Analysis of stem-cell-like properties of human CD161++IL-18Rα+ memory CD8+ T cells

Simone H. C. Havenith1,2, Si La Yong1,2, Sian M. Henson3, Berber Piet2,4, Mirza M. Idu5, Sven D. Koch1,2, René E. Jonkers4, Natasja A. M. Kragten2, Arne N. Akbar3, René A. W. van Lier6 and Ineke J. M. ten Berge1

1Renal Transplant Unit, Department of Internal Medicine, Academic Medical Center, Amsterdam, The Netherlands
2Department of Experimental Immunology, Academic Medical Center, Amsterdam, The Netherlands
3Division of Infection and Immunity, Department of Immunology, University College London, London, United Kingdom
4Department of Respiratory medicine, Academic Medical Center, Amsterdam, The Netherlands
5Department of Surgery, Academic Medical Center, Amsterdam, The Netherlands
6Landsteiner Laboratory, Sanquin Research, Academic Medical Center, Amsterdam, The Netherlands

Correspondence to: Simone H.C. Havenith, Meibergdreef 9 (K0-154), 1105 AZ Amsterdam, The Netherlands; E-mail: s.h.havenith@amc.uva.nl

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Abstract

CD161++IL-18Rα+ human T cells have recently been identified as a new subset of memory cells but their exact role remains unclear. CD161++IL-18Rα+CD8+ T cells express a semi-invariant TCR Va7.2-Jα33, which recognizes the MHC-related protein 1. On the basis of properties including the expression of the ABC-B1 transporter, cKit expression and survival after chemotherapy, CD161++IL-18Rα+CD8+ T cells have been designated as ‘stem’ cells. Here we analyse location and functional properties of CD161++IL-18Rα+CD8+ T cells and question whether they have other traits that would mark them as genuine ‘stem’ cells. CD161++IL-18Rα+CD8+ T cells were found in peripheral blood, spleen and bone marrow but interestingly hardly at all in lymph nodes (LNs), which may possibly be explained by the finding that these cells express a specific set of chemokine receptors that allows migration to inflamed tissue rather than to LNs. In addition to TCR ligation and co-stimulation, CD161++IL-18Rα+CD8+ T cells require cytokines for proliferation. The CD161++IL-18Rα+CD8+ pool contains cells reactive towards peptides, derived from both persisting and cleared viruses. Although CD161++IL-18Rα+CD8+ T cells express the ABC-B1 transporter, they have shorter telomeres and less telomerase activity and do not express aldehyde dehydrogenase. Finally, CD161++IL-18Rα+CD8+ T cells show similarities to terminally differentiated T cells, expressing IFNγ, KLRG1 and the transcription factor Blimp-1. In conclusion, CD161++IL-18Rα+CD8+ T cells lack many features of typical ‘stem’ cells, but appear rather to be a subset of effector-type cells.

Keywords: CD161, MAIT cells, memory stem cells, telomere erosion

Introduction

CD8+ T cells play a crucial role in the defence against viral infection and tumours by specifically recognizing and eliminating infected cells or tumour cells. During immune responses, naïve CD8+ T cells are primed in the lymph nodes, after which they clonally expand to form a large population of cytotoxic effector cells. After the initial response, most cells are rapidly cleared through apoptosis, but a few memory cells survive. These memory cells have the capacity to expand rapidly upon secondary antigen challenge (1). Advances in recent years have made it possible to measure a wide variety of surface and intracellular markers, which has led to the characterization of a multitude of functionally distinct CD8+ T cell subsets (2).

