr. Cresswell (Yale University) [13] to test the binding affinity and stability of the Mart-1₂₇₋₃₅ peptide and Flu (MP₅₈₋₆₆) peptides on A2 molecule. Figure 2a describes the binding abilities of those two peptides to A2 molecules. No significant difference in binding affinity between Mart-1₂₇₋₃₅ peptide and Flu (MP₅₈₋₆₆) peptides were found. There was a possibility that the two peptides in question have different dissociation time but we found no significant difference in dissociation time between Mart-1₂₇₋₃₅ peptide and Flu (MP₅₈₋₆₆) peptides.

MHC-II expression by CD4⁺, CD8⁺ and DC

MHC-II expression on CD4⁺ and CD8⁺ cells were tested before and after stimulation (Figure 2b). Freshly isolated CD4⁺ and CD8⁺ cells were predominantly negative for MHC-II, but a clear spike in MHC-II expression was seen in both activated CD4⁺ and CD8⁺ cells (Figure 2b). Both DC and activated CD8⁺ cells in culture expressed MHC-II but there was a significant difference in MFI of MHC-II expressed by DC than activated CD8⁺ cells (Fig. 2c and 2d).

Characterization of activated CD4⁺ cells induced in the PBL IVCs

Figure 3 shows a representative data with purified CD4⁺ cells

Table 1: Functional characterization of expanded CD8⁺ Mart-1 antigen specific cells after initial stimulation and re-stimulation with appropriate antigen.

Donor	After 1 st week of IVC (IFN γ pg/ml)				After 2 nd week of IVC (IFN γ pg/ml)			
	None	T2	T2+M1	T2+M3	None	T2	T2+M1	T2+M3
1	0.00	20.00	320.00	25.00	0.00	10.00	110.00	30.00
2	0.00	0.00	140.00	10.00	50.00	50.00	210.00	40.00
3	10.00	40.00	200.00	55.00	0.00	50.00	100.00	30.00
4	0.00	40.00	300.00	120.00	0.00	10.00	80.00	0.00
5	50.00	120.00	510.00	125.00	0.00	0.00	110.00	70.00
6	0.00	60.00	220.00	75.00	0.00	0.00	60.00	0.00

Table 2: Functional characterization of expanded CD8⁺ Flu antigen specific cells after initial stimulation and re-stimulation with appropriate antigen.

Donor	After 1 st week of IVC (IFN γ pg/ml)				After 2 nd week of IVC (IFN γ pg/ml)			
	None	T2	T2+Flu	T2+M3	None	T2	T2+Flu	T2+M3
1	0.00	20.00	720.00	125.00	0.00	110.00	650.00	130.00
2	0.00	0.00	440.00	110.00	50.00	50.00	820.00	240.00
3	0.00	140.00	1200.00	255.00	0.00	150.00	900.00	330.00
4	0.00	70.00	300.00	120.00	0.00	110.00	380.00	100.00
5	50.00	220.00	410.00	125.00	0.00	0.00	610.00	190.00
6	0.00	60.00	520.00	185.00	0.00	110.00	1060.00	330.00

Table 3: Percent of CD8⁺ Mart-1 Tetramer positive cells post 1st, 2nd, 3rd and 4th Mart-1₂₇₋₃₅ antigen specific stimulation in Mart-1 PBL IVC.

Donors	7-10 Days of culture (1 st)	14-16 Days of culture (2 nd)	21-24 days of culture (3 rd)	28-30 days of culture (4 th)
1	1.20	3.10	0.02	0.00
2	2.20	0.80	0.00	0.00
3	0.90	1.60	0.00	0.00
4	0.70	4.20	0.10	0.00
5	1.60	2.30	0.00	0.00
6	2.10	1.40	0.00	0.00

Table 4: Percent of CD8⁺ Flu Tetramer positive cells post 1st, 2nd, 3rd and 4th MP₅₈₋₆₆ antigen specific stimulation in Flu PBL IVC.

Donors	7-10 Days of culture 1 st	14-16 Days of culture 2 nd	21-24 days of culture 3 rd	28-30 days of culture 4 th
1	2.40	3.60	3.02	3.40
2	1.70	2.80	2.00	2.00
3	0.90	2.90	1.80	2.50
4	0.70	6.20	5.10	5.30
5	1.90	4.30	2.20	3.10
6	2.20	2.40	3.00	5.60

from the cultures. Significantly higher number of CD4⁺ Foxp3⁺ cells, were detected in PBL IVCs with Mart-1 peptide (Figure 3a). Before the initiation of the IVCs the percentage of CD4⁺ and Foxp3⁺ cells varied from 2.1 to 3.2 in the total PBL (data not shown). When we analyzed for the expression of CD62L, homing receptor and for central memory on these CD4⁺ cells, we found CD62L is much higher in CD4⁺ cells taken from Flu IVCs figure 3b. While comparing the CD4⁺ cells isolated from two different IVCs we found 3 times more of CD4⁺ cells from Mart-1 IVC, expressing OX40. We did not detect any significant difference in expression

of 4-1BB between these CD4⁺ cells taken from two different IVCs (Figure 3b).

