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PRINCIPAL INVESTIGATOR: Dr. Gianpietro Dotti

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, TX 77030

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Dr. Gianpietro Dotti

E-Mail: gdotti@bcm.edu

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14. ABSTRACT

Neuroblastoma (NB) is the most common malignant extracranial tumor of childhood. Since NB appears susceptible to immunotherapies that include monoclonal antibodies and T-cell immune responses elicited by tumor vaccine, we have combined the beneficial effects of both humoral and cell-mediated components of the anti tumor response. We demonstrated indeed that adoptive transfer of Epstein-Barr-virus (EBV)-specific cytotoxic T lymphocytes (EBV-CTLs) genetically modified to express a chimeric antigen receptor (CAR-GD2) targeting the GD2 antigen expressed by neuroblasts persist in the peripheral blood and induce objective tumor responses (including complete remissions). We will now augment the expansion and survival of CAR-GD2 modified EBV-CTLs by coexpressing the IL-7Rα that restores their capacity to respond to homeostatic IL-7. We will also enhance the capacity of these cells to invade solid tumor masses by expressing heparanase (HPSE) that disrupts the non-cellular stromal elements of NB. Experiments will be conducted *in vitro* and *in vivo* in a xenograft mouse model.

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Introduction. In our recent Phase I study we found that the adoptive transfer of Epstein-Barr-virus (EBV)-specific cytotoxic T lymphocytes (EBV-CTLs) genetically modified to express a chimeric antigen receptor (CAR-GD2) targeting the GD2 antigen expressed by neuroblasts, can persist in the peripheral blood for 6 weeks and induce objective tumor responses (including complete remission) or tumor necrosis in 4/8 subjects with refractory/relapsed NB[1]. Although encouraging, this study also revealed that the signal from the transgenic CTLs progressively declined over time in the majority of patients[1] suggesting that the anti tumor effects of these cells could be augmented by prolonging the survival and effector function of the transgenic CTLs, for example by restoring their responsiveness to homeostatic cytokines such as IL-7[2] and inducing a robust $CD8^+$ T cell memory response[3]. Our second approach aims to disrupt the non-cellular stromal elements of NB that may impede access to CAR-modified EBV-CTLs. The ability of tumor-specific CTLs to cross tumor blood vessels is crucial for reaching the tumor cells. Leukocyte extravasation is highly dependent upon the degradation of the components of the subendothelial basement membrane (SBM) and the extracellular matrix (ECM) such as heparan sulfate proteoglycans (HSPGs), fibronectin and collagen[4]. Heparanase (HPSE) is the only known mammalian endoglycosidase degrading HSPGs at distinct HS intra-chain sites[4;5]. Although HPSE is expressed in activated $CD4^+$ lymphocytes, neutrophils, monocytes and B lymphocytes[4;6;7] we have found it to be deficient in cultured T cells and EBV-CTLs.

Key research accomplishments/report outcomes

In Task 1 we proposed to co-express CAR- GD2 and IL-7Rα in EBV-CTLs to improve their expansion and anti tumor effects in response to IL-7, whilst avoiding the expansion of regulatory T cells (Treg) (time frame months 1-24). Because Tregs represent a scanty subset of the $CD4^+$ T lymphocytes, we first optimized a procedure to obtain and expand Tregs *ex vivo* to perform the experiments proposed in Task 1. We have selected naturally occurring Tregs (nTregs) from the peripheral blood of 6 healthy donors based on their $CD25^{bright}$ and CD4 expression using immuno-magnetic selection. Isolated Tregs were then expanded (rTregs) in culture for 3 weeks with OKT3/feeders, IL-2 (50UI/ml) and Rapamycin (1mg/ml), to increase the purity of the expanded population. The suppressive function of these expanded Tregs (rTregs) was confirmed in a CFSE-based suppression assay. PBMCs were stained with CFSE and activated with OKT3 and feeders and rTregs in the absence of any cytokines. After 5-7 days of culture, cells were analyzed by FACS and the dilution of the dye used to measure the divisions of activated PBMCs and thus proliferation. rTregs retained their inhibitory function *in vitro* (***Fig. 1A and B***).