A number of recent studies have described the properties of a new human memory CD8+ T-cell subset characterized by high expression of CD161 and IL-18Rα. The group of Lantz described mucosal-associated invariant T (MAIT) cells, ‘innate-like’ T lymphocytes characterized by the expression of a semi-invariant TCR Va7.2-Jα33, which recognize the MHC-related protein 1 (MR1) (3). MAIT cells can become activated when stimulated with antigen presenting cells that are fed with bacteria and fungi but not with viruses. More recently, Turtle et al. (4) described a CD161++IL-18Rα+ memory CD8+ T-cell subset, which based on the expression of the ABC-B1 transporter and c-KIT, resemble hematopoietic stem
cells (HSC). ABC-B1 transporter expression may endow these cells with resistance to toxic xenobiotics, such as cytostatic drugs (5, 6). Indeed, they are more likely to be resistant to chemotherapy and may play an important role in the protection against latent viral infections during chemotherapy-induced lymphocytopenia (4, 7). Because of their resemblances to HSC, these memory CD8+ T cells have been proposed to be a good candidate for a memory ‘stem’ cell pool, which is able to provide life-long protection without the need to re-encounter antigen (8). Several reports have shown that CD161++IL-18Rαα+ CD8+ T cells express RORγt and can produce IL-17 upon in vitro stimulation (9, 10). As it was demonstrated that MAIT cells express high levels of the ABC-B1 transporter (10), the MAIT and memory ‘stem’ cell pool may represent identical or at least overlapping populations.

We question here whether CD161++IL-18Rαα+ CD8+ T cells have any other stem-cell characteristics. To investigate this, we determined their location and functional properties, including telomere length and telomerase activity.

**Materials and methods**

**Isolation of mononuclear cells from peripheral blood, lymph nodes, bone marrow and lung tissue**

Peripheral blood mononuclear cells (PBMCs) were obtained from buffy coats of healthy blood donors, from blood of kidney transplant recipients or from patients undergoing lung surgery. PBMCs were isolated using standard density gradient centrifugation.

Lymph nodes were collected from kidney transplant recipients during donor kidney implantation. Briefly, lymph node mononuclear cells were isolated from surgical residual material (surrounding the external iliac vessels) of the recipient, gathered during the implantation of the transplanted kidney. The residual tissue removed in this procedure often contains lymph nodes. Directly after kidney transplant surgery, the gathered lymph nodes were chopped in small pieces. A cell suspension was obtained by grinding the material through a flow-through chamber.

Peripheral lung tissue was collected from patients who underwent a lobectomy for a peripheral lung tumour. Lung mononuclear cells were isolated from peripheral lung tissue according to the procedure described by Piet et al. (11). Bone marrow cells were isolated from bone marrow aspirates obtained from the sternum of patients undergoing cardiac surgery. Bone marrow mononuclear cells were isolated using standard density gradient centrifugation techniques. Spleen mononuclear cells were isolated from spleen of post-mortal organ donors. The spleen cells were isolated with a cell strainer followed by density gradient centrifugation. All mononuclear cells gathered were subsequently cryopreserved until the day of analysis.

Where necessary, patients gave written informed consent and the study was approved by the Medical Ethics Committee of the Academic Medical Center in Amsterdam.

**Immunofluorescent staining and flow cytometry**

PBMCs were washed in PBS containing 0.01% (w/v) NaN3 and 0.5% (w/v) BSA. For tetramer staining, PBMCs were first incubated with an appropriate concentration of tetrameric complexes in a small volume for 30 min at 4°C, protected from light. Fluorescent-labelled conjugated mAbs were added and incubated for 30 min at 4°C, protected from light at concentrations according to manufacturer’s instructions. For surface-marker expression analysis, the following antibodies were used in different combinations: CD161 FITC/PE/APC, CD3 PE-Cy7, CD4 AF700, CD8 PerCP-Cy5.5 CD45RA PE-Cy7, Granzyme A PE, Perforin PE, CCR5 PE, CXC3R3 Alexa 488, CDD2L PE, CD24 FITC (BD Biosciences, San Diego, CA, USA), CD45RA PerCP-Cy 5.5, CD3 APC, CD127 PerCP-Cy 5.5, CD3 AF700, IL18-Rαα FITC/PE, CD44 APC, CD166 PE (eBioscience Inc, San Diego, USA), CD8 PE AF610, CD27 APC-APF750, Granzyme B PE (Invitrogen, Paisley, UK), Granzyme K FITC (Immunotools, Friesoythe, Germany), CX3CR1 PE (MBL international corporation), CCR7 PerCP-Cy 5.5, CXC4R6 AF647 (Biolegend, San Diego, USA), CXC4R4 PE, CCR6 FITC (R&D systems, Abingdon, UK), CD133 APC (Miltenyi Biotec, Bergisch Gladbach, Germany) KLRG1 biotin(a gift from Prof. H. Pircher) with GaM APC-Alexa Fluor 750 secondary antibody (Invitrogen). Samples were analysed on a BD FACScanto using FACSdiva Software. Analysis was done using FlowJo Mac.

