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Technical note

In-vitro blockade of the CD4 receptor co-signal in antigen-specific T-cell stimulation cultures induces the outgrowth of potent CD4 independent T-cell effectors

Sarah Vatter[a], Maximilian Schmid[a], Claudia Gebhard[a,b], Carina Mirbeth[a,b], Sebastian Klobuch[a], Michael Rehli[a,b], Wolfgang Herr[a,b], Simone Thomas[a,b,*]

[a] Department of Internal Medicine III, Hematology and Oncology, University Hospital Regensburg, Germany
[b] Regensburg Center for Interventional Immunology, University of Regensburg, Regensburg, Germany

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ABSTRACT

T-cell receptor (TCR) redirected T cells are promising tools for adoptive cancer immunotherapy. Since not only CD8 but also CD4 T cells are key players for efficient antitumor responses, the targeted redirection of both subsets with the same antigen-specific TCR comes more and more into focus. Although rapidly evolving technologies enable the reliable genetic re-programming of T cells, the limited availability of TCRs that induce T-cell activation in both T-cell subsets without CD4/CD8 co-receptor contribution hampers the broad application of this approach. We developed a novel stimulation approach, which drives the activation and proliferation of CD4 T-cell populations capable of inducing effector functions in a CD4-independent manner. Naive-enriched CD4 T cells were stimulated against dendritic cells (DC) expressing allogeneic HLA-DP antigens upon RNA transfection and CD4/HLA interactions were blocked by the addition of CD4 binding antibody. Evolving CD4 T-cell populations were specifically activated independent of the CD4 co-signal and induced strong TCR-mediated IFN-γ secretion as well as cytolysis upon recognition of leukemia cells expressing HLA-DP antigen. Our novel stimulation approach may facilitate the generation of CD4 T cells as source for co-receptor independent TCRs for future immunotherapies.

1. Introduction

Over the last decade, adoptive immunotherapy with T-cell receptor (TCR) engineered T cells came more and more into focus as a promising therapeutic option for the treatment of relapsed or refractory acute leukemias (Morris and Stauss, 2016). In this context, most investigators and clinical trials have focused on the redirection of CD8 T cells with tumor-antigen specific TCRs isolated from HLA-class I restricted CD8 T cells (Fesnak et al., 2016). However, there is an increasing evidence that also CD4 T cells play a key role in anti-tumor immunity and that the adoptive transfer of both CD4 and CD8 subsets induces efficient antitumor responses (Perez-Diez et al., 2007). It has also been shown that CD4 T cells are not only restricted to their helper T cell fate but also mediate cytotoxicity against leukemia cells (Herr et al., 2017; Stevanovic et al., 2012). Therefore, it would be attractive to transfer HLA-class I-restricted TCRs not only into CD8 but also into CD4 T cells to redirect both subtypes to interact synergistically against the same tumor target (Kuball et al., 2005). Since HLA-class I molecules are widely expressed in most tissues, HLA-class I restricted TCRs can induce unwanted on-target toxicity. In contrast, HLA-class II restricted TCRs isolated from leukemia-reactive CD4 T cells might exert less toxicity after transfer in CD4 and CD8 T cells as the expression of HLA-class II molecules is largely restricted to cells of hematopoietic origin. Nevertheless, a prerequisite for both strategies is the availability of TCRs that induce full T-cell activation without contribution of the CD4 or CD8 co-receptor and so far, only few TCRs are characterized by a co-receptor independent function (Kuball et al., 2005; Thomas et al., 2015).

Several investigators including ourselves have previously shown, that allogeneic HLA-DPB1 class II-alleles are powerful leukemia rejection antigens for CD4 T-cell therapy following allogeneic hematopoietic stem cell transplantation (Herr et al., 2017; Rutten et al., 2008; Stevanovic et al., 2012) and that allo-HLA-DPB1 reactive cytolytic CD4 T-cell populations can be reliably generated from naive-enriched CD4 T cells by *in vitro* stimulation methods (Herr et al., 2017). Based on this

Abbreviations: AML, acute myeloid leukemia; ELISpot, enzyme-linked immunosorbent spot; LCL, Epstein-Barr virus-transformed B-lymphoblastoid cell lines; HLA, human leukocyte antigen; IFN, interferon; IL, interleukin; mAb, monoclonal antibody; DC, monocyte-derived dendritic cell; PBMC, peripheral blood mononuclear cell; TCR, T-cell receptor

* Corresponding author at: University Hospital Regensburg, Department of Internal Medicine III, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany.
E-mail address: simone.thomas@ukr.de (S. Thomas).

