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Antigen-specific recognition is critical for the function of regulatory $CD8^+CD28^-$ T cells

P. Renner, F.C. Popp, E. Eggenhofer, P. Slowik, P. Piso, E.K. Geissler, H.J. Schlitt, M.H. Dahlke *

Department of Surgery, University of Regensburg, Regensburg, Germany

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ABSTRACT

The immunomodulatory properties of CD8 T cells with regulatory phenotype have become evident. It remains unclear whether the immunomodulatory function of $CD8^+CD28^-$ T cells requires antigen-specific TCR interaction with major histocompatibility complex class I (MHC I). We have isolated naïve $CD8^+CD28^-$ T suppressor cells (Tsup) from H2-Kk Des-TCR mice that express a transgenic, MHC class I-restricted, clonotypic TCR against an allogeneic MHC class I molecule (H2-Kb) plus self-peptide. These cells were compared to B10.BR wild type (w/t) $CD8^+CD28^-$ T cells and to naïve $CD4^+CD25^+$ regulatory T cells (Treg) of the same strains. Des CD8 effector T cells proliferated more readily when stimulated by H2-Kb splenocytes than w/t controls, whereas Des CD4 T cells showed the same alloresponse as w/t cells. Activation and proliferation of B10.BR CD4 T cells stimulated by H2-Kb APC were suppressed more effectively by Des $CD8^+CD28^-$ T cells than by w/t $CD8^+CD28^-$ T cells. On the contrary, Des $CD4^+CD25^+$ T cells inhibited T cell proliferation less effectively than w/t $CD4^+CD25^+$ T cells. In conclusion, we demonstrate that the function of naive Tsup is strongly enhanced by antigen recognition. Therefore we expect that Tsup are possible candidates for antigen-specific immunosuppressive therapy.

1. Introduction

Regulatory T cells with a variety of phenotypes have been described to control the balance between immunity and tolerance in the periphery [1–3]. Most of these cells belong to the CD4 compartment, like the prototypic $CD4^+CD25^+$ T cells (Treg). However, T cells with a regulatory function have also been identified within the CD8 subset [4,5]. The functional relevance of so-called $CD8^+CD28^-$ T suppressor cells (Tsup) has been suggested in disease models [6] and clinical scenarios [7,8]. Their physiological role as well as the exact mechanism by which they down-regulate the immune response, however, remain unclear. Recognition of target antigen by a specific TCR is the central dogma of the adaptive, cell-mediated immune response. Thus, one might expect that not only initiation, but also subsequent down-regulation of the specific immune response by specialized suppressor T cells should be antigen-specific. While it has been described that activation, but not maintenance of $CD4^+CD25^+$ T cell function is dependent on antigen recognition [9], it remains controversial whether the same is true for $CD8^+CD28^-$ suppressor T cells.

$CD8^+CD28^-$ T suppressor cell subsets with antigen-specific function have been described [10]. These antigen-specific $CD8^+CD28^-$ T cells inhibit CD4 responses in a MHC class I-restricted manner. Generation of tolerogenic dendritic cells (DC) by antigen-specific $CD8^+CD28^-$ T cells has been suggested to be the dominant mechanism through which the alloresponse is down-regulated [11]. Tolerized DC, in turn, can then anergize allospecific CD4 cells. Contradicting these findings, other studies have shown that CD8 suppressor cells do not express FoxP3 and do not require antigen recognition but rather suppress the alloresponse in an IFN-γ dependent manner [12].

To determine the role of antigen in Tsup function, we used a well-characterized TCR-transgenic murine system in which antigen-specific Tsup can be isolated [13], and investigated the requirement of antigen recognition for the function of $CD8^+CD28^-$ and $CD4^+CD25^+$ Des T cells. We demonstrate here that antigen-specific Tsup are more efficient suppressors of T cell proliferation in presence of their respective antigen when compared to wild type controls. $CD4^+CD25^+$ naive regulatory cells of the same strain that cannot bind antigen through the transgenic receptor are less effective. Thus, we believe that CD8-mediated control of the immune response carries the principal potential for antigen-specific immunosuppression.

2. Methods

2.1. Mice

6–8 week old C57BL/6 mice (H2-Kb haplotype) were purchased from Charles River Laboratories (Wilmington, MA, USA). B10.BR (H2-Kk haplotype) and Des-TCR mice [13] (H2-Kk haplotype, transgenic TCR) were a kind gift of B. Arnold (Heidelberg, Germany). All mice were bred in our institute's animal facility under standard conditions. All animal experimentations were carried out in accordance with the

* Corresponding author. Department of Surgery, University of Regensburg, Franz Josef Strauss Allee 12, 93042 Regensburg, Germany. Tel.: +49 941 944 6914; fax: +49 941 944 6858.
E-mail address: marc.dahlke@klinik.uni-regensburg.de (M.H. Dahlke).