**Tetramer complexes**

The following HLA-peptide tetramer complexes were kindly provided by Anja ten Brinke (Sanquin, Amsterdam, the Netherlands): HLA-A1 tetramer loaded with the CMV pp65-derived YSEHPTFTSQY, HLA-A2 tetramer loaded with the CMV pp65-derived NLVPMTAVT peptide, HLA-B7 tetramer loaded with the CMV pp65-derived TPRVTGGGAM peptide, HLA-B35 tetramer loaded with the CMV pp65-derived IPSINVHHY peptide, HLA-A2 tetramer loaded with the CMV IE1-derived VLEETSVML peptide and HLA-A2 tetramer loaded with the FLU-derived GILGFVFTL peptide.

**Cell sorting**

One day prior to cell sorting, PBMCs were thawed and labelled with CFSE. For isolation of CD161++IL-18Rαα+ memory CD8+ T cells and CD161++IL-18Rαα+ memory CD8+ T cells, cells were labelled with CD161 APC, CD45RA PE-Cy7, CD8 PerCP-Cy5.5 (BD Biosciences), IL18-Rαα PE (eBioscience) and CD27 APC af750 (Invitrogen). For isolation of CD4+ T cells and monocytes, cells were labelled with CD4 APC and CD14 PE (BD Biosciences). For isolation of mDCs and pDCs, cells were labelled with Lineage marker FITC (Immunosource, Schilde, Belgium), BDCA-1 APC and BDCA-4 PE (Miltenyi Biotec) cells were sorted on a FACSAria (BD Biosciences). Purity of the obtained sorted cells was verified using flow cytometry and was at least 95%. Sorting strategy and gating are depicted in Supplementary Figure 1, available at International Immunology Online.

**CFSE labeling**

PBMCs were pelleted and resuspended in PBS at final concentration of 5-10 10E6 cells/ml. Next, cells were labeled in 0.5 uM (final concentration) of CFSE (Molecular Probes Europe) in PBS for 10 minutes at 37 C. Cells were washed and subsequently resuspended in IMDM (Life Technologies) containing 10% fetal calf serum, 100U ml sodium penicillin
Stimulation and cell culture

Cells were cultured in IMDM (Life Technologies, Gaithersburg, MD) containing 10% fetal calf serum, 100 U ml⁻¹ sodium penicillin G (Brocades Pharma BV, Leiderdorp, The Netherlands), 100 µg ml⁻¹ streptomycin sulphate (Invitrogen) and 0.0035% 2-ME (Merck). For stimulation, we added soluble 1 µg ml⁻¹ anti-CD3 (clone 1XE, Sanquin, Netherlands) and soluble 5 µg ml⁻¹ anti-CD28 (clone 15E8, Sanquin) to the cell culture in absence or presence of various different cytokines; 50 U ml⁻¹ IL-2 (Biostat AG, Dreieich, Germany), 10 ng ml⁻¹ IL-7 (Sanquin), 5 ng ml⁻¹ IL-12, 10 ng ml⁻¹ IL-15 (R&D systems, UK), 50 ng ml⁻¹ IL-18 (R&D systems, UK), 25 ng ml⁻¹ IL-21 (Zymogenetics, Seattle, USA). Furthermore, sorted CD161⁺IL-18Rα and CD161⁺IL-18Rα memory CD8⁺ T cells were stimulated with CMV, EBV and FLU peptides in a one-to-one ratio.