CD4⁺ cells from the PBL IVCs with Mart-1 and Flu were purified by positive selection with magnetic beads. These CD4⁺ cells were then re-stimulated with plate bound anti-CD3 or with autologous DC (responder: stimulator = 10:1). Figure 4 describes functional profile of CD4⁺ cells taken from the PBL cultures by intracellular staining. We found significantly higher levels of IL-10 synthesized by CD4⁺ cells taken from Mart-1 IVC

Figure 1: Expansion of Flu MP₅₈₋₆₆ and Mart-1₂₇₋₃₅ antigen specific tetramer positive cells in CD8⁺ IVC. Flu MP₅₈₋₆₆ and Mart-1₂₇₋₃₅ antigen specific tetramer positive CD8⁺ cells at baseline (a) and post first two stimulations with autologous APC in an antigen specific manner (b). Intracellular IFN γ expression by expanded Flu MP₅₈₋₆₆ (c) and Mart-1₂₇₋₃₅ (d) tetramer positive CD8⁺ cells on co-culture with T2A2 cells pulsed with either control peptide (MAGE-3₂₇₁₋₂₇₉) or cognate peptide (Flu MP₅₈₋₆₆ or Mart-1₂₇₋₃₅), respectively. Antigen specific IFN γ release in the supernatant was estimated ~16 hours post co-culture by ELISA (e).

than from Flu IVC. IFN- γ , IL-2 and TNF- α , synthesized by these CD4⁺ cells purified from two different IVCs (Mart-1 and Flu) were comparable (Figure 4).

CD25, FoxP3 and IL-10 expression in CD4⁺ cells derived from TIL and PBL- IVC

We also looked at the regulatory phenotype of CD4⁺ cells from freshly obtained tumor tissues (TILs) and PBL tumor IVC in the

presence of IL-2. We found 35.5 % TIL derived CD4⁺ cells were CD25 positive (Figure 5a upper panel). Around 12.1 % of these cells were FoxP3 positive while 13.2 % of these cells were IL-10 positive (Figure 5a upper panel). In case of PBL + tumor IVC derived CD4⁺ cells about 34.7 % were CD25⁺, 16.8 % were FoxP3 positive and a total of 62.8 % CD4⁺ cells were IL-10 positive (Figure 5a lower panel). Further analysis of PBL + autologous tumor IVC derived CD4⁺CD25⁺ cells revealed that 42.1 % of these

Figure 2: Binding affinity of Mart-1₂₇₋₃₅ and Flu (MP₅₈₋₈₆) peptides with HLA A2 and MHC II expression on activated T cells. MFI of Mart-1₂₇₋₃₅ and Flu (MP₅₈₋₈₆) peptide bound HLA A2 molecule on T2 cells were analyzed at 0-4 hour time point (a). MHC II expression was checked by FACS on freshly isolated and activated lymphocytes peripheral (b). The FSC and SSC of activated CD8 and DC's were different. Hence a graph was plotted to show difference between MHC II expression by activated CD8 and DC's. Ratios between MFI of MHC II stained and unstained sample from three individual was used to plot the graph (c). Histogram shows the MFI shift in activated CD8 cells and DC's after staining them with MHC II antibody (d).

cells were Foxp3⁺ and 80.0 % of these Foxp3⁺ cells were IL-10 positive (Figure 5b). Around 38 % of CD4⁺CD25⁺ cells were both IL-10 and Foxp3 positive. From this study it was clear that a significant number of these CD4⁺CD25⁺ cells stained for IL10 and not all the IL-10 producing CD4⁺CD25⁺ cells from PBL tumor IVC were Foxp3 positive (Figure 5b).