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2.2. Flow cytometry and antibodies

For flow cytometry analysis, cells were washed and resuspended in phosphate-buffered saline (PBS). Cell suspensions were incubated with monoclonal antibodies at 4°C for 20 min with combinations of saturating amounts of PacificBlue™-, fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, AlexaFluor™ 700-, PerCP-, PE-Cy5-, allophycocyanin (APC)- and APC-Cy7-conjugated antibodies purchased from Becton Dickinson (Heidelberg, Germany) or eBioscience (San Diego, USA). Clonotypic Désiré antibody [29,30], specific for the transgenic TCR was generated in-house. For the detection of living cells, propidium iodide (Sigma Aldrich, St. Louis, USA) or DAPI was added directly before analysis. Cells were analyzed on a FACS Calibur or LSR-II flow cytometer (Becton Dickinson) using either CellQuest or FACSDiva Software (Becton Dickinson).

2.3. Cell purification

$CD4^+CD25^+$ T regulatory cells were isolated and purified from spleen single cell suspensions using a commercial kit (Miltenyi, Bergisch Gladbach, Germany) according to the manufacturer's protocol, or by staining of splenocytes with CD25-PE and CD4-APC antibodies followed by cell sorting with a FACSAria flow cytometry sorter (Becton Dickinson, Heidelberg, Germany).

For purification of $CD8^+CD28^-$ cells, single cell suspensions of splenocytes were first stained with CD28-PE antibody and subsequently depleted from CD28 positive cells using paramagnetic anti-PE microbeads (Miltenyi, Bergisch Gladbach, Germany). In the next step, cells were positive-selected for $CD8^+$ using anti-CD8-beads in the same system (Miltenyi, Bergisch Gladbach, Germany). For mixed lymphocyte cultures, the same technique was applied to purify responder cells with anti-CD4-beads, and to deplete stimulator cells of $CD90^+$ cells with anti-CD90-beads (Miltenyi, Bergisch Gladbach, Germany).

2.4. CFSE staining and mixed leukocyte reactions (MLR)

Allogeneic mixed leukocyte reactions were performed with 10^5 magnetically purified $CD4^+$ or $CD8^+$ splenocytes from either B10.BR or Des-TCR donors as responders. To observe proliferation events, responder cells were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen, Karlsruhe, Germany) using a protocol described before [14]. In brief, cells were washed twice in FCS-free medium and then incubated with 5 µmol CFSE per 10^6 cells for five minutes at 37°C. The labelling procedure was stopped by adding FCS containing medium. As stimulators, 2×10^5 irradiated (30Gy), CD90-depleted C57Bl/6 splenocytes were used. Responder cells stimulated by CD90-depleted syngeneic cells served as controls. MLRs were carried out in 96-well round bottom plates in a total volume of 200 µl RPMI (Gibco) cultivation medium (supplemented by 0.1% 50 mM 2-mercaptoethanol, 1% MEM-non-essential amino-acids, 2% 1 M HEPES (pH 7.36–7.39/NaOH), 1% 100 mM sodium-pyruvate solution, 1% 100× Antibiotic/Antimycotic, 1% MEM Vitamin solution, 1% 200 mM L-glutamine and 10% heat-inactivated FCS) and analyzed by flow cytometry after five days of culture.

For ^{3}[H]thymidine incorporation assays, 0.5 µCi ^{3}[H]thymidine (PerkinElmer, Boston, USA) per well was added 18 h prior to termination of the co-culture. Cells were harvested onto a microfibre plate (Wallac, Turku, Finland) and radioactivity was measured using a liquid beta scintillation counter (Wallac, Turku, Finland).

2.5. Software

Flow cytometry data was computed by FlowJo V7.1.3 software (TreeStar, Inc., Ashland, OR, USA). A students' *t*-test was used for statistical comparison (GraphPad Software Inc., La Jolla, CA, USA). $p<0.05$ was considered statistically true.