Telomere length measurement by flow-FISH

Telomere length of CD161⁺IL-18Rα and CD161⁺IL-18Rα memory CD8⁺ T cells was measured using a modified version of the flow-fluorescence in situ hybridization (FISH) method that was previously described (13). First, cells were stained with mAb to CD3V500, CD8V450, CD161FITC (BD), CD27AF700 (eBioscience), CD45RA-NC605 (Invitrogen). After staining for 30 min, the cells were washed in PBS and fixed in BS3 (final concentration 1 nM) (Pierce, Rockford, USA) for 30 min at 4°C. The reaction was quenched using 1 ml of 50nM Tris (pH 7.2) in PBS and incubated in the dark for 20 min at room temperature. After being washed with PBS followed by hybridization buffer, cells were incubated in 0.75 µg ml⁻¹ of the telomeric probe conjugated to Cy5 (Cambridge research biochemicals, Cleveland, UK). Samples were then heated for 10 min at 82°C, rapidly cooled, and left to hybridize in the dark at room temperature for 1 h. Samples were washed in post-hybridization buffer followed by PBS and analysed immediately by flow cytometry (Fortessa, BD). All samples were run in triplicate. We calculated fold changes of mean fluorescence intensity (MFI) of the different CD8⁺ T cell subsets, the MFI of CD27⁺CD45RA⁻ naïve T cells was set to 1.

Measurement of telomerase activity

Sorted CD161⁺IL-18Rα and CD161⁺IL-18Rα memory CD8⁺ T cells were stimulated for 7 days with 10 ng ml⁻¹ IL-15, after which 2 x 10E5 cells were snap-frozen. Telomerase activity was determined using the telomeric repeat amplification protocol (TRAP; TRAPeze telomerase detection kit; Serologicals, distributed through Chemicon Europe) as previously described. PCR was performed with samples adjusted to 500 Ki67⁺ cells per reaction. Ki67⁺ cells were identified using αKi67 PE Ab (BD) (14).

Aldehyde dehydrogenase activity

Aldehyde dehydrogenase (ALDH) activity was measured using the commercial reagent ALDEFLUOR (STEM-CELL Technologies Inc, Vancouver, BC, Canada). The ALDEFLUOR substrate (BAAA) is taken up by live cells through passive diffusion, whereupon it is converted in the cytoplasm into a fluorescent molecule (BAA-) under the action of ALDH. In the assay buffer, ABC-transporter inhibitors are present, which prevent active efflux. As a control, ALDH activity was blocked using diethylaminobenzaldehyde (DEAB). Cells with high ALDH activity can be identified by green fluorescence (BAA-) on the flowcytometer.

Quantitative PCR

Total RNA was isolated using a standard RNA isolation kit (Invitek, Berlin, Germany) and reverse transcribed to cDNA using random hexamers and Superscript II reverse transcriptase (Roche Diagnostics). Quantitative PCR was performed using the StepOnePlus RT–PCR system (Applied Biosystems) using Express SYBR GreenER (Invitrogen). Primer sequences are available on request.

Statistical analysis

Statistical analysis of paired samples was done by two-tailed Mann–Whitney signed rank test with a 95% confidence interval. Non-paired samples were analysed with two-tailed Mann–Whitney test with a 95% confidence interval.
Strikingly, CD161++IL-18Rα+ CD8+ T cells were virtually absent in LN (Fig. 1B and C). Also in the lung, we observed a low frequency of this population (Fig. 1B and D).

The absence of CD161++IL-18Rα+ CD8+ T cells from LN might be due to specific chemokine receptor expression. Indeed, we found expression of CCR6, CXCR6 and CCR5 but...
not of CXCR4, CX3CR1, CXCR3 or CCR7 on CD161++IL-18Rα+ CD8+ T cells (Fig. 1E).

Thus, CD161++IL-18Rα+ CD8+ T cells do not home to resting lymph nodes but may be capable of migrating to inflamed tissue.