Cytokine ELISA with CD4⁺ cells from PBL tumor IVC on re-stimulation

PBL tumor IVC derived purified CD4⁺ cells did not secrete either IL-2 or IL-4 when stimulated with anti CD3 antibody. But these cells did secrete low levels of IFN γ and TNF α (Figure 6a). These CD4⁺ cells upon stimulation with anti CD3, secreted very high levels of IL-10. We also found PBL tumor IVC derived purified CD8⁺ cells secreted insignificant amount of IL-4 and no IL-10 when stimulated with anti CD3 antibody, but were also found to secrete IL-2, IFN γ and TNF α when re-stimulated with anti CD3 antibody (Figure 6a). We then looked at the cytokine secretion profiles of CD8⁺ and CD4⁺ cells taken out from mixed

culture and again mixed together in different CD8:CD4 ratios along with anti CD3 antibody. As we increased the number of CD4⁺ cells in the cultures of fixed number of CD8⁺ cells stimulated with anti-CD3 we did not detect any significant increase in IFN γ or TNF α secretion by CD8⁺ cells by intracellular cytokine staining indicating no further help to those CD8⁺ cells. On the contrary at 1:1 ratio (CD8:CD4) the CD4⁺ cells got additional stimulation and significantly increased the level of IL-10 in the cultures (Figure 6a). A dose dependent response of IL-10 secretion by CD4⁺ cells was observed when CD4⁺ cells were mixed in the cultures of CD8⁺ cells with anti CD3 antibody stimulation (Figure 6a). In the cultures where equal numbers of CD8⁺ and CD4⁺ cells were present, CD4⁺ cells secreted significantly higher levels of IL-10 (difference mean ~ 200 pg/ml) which is significant ($p < 0.05$) than that of those secreted by same number of CD4⁺ cells alone upon stimulation with anti CD3 antibody (Figure 6a). These CD4⁺ cells possibly got additional stimulation provided by CD8⁺ cells that helped resulting in increased secretion of IL-10.

Figure 3: Phenotypic characterization of CD4⁺ cells isolated from Flu MP₅₈₋₆₆ and Mart-1₂₇₋₃₅ PBL IVC. CD4⁺ cells were isolated from Flu MP₅₈₋₆₆ and Mart-1₂₇₋₃₅ antigen specific PBL IVCs after 4th stimulation. Phenotypic analysis of the purified CD4⁺ cells from PBL IVC were performed by staining these cells with CD4⁺, CD25 and Foxp3 (a) and CD62L, OX40 and 4-1-BB (b).

Cytokine profile of CD4⁺ cells in freshly isolated TILs

We analyzed lymphocytes from freshly isolated from melanoma tumor tissue before putting them in culture. We observed that significant number of CD4⁺ cells were positive for IL-10. So we asked, if CD4⁺ cells at the tumor site in the presence of very high number of autologous tumor load give a similar response in terms of IL-10 production (Figure 6b). We gated the tumor tissue single cell suspension first for lymphocytes and then sub gated them for CD4⁺ cells. Analysis of CD4⁺ cells showed that they were negative for IFN γ , TNF α , IL-2 and IL-4 cytokines (Figure 6b). But 29.2 % of these CD4⁺ cells were positive for intracellular IL-10 (Figure 6b).

Discussion

CD4⁺CD25⁺ and Foxp3⁺(or FoxP3 negative) regulatory T cells (Tregs) play a critical role in all aspects of immune responses and the control of immune homeostasis [6]. It has become clear that the major population of Foxp3⁺ Treg is generated in the thymus, previously known as nTreg now it is called tTreg [3, 14]. It is also clear that certain percentage of Tregs

Figure 4: Functional characterization of CD4⁺ cells isolated from Mart-1₂₇₋₃₅ and Flu MP₅₈₋₆₆ PBL IVC. CD4⁺ were isolated from Mart-1₂₇₋₃₅ and Flu MP₅₈₋₆₆ antigen specific PBL IVC after 4th. These CD4⁺ cells were left un-stimulated, stimulated with autologous APC and stimulated with anti-CD3 antibody, respectively. Intracellular level of cytokines IFN γ , TNF α , IL-2, IL-4 and IL-10 were tested by FACS.

Figure 5: Comparative phenotypic analysis of CD4⁺ cells isolated from TIL and PBL Tumor IVC. Autologous tumor cell line was generated from surgically removed tumor tissue from a melanoma patient. TILs obtained from the single cell suspension from the tissues were tested. PBL tumor IVC was setup with autologous tumor line in the presence of 100 U/ml IL-2, and allowed to proliferate for 2-3 weeks before we used them. We looked at the expressions of CD25, Foxp3 and IL-10 on TIL and PBL tumor IVC derived CD4⁺ cells (a). We further gated CD4⁺CD25⁺ cells from PBL tumor IVC and reanalyzed the data for Foxp3 and IL-10 expression (b).