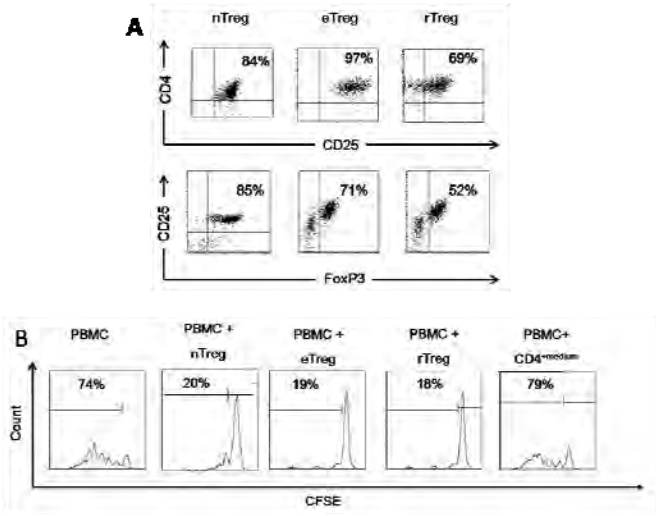

Figure 1. Functional characterization of freshly isolated and ex vivo expanded Tregs*. (A) Flow cytometry dot plots of nTregs, eTregs (Tregs generated from $CD4^+CD25^-$ cells) and rTregs from 1 representative donor, stained with anti-CD4, anti-CD25 and anti-FoxP3 fluorochrome-conjugated mAbs. (B) The suppressive activity of nTregs, eTregs, rTregs, and control $CD4^{+medium}$ T cells was evaluated using a CFSE-based assay in which PBMCs labeled with CFSE were stimulated with irradiated allogeneic feeders and OKT3 in the presence or in the absence of Tregs. nTregs, eTregs and rTregs retained inhbitory activity.*

Although the isolation and expansion of functional inhibitory Tregs seem feasible, we have to take into account that these cells need appropriate tissue trafficking to perform the proposed *in vivo* experiments. We have then elucidated the chemokine receptor profile of nTregs and compared it to *ex vivo* expanded rTregs to assess if and how culture conditions may affect this expression pattern, as modifications in Treg migration properties would have important implications. Our data show that *ex vivo* expanded Tregs express CCR7, suggesting that they should migrate to lymph nodes. By contrast, the expression of CCR4 and CCR5 receptors is suppressed as compared toTregs. (***Fig. 2***).

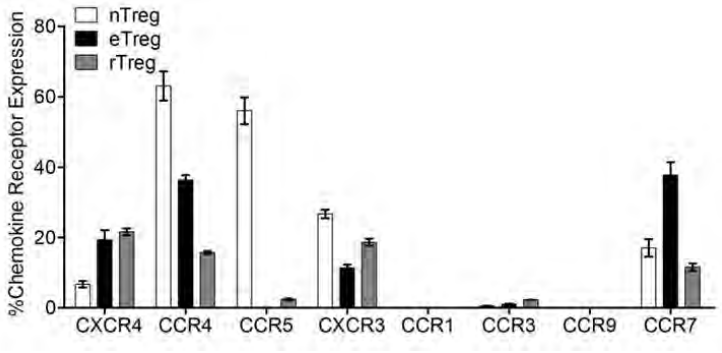

Figure 2. Chemokine receptors expression profile by differentially expanded Tregs. Analysis of chemokine receptors in freshly isolated nTregs and ex vivo expanded eTregs and rTregs. Shown are nTregs isolated from PBMCs of healthy donors, eTregs and rTregs, stained with anti-CXCR4, CCR4, CCR5, CXCR3, CCR1, CCR3, CCR9 and CCR7 fluorochrome-conjugated mAb. Bars (white for nTregs; black for eTregs and gray for rTregs) represent mean ± SD of 5 experiments.

The differential chemokine receptor profile in expanded Tregs correlated with their impaired capacity to migrate towards the specific chemokine TARC (CCL17), RANTES (CCL5) and MIP-1α (CCL3), suggesting that these cells may have tissue specific migration (***Fig. 3***) (manuscript in preparation).