**CD161++IL-18Rα+ memory CD8+T cells have stringent activation requirements**

We considered that memory T cells with stem-cell characteristics would have the potential to expand vigorously in response to antigen stimulation. However, in contrast to CD161++IL-18Rα+ memory CD8+ T cells, purified CD161++IL-18Rα+ memory CD8+ T cells did not proliferate when stimulated for 3 days with a combination of anti-CD3 and anti-CD28 antibodies (Fig. 2A). Apparently, CD161++IL-18Rα+ memory CD8+ T cells cannot be activated by a TCR stimulus and co-stimulation alone, but need a third signal to proliferate. In a transwell system, with a membrane impermeable for cells, we found that cell–cell contact contributes, but was not necessary for CD161++IL-18Rα+ memory CD8+ T-cell proliferation (Fig. 2B). To characterize the cell type that provides the third signal, we added sorted CD4+ T cells, monocytes or a combination of both to the stimulation (Supplementary Figure 1C, available at International Immunology Online.). We found the CD4+ T cells are necessary in order to provide help for the CD161++IL-18Rα+ memory CD8+ T cells (Fig. 2C). Adding B cells alone or in combination with CD4+ T cells and/or monocytes to the stimulation had no effect on proliferation (data not shown). CD4+ T cells might support division by the secretion of cytokines. Indeed, addition of IL-2 in combination with IL-21 to the cultures had the same effect as the addition of CD4+ T cells (Fig. 2D).

Interestingly, the addition of IL-15, IL-12, IL-18 or IL-19 in combination with IL-12, to TCR stimulation and co-stimulation, significantly increased proliferation of CD161++IL-18Rα+ CD8+ T cells (Fig. 2E). IL-15, IL-18 and IL-12 are all cytokines produced by myeloid dendritic cells. Purified myeloid dendritic cells (Lin-, BDCA-1+) did not provide the additional signal, but modestly enhanced the effect of CD4+ T cells (Fig. 2F). With plasmacytoid dendritic cells (Lin-, BDCA-4+), this additional effect was not seen.

Finally, we used the homeostatic cytokines IL-7 and IL-15 in absence of TCR stimulation. When stimulating CD161++IL-18Rα+ memory CD8+ T cells with IL-15 for 7 days we observed significantly more proliferation; a trend which was also seen with IL-7 (Fig. 3G).

In summary, CD161++IL-18Rα+ memory CD8+ T cells do not proliferate upon TCR stimulation and co-stimulation but require an additional signal to proliferate, which can be delivered either by CD4+ T cells, most likely through the production of cytokines, or by the exposure to the T,1-inducing cytokines IL-12 and IL-18.

The CD161++IL-18Rα+ memory CD8+ T-cell pool contains virus-specific cells

Contradictory data have been published on the existence of virus-specific cells within the CD161++IL-18Rα+ CD8+ T cells (3, 4, 9, 10). In fact, when the CD161++IL-18Rα+ memory CD8+ T cells were only to contain invariant MAIT cells, then the presence of a broad repertoire of virus-specific cells would be unlikely.

To address this point, we measured binding to viral peptides presented by HLA class-I tetrameres. Tetramer stainings were preformed directly ex-vivo using large cell numbers (>5 million lymphocytes). This revealed small but clearly distinguishable populations of both CMV- and FLU-specific CD8+ T cells within the CD161++IL-18Rα+ subset, (Fig. 3A and B). To substantiate the presence of these cells, sorted CD161++IL-18Rα+ cells were stimulated for 7 days with CMV- or FLU-peptide in the presence of IL-2 and irradiated CD8 cells. Virus-specific populations could be expanded from the CD161++IL-18Rα+ CD8+ pool (Fig. 3A and B). Furthermore, in a mixed lymphocyte culture CD161++IL-18Rα+ CD8+ T cells responded, but when compared to CD161++IL-18Rα+ conventional memory CD8+ T cells (Fig. 3C), a lower percentage of the CD161++IL-18Rα+ memory CD8+ T cells (Fig. 3D) were found to be allo-responsive.