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approach, we tested herein whether CD4 blockade facilitates the targeted generation of CD4 co-receptor independent CD4 effector T cells recognizing allogeneic HLA-DPB1 alleles. We hypothesized that the CD4 blockade preferentially induces the activation of CD4 T-cells with strong TCR/HLA interactions, eventually driving the outgrowth of CD4 T cells acting without CD4 contribution. We show herein that the addition of a CD4 antibody to the T-cell stimulation cultures indeed results in the outgrowth of CD4 co-receptor independent CD4 T cells that are characterized by specific secretion of IFN-γ and cytolytic effector function upon recognition of primary leukemia cells expressing allogeneic HLA-DPB1 alleles.

2. Materials and methods

2.1. Cells and antibodies

Peripheral blood mononuclear cells (PBMCs) and acute myeloid leukemia (AML) blasts were isolated by standard Ficoll density separation from leukocyte reduction system (LRS) cones of healthy donors and AML patients after written informed consent in accordance with the Declaration of Helsinki and upon approval by the local ethics committee. CD4 T-cells were isolated from PBMCs by immunomagnetic beads technology (Miltenyi Biotec, Germany) and subsequently enriched for naive- ($CD3^+/CD4^+/CD45RA^+$) subsets by flow cytometric cell sorting. High-resolution HLA-DP genotyping was performed according to standard procedures. EBV-transformed B-lymphoblastoid cell lines (LCL) and monocyte-derived DCs were generated by standard protocols as described (Thomas et al., 2012). Flow cytometry was performed on FACS Calibur (BD Biosciences, Germany) and analyzed using FlowJo 7.6.5 software. Flow cytometric cell sorting was performed on FACS Aria (BD Biosciences). Fluorochrome labeled or unlabeled monoclonal antibodies (mAb) were used in accordance to the manufacturers' specifications including anti-human CD3 (UCHT1), CD4 (RPA-T4), CD45RA (HI100) (all BD Biosciences), and HLA-DP (B7/21) (Leinco Technologies, USA). Monoclonal and purified mAb targeting CD4 (RPA-T4) (BioLegend, USA), HLA-class I (W6/32), HLA-DQ (SPV-L3), HLA-DR (HB55), HLA-DP (HB55) were added at saturating concentrations. Isotype control was mouse IgG1κ (P3.6.2.8.1) (Thermo Fisher Scientific, USA).

2.2. RNA transfection

HLA-DPA1 and HLA-DPB1 encoding RNA were synthesized by *in vitro* transcription from pGEM4Z vectors containing full-length HLA-DPA1*01:03, -DPB1*02:01, -DPB1*03:01, or -DPB1*04:01 genes using the mMESSAGE mMACHINE T7 Ultra kit (Thermo Fisher Scientific) (Thomas et al., 2012). RNA electroporation of DCs was performed as described (Thomas et al., 2012) by applying a square wave pulse of 400 V/5 ms. HLA-DP expression was measured 12-14 h after electroporation by flow cytometry and aliquots of DC allo-HLA-DP transfectants were frozen until usage.

2.3. T-cell stimulation cultures

Allo-HLA-DP reactive CD4 T cells were expanded from naive-enriched ($CD45RA^+$) CD4 T-cells as previously described (Herr et al., 2017). In brief, naive-enriched CD4 T-cells (1×10^3/well) were weekly stimulated with irradiated (70 Gy) HLA-DPA1/DPB1 mismatch transfected autologous DC at a T-cell to DC ratio of 20:1 in 96-well microtiter plates containing AIM-V medium supplemented with 10% human serum, recombinant human interleukin (IL)-12 (1 ng/mL), IL-7 (5 ng/mL) (R&D Systems, Germany) and IL-15 (5 ng/mL) (PeproTech, USA). T cells were re-stimulated weekly with allo-HLA-DP^+ DCs at a T-cell to DC ratio of 20:1. From day 14 onwards, IL-12 was replaced by IL-2 (50-100 IU/mL; Proleukin, USA). For blocking of CD4/HLA-class II interaction, CD4 T cells were pre-incubated for 1 h with purified anti-human CD4 mAb (RPA-T4) at saturating concentrations (1 μg/mL) on day 0 and before addition of DC. Afterwards, CD4 mAb was added weekly (1 μg/mL) during re-stimulation. From day 28 of culture, T cells were re-stimulated without CD4 blocking mAb and were transferred into 48- or 24-well plates.