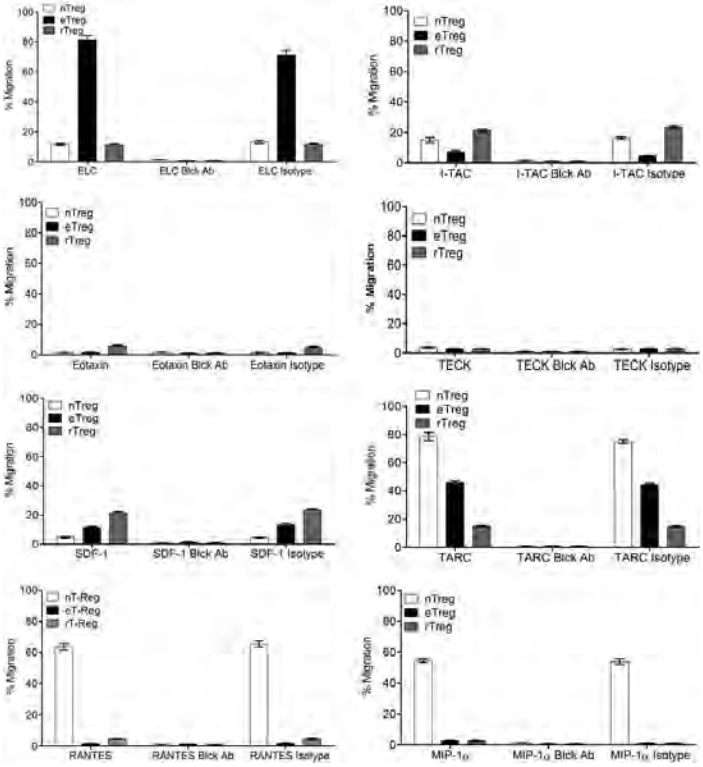

Figure 3. Differential migration capacity of ex vivo expanded Tregs. *The migration of nTregs (white bars), eTregs (black bars), and rTregs (gray bars) towards (A) ELC (CCL19), (B) Eotaxin (CCL11), (C) TECK (CCL25), (D) I-TAC (CXCL11), (E) SDF-1 (CXCL2), (F) TARC (CCL17), (G) RANTES (CCL5) and (H) MIP-1α (CCL3) was evaluated using a transwell migration assay. Specificity of the migration assay was confirmed using blocking antibodies and isotype controls. The data are represented as mean ±SD for Tregs isolated or expanded from 5 healthy donors. Inset shows the receptor-ligand specificity.*

We have then used the expanded rTregs to evaluate whether IL-2 and IL-7 can sustain the proliferation of EBV-CTLs genetically modified to express IL-7Rα$^+$ without providing a survival advantage to Tregs. Therefore, we labeled EBV-CTLs with CFSE and cultured them with autologous LCL (at a CTL:LCL ratio of 2:1) and rTregs (at a CTLs:rTregs ratio of 1:1) in the presence of IL-2 (50U/ml), since this cytokine is conventionally used to sustain CTLs function and proliferation. CTL proliferation was evaluated by day 7 by FACS analysis. As shown in **Fig. 4**, the proliferation of CTLs in response to LCL, in the presence of IL-2 (77%±19%) was significantly inhibited in the presence of rTregs (39%±22%; p<0.05). This confirms the overall negative effects of Tregs in the presence of IL-2. Indeed, IL-2 is known to sustain Tregs expansion and function .

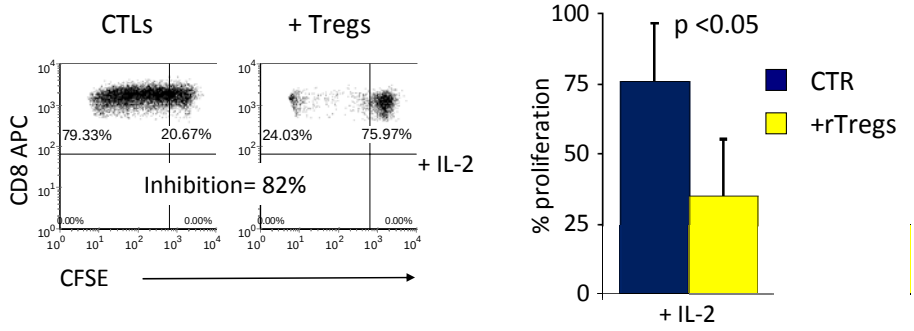

Figure 4. Inhibitory effects of Tregs and IL-2 on EBV-CTLs proliferation. *Tregs were tested for their ability to inhibit CTL proliferation in response to autologous LCL and IL-2 using a CFSE-based assay. The plots show the proliferation of EBV-CTLs in the presence of IL-2 only (left plot) or in the presence of IL-2 and Tregs (right plots) in 1 representative donor. The graph summarizes (mean ± SD) the percentage of proliferating CTL in 6 donors. The blue bar indicates CTLs proliferation in the presence of LCL and IL-2 while the yellow bar represents CTLs proliferation in the presence of LCL, IL-2 and rTregs.*

We next assessed if this inhibition mediated by rTregs occurred also for EBV-CTLs genetically modified to express the IL-7Rα to make them responsive to IL-7. Therefore, we labeled IL-7Rα$^+$ EBV-CTLs with CFSE, cultured them with autologous LCL (2:1 ratio) and Tregs (ratio 1:1) in the presence of IL-2 (50U/ml) or IL-7