Together, these data indicate that CD161++IL-18Rα+ CD8+ T cells contain virus-specific cells.

CD161++IL-18Rα+ CD8+ T cells predominantly have a Th1 cytokine-producing profile

In view of the fact that memory stem cells would be unlikely to produce effector molecules, we went on to test cytokine secretion and granzyme expression by CD161++IL-18Rα+ CD8+ T cells.

Upon stimulation of sorted cells with anti-CD3 and anti-CD28 antibodies in combination with IL-12 and IL-18, CD161++IL-18Rα+ memory CD8+ T cells like CD161-IL-18Rα+ CD8+ T cells were capable of producing IFN-γ, TNF-α, CXCL10 (IP-10), CCL3 (MIP-1β), CCL4 (MIP-1α) and CCL5 (Rantes) (Fig. 4A). CD161++IL-18Rα+CD8+ T cells produced very little IFN-γ when stimulated with anti-CD3 and anti-CD28 antibodies alone, whereas CD161++IL-18Rα+ memory CD8+ T cells made more, which is in accordance with the different activation requirements of these cells. Various type 2 cytokines, as well as IL-17 and IL-10, were hardly detectable in the supernatant of either CD161--IL-18Rα+ memory CD8+ T cells or CD161++IL-18Rα+ memory CD8+ T cells (Fig. 4B and C).

CD161++IL-18Rα+ CD8+ T cells contain granzyme A and granzyme K but no granzyme B (Fig. 4E), which coincides with their CD27--CD45RA+ memory phenotype (15–17). Furthermore, they express KLRG1 (Fig. 1E), a marker expressed on antigen-experienced T cells that is described to be a marker for senescence (18) and which might play an inhibitory role (19).

To analyse the molecular basis for the differentiation state of the CD161++IL-18Rα+ CD8+ cells, we investigated the expression of the key transcription factors Blimp-1, T-bet, Eomes and ZNF683, which are known to be differentially, regulated between memory and effector cells (20, 21). We observed significantly more Blimp-1 to be expressed on CD161++IL-18Rα+ memory CD8+ T cells than CD161-IL-18Rα+ cells (Fig. 5A), T-bet and Eomes were expressed equally on both subsets (Fig. 5B and C). ZNF683 was not expressed at all in the CD161++IL-18Rα+ memory CD8+ T cells (Fig. 5D).
Fig. 2. CD161⁺⁺IL-18Rα⁺⁺ memory CD8⁺ T cells require a 'third' signal to start proliferating. Sorted CFSE-labelled CD161⁺⁺IL-18Rα⁺⁺ CD27⁺CD45RA⁻ CD8bright cells (black bars) and CD161⁻ IL-18Rα⁻ CD27⁺CD45RA⁻ CD8bright cells (grey bars) were stimulated with anti-CD3/anti-CD28 for 3 days: (A) in the presence and absence of CD8⁻ cells, (B) in a transwell system using a 0.4 µm pore filter, in the presence of CD8⁻ cells, (C) in the presence of sorted CD4⁺ T cells (CD4⁺CD14⁻), monocytes (CD14⁺) and a combination of the latter, (D) in the presence of IL-2, IL-21 and a combination of the latter, (E) in the presence of IL-7, IL-15, IL-12, IL-18, the combination of IL-12 and IL-18; and the combination of IL-12 and IL-18 in absence of anti-CD3/anti-CD28 and (F) in the presence of CD4⁺ T cells in combination with mDCs and/or pDCs. (G) Sorted cells stimulated for 7 days with IL-7 and IL-15. In all figures, bars depict mean and error bars depict SD and all figures are representative for three independent experiments. On the Y-axis, the proliferation rate is expressed in precursor frequency. Statistically significant differences were determined by the Mann-Whitney test. *P < 0.05, **P < 0.01, ***P < 0.005, ns = difference is not significant.
Overall, CD161++ IL-18Rα+ CD8+ T cells predominantly display phenotypic characteristics of differentiated Th1 cytokine-producing cells that are antigen experienced in vivo. However, based on the expression of CD27 and the absence of both CX3CR1 and granzyme B, they are markedly distinct from effector-type cells that are prototypic for the cellular response against CMV (21, 22).