Figure 6: Characterization of CD4⁺ cells isolated from PBL IVC or TILs. IVCs with PBL and autologous tumor were setup in the presence of 100 U/ml IL-2. Two weeks after culture, CD8⁺ T cells (> 99 % purity) were separated from the culture using Dynal beads while remaining cells were CD4⁺ (>93% purity). Functional characterization of these CD8⁺ cells (2×10^5 cells/well) or CD4⁺ cells (2×10^5 cells/well) or CD8⁺ and CD4⁺ cells mixed together (CD8:CD4 ratio of 10:1, 5:1, 2:1 and 1:1) was performed with or without stimulation with anti CD3 antibody (1 μ g/well). Culture supernatants were collected and analyzed for cytokines by ELISA (a). Surgically removed melanoma tissue sample was used to generate single cell suspension of Infiltrating Lymphocytes (TIL). Intracellular cytokine (IL-10, IL-4, IL-2, IFN γ and TNF α) staining was done immediately after isolation of TIL from tumor tissue and analyzed by FACS. The samples were first gated for lymphocytes and then sub gated for CD4⁺ cells (b).

is generated from T-conventional (Tconv) cells in peripheral sites. It has been proposed that such cell populations be termed peripherally derived Tregs (pTregs) [15]. Specificity of tTregs is not established. Although there could be a specific reason for induction of pTregs but how these cells are induced in the periphery, is also not known [14]. It remains unclear whether *in vitro* induced iTregs and peripherally induced pTregs are similar and that might use the same mechanism for suppression [3, 6] of effector immune response. In this article we tried to address the important issues regarding the induction and functions of these pTreg and/or iTreg subpopulations in an *in vitro* set up. A detailed characterization of these subpopulations has important clinical implications in cellular immunotherapy for cancer [3, 6, 14]. Use of tumor associated antigen specific CD8⁺ CTL have shown considerable promise in translational tumor immunotherapy [16]. Variety of intrinsic regulatory processes within T cells as well as a number of microenvironment-based extrinsic mechanisms have turned out to be serious impediments in achieving robust and sustained outcomes of CTL mediated immunotherapy for cancer [16]. A great deal of effort is underway to understand to overcome these impediments. Polyclonal tTreg cells do not always influence the immune response by mediating immune suppression or completely shutting down T-cell activation. Thus, polyclonal tTreg cells would not necessarily lead to global immune suppression or the inhibition of effector responses to all antigens [3]. Polyclonal tTregs might specifically target trafficking pathways and not allowing immunity to develop in lymphoid organs. By limiting the number of potentially auto-reactive T cells that are allowed to migrate and enter tissues. That could be the reason for the existence of tumor specific CD8⁺ CTL in tumor tissues. In such conditions a specific Treg cells might be induced from conventional CD4⁺CD25⁻ T cells to work against those auto-reactive T cells that are generated in the periphery [4, 5, 17-20].

We have made an attempt to understand the functional properties of the CD4⁺ cells isolated from the co-cultures with unfractionated PBL. IL-10 secreted by these cells could dampen the antigen presenting ability of DC as it is already known that iTreg cells suppress DC function and subsequently do suppress T effector function via IL-10 [5, 12, 21].

Naive and activated T cells are known to express different adhesion molecules and are thought to exhibit different migratory patterns that result from their expression of discrete adhesion molecules. Two known adhesion molecules that have been associated with differentiating the naive and activated/memory T cells are CD62L (L-selectin) and CD44 (H-CAM) [22, 23]. In our *in vitro* CD8⁺ CTL generation experiments against Mart-1 and Flu we found a marked difference in lymphoid homing receptor expression. It has been demonstrated previously that naive T cells express a CD62L^{hi}CD44^{lo} phenotype, whereas memory T cells exhibit a CD62L^{lo}CD44^{hi} phenotype [22, 23]. In this regards increased number of CD62L expressing CD4⁺ cells from Flu IVC, could be indicative of memory cells against Flu matrix protein derived antigens, as most of us are exposed to Flu antigen at least once in life time where as Mart-1 being a self antigen no such thing as memory cells were seen.

Analysis of regulatory phenotype of lymphocytes from tumor tissue (TILs) or PBL IVC derived CD4⁺ cells revealed that, a substantial population of these cells were CD25⁺. The number of CD4⁺ cells expressing CD25 or Foxp3 from those two cultures (TIL or PBL + Tumor IVC) were comparable (Figure 5a). This can be explained by the fact that in both conditions (TIL or PBL+Tumor IVC) CD4⁺ cells have already seen the autologous tumor antigen. So in either case the CD4⁺ T cells have seen the tumor antigen and behaved as T regulatory cells to prevent tumor (auto) destruction. The expression of Foxp3 has been shown in both tumor-infiltrating Tregs and melanoma cells by immune histochemical analysis of human melanoma tissue sections [24]. In humans CD4⁺CD25⁻ effector T cells can also up regulate Foxp3 expression transiently [4, 20].