(5ng/ml) and assessed their proliferation by day 7 by FACS analysis. Similarly to control (NT) EBV-CTLs, the proliferation of IL-7Rα$^+$ EBV-CTLs in response to autologous LCL and IL-2 (71±14%) was significantly reduced in the presence of rTregs (31x±6%, p<0.05) (**Fig. 5**). When IL-7 was added to the culture, the proliferation of IL-7Rα$^+$ EBV-CTLs was similar to that of IL-7Rα$^+$ EBV-CTLs cultured in the presence of IL-2 (74±16%). However, in the presence of IL-7, the proliferation of IL-7Rα$^+$ EBV-CTLs was significantly retained even in the presence of Tregs (58±10%; p< 0.05) (**Fig. 5**).

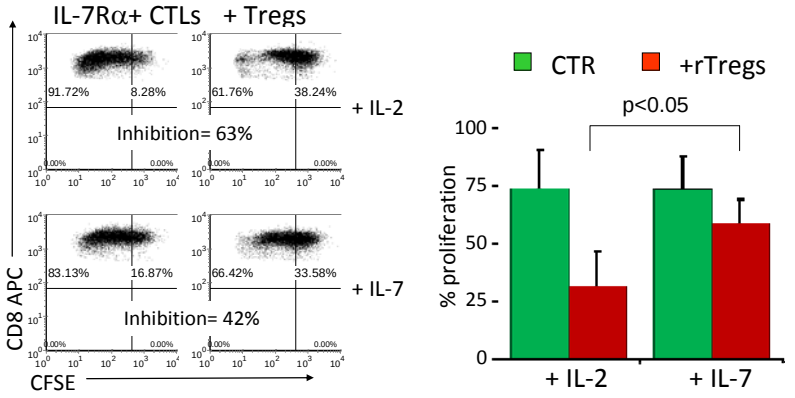

Figure 5. The inhibitory effects of Tregs on the proliferation of IL-7Rα$^+$ EBV-CTLs are mitigated in the presence of IL-7. *Tregs were tested for their ability to inhibit IL-7Rα$^+$ EBV-CTLs proliferation in response to autologous LCL and IL-2 or IL-7, using the CFSE-based assay. The upper plots show the proliferation of IL-7Rα$^+$ EBV-CTLs in response to autologous LCL, in the presence of IL-2 only (left plot) or in the presence of IL-2 and Tregs (right plots) in 1 representative donor. The graph summarizes (mean ± SD) the percentage of proliferating CTLs in 6 donors. The green bars indicate CTLs proliferation in the presence of autologous LCL and the indicated cytokine. The red bars indicate CTL proliferation in the presence of autologous LCL and the indicated cytokine and rTregs.*

We will continue the experiments combining the expression of IL-7Rα with the expression of the CAR specific for the GD2 antigen.

Task 2. To evaluate the contribution of IL-7R α ligation and co-stimulation from viral-infected target cells on th e development of lo ng-lived memory CAR-GD2-mofied EBV-CTLs in a hu manized SCID mouse model previousl y engrafted w ith human hematopoietic stem cells (time frame months 12-48). We are currently engrafting the first set of mice using human derived CD34$^+$ cells.

Task 3: To co-express CAR-GD2 and HPSE in EBV-CTLs and determine the consequent modulation of NB tissue infiltration and killing (time frame 1-48). Leukocyte extravasation in response to inflammation has been extensively studied. It is highly dependent upon the degradation of the components of the subendothelial basement membrane (SBM) and the extracellular matrix (ECM) such as heparan sulfate proteoglycans (HSPGs), fibronectin and collagen. Heparanase (HPSE) is the only known mammalian endoglycosidase degrading HSPGs at distinct HS intra-chain sites. To investigate the regulation of HPSE expression in human T lymphocytes, we evaluated the expression of HPSE protein in freshly isolated PBMCs and selected CD4$^+$ and CD8$^+$ lymphocytes. As shown in **Fig. 6**, HPSE was rapidly upregulated in human CD4$^+$, but not CD8$^+$ lymphocytes after activation with OKT3/CD28 mAbs. These data parallel previous observations in rat lymphocytes.