CD161++ IL-18Rα+ memory CD8+ T cells have shorter telomeres and less telomerase activity than the 'classical' memory CD8+ T cells.

When cells differentiate, they lose their self-renewing capacity, telomere length and telomerase activity. Stem cells can selectively preserve their telomeres by high telomerase activity. Here, we questioned whether the CD161++ CD8+ T cells also possess this quality.

To analyse telomere length, we performed a FACS-based in situ hybridization technique. Despite the comprehensive hybridization protocol, we were still able to distinguish CD161++ memory CD8+ T cells from the CD161+ memory CD8+ T cells (Fig. 6A). We calculated a relative telomere length of the different CD8+ T cell subsets as fold change compared with the naïve CD8+ T cells. As expected, naïve CD8+ T cells had the highest mean fluorescent intensity for the telomere Cy5 probe, meaning the longest telomere length, followed by memory cells and subsequently the effector cells with the shortest telomere length (Fig. 6B and C). CD161++ memory CD8+ T cells consistently had a shorter relative telomere length than the CD161+ memory CD8+ T cells (P = 0.02) (Fig. 6B and C).

Using the TRAP assay, telomerase activity was studied after 7 days of stimulation with IL-15. We observed that upon stimulation with IL-15, CD161++IL-18Rα+ memory CD8+ T cells had...
Fig. 4. CD161++ IL-18Rαα memory CD8+ T cells have a Th1 profile. Sorted CD161++IL-18Rαα−CD27−CD45RA− CD8bright cells (black bars) and CD161−IL-18Rα−CD27−CD45RA− CD8bright cells (grey bars) were stimulated with anti-CD3/anti-CD28 for 3 days in the presence or absence of the combination of IL-12 and IL-18 or with the combination of IL-12 and IL-18 alone. On the supernatants of this experiment, we performed luminex analysis. The y-axis shows the concentration of (A) IFN-γ, TNF-α, CXCL10, CCL3, CCL4 and CCL5, (B) IL-4, IL-5 and IL-13 and (C) IL-17 and IL-10. Triangles on top of the bars in (A) indicate that these values were found to be above the detection rate. In all figures, mean and SD are shown and all figures are representative for three independent experiments.
a significantly lower telomerase activity than the CD161−IL-18Rα− memory CD8+ T cells (P = 0.01) (Fig. 6D and E).

In summary, CD161++IL-18Rα+ memory CD8+ T cells have shorter telomeres and less telomerase activity compared with ‘classical’ memory CD8+ T cells and thus, in this respect, do not resemble stem cells.

CD161++IL-18Rα+ memory CD8+ T cells do not express ALDH

ALDHs are a family of enzymes involved in detoxifying a wide variety of aldehydes (23). ALDH was first recognized as a marker for HSC and is currently also used as a marker for cancer stem cells (24). To investigate whether CD161++IL-18Rα+ memory CD8+ T cells express ALDH, we performed the ALDEFLUOR assay. Within the total PBMC fraction, we were able to detect a small population of ALDH positive cells (Fig. 7A), but we did not observe any ALDH positive cells within the CD161++IL-18Rα+ memory CD8+ T cells (Fig. 7B). As a control, we added diethylaminobenzaldehyde (DEAB), which blocks the ALDH activity.

In conclusion, CD161++IL-18Rα+ memory CD8+ T cells are not able to detoxify aldehydes using ALDH, again lacking similarity with classical stem cells.

Discussion

CD161++IL-18Rα+ memory CD8+ T cells form a distinct subset of CD8+ T cells that resemble HSC by over-expressing the ABC-B1 transporter and cKit (4). Apart from these two features, we did not identify any other characteristics that mark these cells as stem cells. Rather, our data suggest that this population mainly contains T\(_1\) cytokine-producing memory-type cells with very stringent activation requirements that, based on chemokine receptor expression, have the ability to migrate to inflamed tissue.