Although this work was done with a small group of donors, a difference in pathways for the induction of CD4⁺pTreg (or iTreg) phenotype (difference in number or function) are clearly seen when antigen specific CTLs (with Mart-1₂₇₋₃₅ and Flu₅₈₋₆₆ peptides) were induced. In this study CTL were generated using identically matured autologous DC. When Flu specific CTLs were induced in long term cultures with total PBL, CD8⁺CTL remained active for significantly longer period. CD4⁺ cells in cultures were found to secrete higher amounts of helper cytokine IFN γ than IL-10. Since all other conditions remained same, probable indication goes towards the difference in type of antigens. Previously we have shown that CD4⁺ clones, derived from melanoma involved lymph nodes or PBL stimulated by autologous melanoma cells selectively down regulated the induction of cytotoxic immune response against the autologous melanoma cells [7]. We have also shown that repetitive *in vitro* stimulation with antigen loaded APC could lead to the emergence of non cytolytic CD4⁺ T cells secreting suppressor factor in culture supernatant. This inhibitory effect of supernatant factor could be abrogated by neutralizing IL-10 with anti IL-10 antibody [12].

Expression of MHC II molecules by activated T cells from humans is regulated by CIITA-PIII subtype of the class II trans activator (CIITA) [25]. Direct *ex vivo* expression of MHC-II in the context of CD [25] high identifies a mature, functionally distinct regulatory T cell population involved in contact dependent *in vitro* suppression [26]. So circulating human peripheral blood derived T cells can express MHC class II [27, 28], but mice T cells cannot transcribe CIITA required for MHC II expression [29, 30]. It has been shown earlier that MHC II DR on human T cells, primarily being regarded as a marker for activation [30 – 32]. The expression of class II on CD4⁺ T cells is inducible and Class II⁺ CD4⁺ T cells can present antigen in the absence of APC [33]. It has been reported that suppression of TCR transgenic CD8⁺ T cells stimulated with soluble peptide was mediated via a T-T interaction with activated CD4⁺CD25⁺ T cells [34]. Antibody blocking of MHC II on human activated regulatory T cells abrogated their suppressive potential [35]. In our study we also found that naive CD8⁺ cells do not express MHC-II molecules. After stimulation with DC pulsed with HLA A2 binding peptides or tumor cells expression of MHC-II molecule (newly synthesized) was seen. MHC-II expressed by activated CD8⁺ cells were not simply shaded off MHC-II molecules from the DCs in

the cultures (Figure 2b). Purified CD8⁺ CTL from the cultures maintained their expression of MHC-II molecules as long as they survived (4-8 weeks) in cultures. Newly expressed MHC II on CD8⁺ activated cells and the additional stimulatory effect of such cells on expanding CD4⁺ cells (Figure 6a) indicated that activated CD8⁺ CTL could also provide important signal for the outcome of the immune reaction (rejection or tolerance). The IL-10 secreted by CD4⁺ cells was able to suppress CD8⁺ CTL expansion in IVC with PBL plus autologous tumor. It has been reported earlier that iTregs could regulate CTL response through IL-10 *in vitro* and also *in vivo* [3, 5, 12, 21, 26]. Further detailed work is needed to understand the mechanism behind this differential activation of induced Treg (or pTregs) cells and the role of MHC-II⁺ CD8⁺ CTLs to that.

Tumor associated peptide antigen pulsed DC or whole tumor cells when presented to autologous lymphocytes in the *in vitro* cultures, induced a class of CD4⁺CD25⁺ cells that secreted significant amount of IL-10 and controlled the expansion of CD8⁺ CTLs. A similar picture was seen *in vivo*, where we analyzed freshly isolated lymphocytes from the surgically removed tumor tissues. Whether peptide antigens or tumor cells when used to stimulate autologous lymphocytes in cultures a clonal (oligo or polyclonal) population of CD4⁺ were induced that secreted significant amount of IL-10 and controlled CTL expansion. An identical *in vivo* picture was seen when we analyzed freshly isolated lymphocytes from surgically removed tumor tissues. Hence the overall negative regulation is maintained by induced CD4⁺CD25⁺ cells (possibly) pTregs via IL-10, and MHC-II⁺ CD8⁺ activated T cells could also provide signals through newly synthesized MHC molecules in the *in vivo* micro environment and *in vitro* cultures.

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