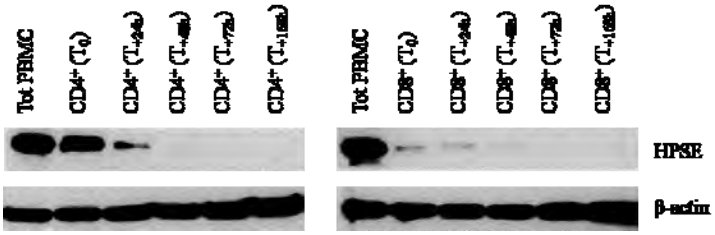

Figure 6. Expression of human heparanase. *Western blot analysis on PBMC, CD4$^+$ and CD8$^+$ sorted PMBC and CD4$^+$ and CD8$^+$ OKT3/CD28 blasts.*

To evaluate whether the ectopic expression of HPSE in T cells enhances their capacity to invade the Matrigel layer, we cloned the full length human HPSE (NM-006665) into the SFG retroviral vector. The SFG vector also contains an IRES element to express the selectable marker eGFP [SFG.HPSE(I)GFP]. Activated T lymphocytes were then transduced with this vector and their Matrigel invasion capacity was evaluated. **Fig. 7** shows that SFG.HPSE(I)GFP$^+$ T cells have consistently enhanced invasion capacity as compared to control cells.

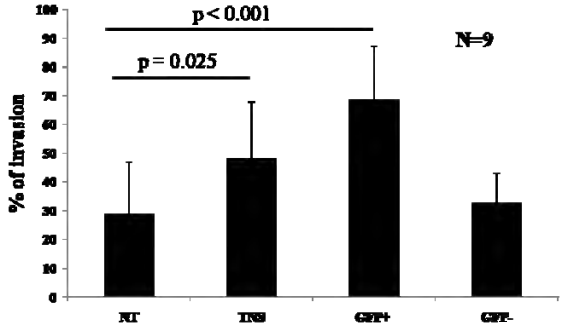

Figure 7. Invasion properties of T cells transduced with the SFG.HPSE)I)eGFP vector. *The invasion capacity of OKT3/CD28 blasts was assessed by using a BD BioCoat Matrigel™ invasion chamber (2.5*10^5/well). Ten percent FBS medium using was used as chemoattractant. Data summarize 9 experiments.*

We then generated a bicistronic vector encoding both CAR-GD2 and HPSE and evaluated the function of both genes in activated T lymphocytes. As shown in **Fig. 8**, T cells expressing both HSPE and the CAR-specific for GD2 have improved invasion capacity as compared to control NT cells or T cells expressing the CAR alone (left panels), and retained cytotoxic activity against LAN-1 neuroblastoma cells GD2$^+$.

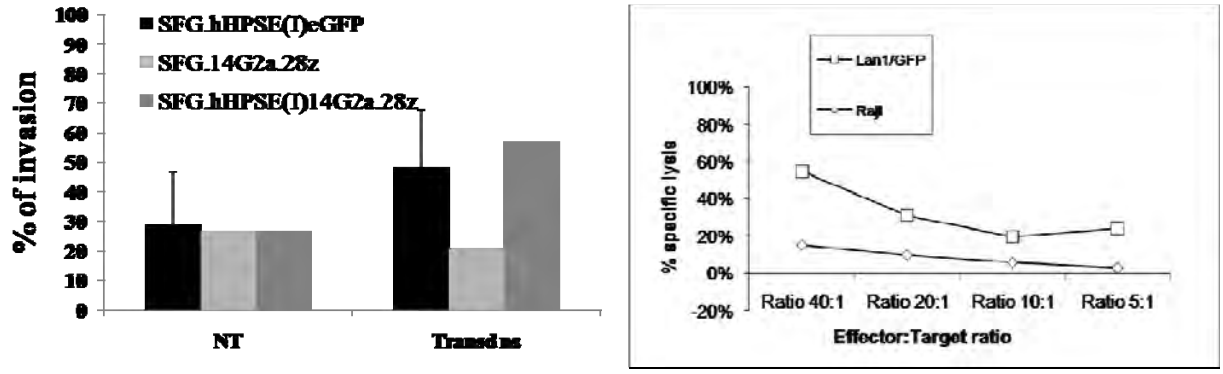

Figure 8. Invasion and cytotoxic activity of T cells co-expressing HPSE and CAR GD2-specific*. Left panel. Evaluation the invasion capacity of OKT3/CD28 blasts through BD BioCoat Matrigel™ invasion chamber (2.5*10^5/well); 10% FBS medium using as chemoattractant. Right panel shows cytotoxic activity of T cells co-expressing both HPSE and CAR GD2-specific.*

Conclusions. We have generated functional Tregs and found that EBV-CTLs in which IL-7 responsiveness has been restored are resistant to the inhibitory effects of Tregs. We have also found that overexpression of HPSE enhances the invasion of T cells. In addition HPSE can be coexpressed with a GD2-specific CAR that confers antigen specific cytotoxic function to T cells. Based on these data we will continue the project as described in our original specific aims.

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