3. Results

3.1. Expression of transgenic T Cell Receptors in the Des-TCR system

All CD4 and CD8 lymphocytes purified from the Des-TCR mice express the transgenic TCR (Fig. 1 A). However, due to incomplete allelic exclusion of the TCR alpha chain, a significant subset of Des-TCR T cells expresses two different alpha chains: one being rearranged endogenously and the other representing the product of the transgene [15]. As a result, in addition to the transgenic Des-TCR, some CD8 T cells and all CD4 T cells express an endogenously rearranged TCR of unknown specificity. $CD4^+$ T cells do not develop in Des-TCR RAG-1−/− mice, suggesting that positive selection of these cells occurs via the endogenous TCR. However, the overall number of Des-TCR CD4 cells is decreased (Fig. 1B) and these CD4 T cells have an impaired TCR repertoire because of their lower frequency of naturally recombining, CD4-dependent TCR. Furthermore, it has to be considered that Des-TCR CD4 T cells co-express high levels of fully transgenic TCR and lower levels of natural TCR although they do not express the costimulatory machinery to function through MHC class I.

3.2. The transgenic Des-TCR CD8 response towards H2-Kb alloantigen is more effective than the w/t response, whereas both transgenic Des-TCR CD4 T cells and B10.BR w/t controls proliferate moderately upon stimulation with H-2Kb alloantigen

To compare the extent of the proliferative response of transgenic versus wild type CD8 cells after stimulation with H-2Kb alloantigen, we stimulated purified Des-TCR and B10.BR CD8 cells with irradiated H-2Kb positive CD90-depleted splenocytes in vitro. The proliferative response was analyzed by CFSE dilution. Five days after stimulation, all CD8+ transgenic Des-TCR cells had undergone several divisions, whereas only a fraction of wild type cells proliferated upon alloantigen stimulation (Fig. 2A). The CD25 expression of transgenic CD8 T cells was significantly higher than that of their wild type counterparts indicating greater activation by alloantigen (Fig. 2B).

In contrast to CD8 T cells, proliferation of purified Des-TCR CD4 T cells activated by H2-Kb positive splenocytes did not result in a significantly different response compared to wild type controls in a thymidine incorporation assay (Fig. 3A). Data from the radioactive incorporation assay was additionally confirmed by a CFSE dilution assay. Here, several population doublings for Des-TCR and wild type CD4 T cells were observed, although, in this more sophisticated assay, the CD4 Des-TCR response was less pronounced (Fig. 3B). In addition, CD25 expression of CD4 Des-TCR cells was decreased compared to their wild type controls (Fig. 3C), indicating weaker overall activation.

3.3. Transgenic $CD8^+CD28^-$ Des-TCR T cells suppress responder cell proliferation upon H2-Kb stimulation more effectively than w/t $CD8^+CD28^-$ T cells

After having outlined that both CD4 and CD8 T cells of Des-TCR and wild type origin proliferate upon TCR stimulation by alloantigen, whereby the response of transgenic CD8 cells was markedly increased, we further investigated whether the regulatory potential of the transgenic cell population was also stronger than that of their wild type counterpart. For this purpose $CD8^+CD28^-$ T cells of Des-TCR and wild type origin were purified and tested for their ability to inhibit the proliferation of allogeneic CFSE-labeled B10.BR CD4 responder T cells responding to H2-Kb+ stimulators.

In this MLR, $CD8^+CD28^-$ suppressor T cells suppressed CD25 up-regulation and proliferation of CD4 responder cells independently of their origin. Interestingly, wild type $CD8^+CD28^-$ T cells suppressed responder cell proliferation in a CFSE dilution assay significantly less than Des-TCR $CD8^+CD28^-$ cells and never reached baseline levels (Fig. 4A). The effect of transgenic cells was more pronounced and not dose dependent in the chosen fractions, indicating a considerable higher suppressive activity of this TCR-transgenic population (Fig. 4B). This observation was confirmed in a thymidine incorporation assay (Fig. 4C). Here, radioactive thymidine uptake was clearly higher when wild type Tsup were used. In addition a decrease in CD25 up-regulation of responding CD4 cells could be outlined (data not shown).

3.4. In contrast to TCR-transgenic Des-TCR $CD8^+CD28^-$ cells, CD4 + CD25 + Des-TCR cells do not suppress the CD4 alloresponse versus H2-Kb

To further follow our hypothesis that interaction between the transgenic Des-TCR and H2-Kb governs the regulatory function of CD8 cells, we isolated Des-TCR and wild type $CD4^+CD25^+$ T cells. These Des $CD4^+CD25^+$ T cells cannot be fully activated by antigen through the transgenic TCR because CD4 cells lack the costimulatory machinery to function in a MHC class I-restricted fashion. Thus, if $CD4^+CD25^+$ T cells regulate the alloresponse through antigen recognition, DES cells can only function through their endogenously rearranged TCR, which is less frequent on DES T cells. In a corresponding MLR, B10.BR $CD4^+$ responder cells strongly proliferate when stimulated with allogeneic H2Kb+splenocytes. And indeed, this proliferation can be significantly suppressed by wild