2.4. Functional assays and TCR-Vβ analysis

Standard ^{51}Chromium-release and ELISpot assays were performed in duplicates as described before (Dörrschuck et al., 2004). For split-well ELISpot assays 50 μL of each well from the T-cell/DC-stimulation culture were co-incubated with untransfected or allo-HLA-DPA1/DPB1 transfected autologous DCs (5×10^4) for 20 h. For blocking experiments, purified anti-human CD4 HLA-class I, HLA-DR, HLA-DQ or HLA-DP mAb (2.5 μg/well) were added. TCR clonotypes of CD4 T cells were analyzed by high-throughput sequencing of TCR−Vβ CDR3 regions using SMARTer cDNA synthesis Kit (Clontech, USA) in accordance with the manufacturers' specification followed by subsequent barcode-based TCR-DNA amplification. Amplified TCR libraries were sequenced on MiSeq (Illumina, USA). Consensus alignment for the major clonotype was done using the software muscle v.3.8.31, TCR nomenclature was specified in accordance with the IMGT numbering system (http://www.imgt.org/).

3. Results

3.1. CD4 receptor blockade promotes the outgrowth of allo-HLA-DP specific CD4-independent T-cells

To allow for the generation of antigen-specific CD4 T-cells from PBMCs of healthy donors, we utilized our well-established stimulation approach, in which naive ($CD45RA^+$) enriched CD4 T cells were stimulated with autologous DCs expressing allogeneic HLA-DP alleles upon RNA electroporation (Herr et al., 2017). This stimulation protocol resulted in a reliable and robust outgrowth of CD4 T-cell populations that specifically recognize target cells (including primary leukemia blasts) expressing the allogeneic HLA-DP antigen that was used in stimulation culture (Herr et al., 2017). To promote the outgrowth of CD4 T cells that were activated without the contribution of the CD4 co-signal, we blocked the engagement of the CD4 co-receptor and the HLA-DP molecule on the target cell by the addition of a CD4 specific blocking mAb. For this, naive-enriched CD4 T cells isolated from 5 different healthy donors (donor 129, 140, 166, 167 and 199) were pre-incubated with CD4 blocking mAb in 96-well plates and were then stimulated with autologous DC expressing allogeneic HLA-DPA1/DPB1 antigens. T cells were re-stimulated weekly by the addition of allo-HLA-DP^+ DCs. CD4 blocking mAb was supplemented weekly until day 28 of culture. After two rounds of re-stimulation (d14 + 5 of culture) CD4 T-cell populations from individual microcultures were analyzed in IFN-γ split-well ELISpot assays for reactivity to allo-HLA-DP transfected or untransfected (mock) autologous DC. Overall, the numbers of CD4 T-cell populations specifically responding to allogeneic HLA-DP while ignoring autologous DC was lower in microcultures stimulated in the presence of CD4 blocking mAb compared to cultures without CD4 blockade. For example, in allo-HLA-DPB1*04:01 stimulation cultures from donor 166 a total of 23 allo-HLA-DP reactive CD4 T-cell populations could be generated in the presence of CD4 blocking mAb, whereas 40 CD4 T-cell populations were obtained without CD4 blockade (Fig. 1A, **left part**). In microcultures from 6 different T-cell/allo-HLA-DP combinations that were stimulated in the presence of CD4 blocking mAb, the numbers of CD4 T-cell populations that specifically reacted to allo-HLA-DPB1 transfected autologous DCs differed between the analyzed donors (range 30-48 CD4 populations per 96-well plate) (Fig. 1A, right). The stimulation procedure did also drive the proliferation of CD4 T cell cultures that reacted to untransfected autologous DC (Fig. 1A, left and right), which were not further re-stimulated. Next, we determined