We showed that CD161++IL-18Rα+ memory CD8+ T cells reside in peripheral blood, bone marrow and spleen, but significantly less in lymph nodes. Dusseaux et al. have demonstrated that they are present in significant numbers in intestinal tissue and that their presence is even higher in the liver (10). Furthermore, the chemokine receptor expression pattern of CD161++IL-18Rα+ memory phenotype CD8+ T cells, CCR5\(^+\), CCR6\(^-\), CXCR6\(^+\) and CCR7\(^-\), makes these cells well equipped to directly migrate to inflamed tissue (25–27). The ABC-B1 transporter may be highly relevant for the functioning of these cells at these sites as it can actively protect these cells against toxic substances they encounter in the liver, gut and at sites of inflammation.

Contradictory reports have been published regarding the pathogen specificity of CD161++IL-18Rα+ CD8+ T cells (3, 4, 10, 28). We here demonstrate that when analysing large amounts of lymphocytes (>5 × 10\(^E6\)), there are few but detectable FLU- and CMV-specific cells present within the CD161++IL-18Rα+ CD8+ T cell subset. Further, after 7 days of stimulation with viral peptides and IL-2, a clear population of virus-specific cells is demonstrable. From this, we conclude that CD161++IL-18Rα+ CD8+ T cells contain virus-specific cells, but in very small numbers. At this moment, we cannot exclude that a few of the semi-invariant T cells also recognize viral peptides presented by classic MHC class I molecules or alternatively that the CD161++IL-18Rα+ memory CD8+ T cell population is heterogeneous and contains low numbers of classical virus-specific cells.

Strikingly, we found that CD161++IL-18Rα+ memory CD8+ T cells are not easily activated. Besides TCR stimulation and co-stimulation, they required a ‘third signal’ to proliferate. This could be provided by the \(\gamma\)\(\delta\) cytokines IL-2, IL-21 or IL-15 or the T\(_1\)-inducing cytokines IL-12 and IL-18. Recently, it was shown that TCR signalling regulation in CD161++CD8+ T cells could be overcome by innate stimulation through CD28 or cytokines (29). Upon activation of the CD161++IL-18Rα+ memory CD8+ T cells predominantly had a T\(_1\) cytokine-producing profile. Various other studies have demonstrated that
Fig. 6. CD161++ IL-18Rα memory CD8+ T cells have more telomere erosion. (A) Gating strategy of lymphocytes, CD3+CD8+ cells and CD27-CD45RA− naïve, CD27+CD45RA− memory and CD27− effector CD8+ T cells and CD161+ memory CD8+ T cells. (B) Histogram of Telomere Cy5 expression gated on naïve (black line), memory (dotted line) and effector CD8+ T cells (interrupted line) in the left graph and in the second graph, CD161-IL-18Rα− (black line) and CD161++IL-18Rα+ (dotted grey line) memory CD8+ T cells (right graph). (C) Telomere length expressed as fold change of the geomean as compared to naïve CD8+ T cells. (D) Sort purified CD161++IL-18Rα+ memory CD8+ T cells (+) and CD161− IL-18Rα− memory CD8+ T cells (−) of five healthy donors (HD1 to 5) were stimulated with IL-15. The samples were collected 7 days after stimulation and were adjusted to 500 proliferating Ki67+ cells per reaction for analysis of telomerase activity, which was determined using the TRAPeze telomerase detection kit. The negative control (−control) consists of the PCR mix and template with no cell extract added. The positive control (+control) consists of telomerase-positive control cells provided with the TRAPeze kit. (E) Relative telomerase activity of CD161−IL-18Rα− (black squares) compared to CD161++IL-18Rα+ (grey squares) memory CD8+ T cells. In (C) mean and SD are shown. (B and C) are representative for seven independent experiments. Statistical analysis; Wilcoxon signed rank test, *P < 0.05.
CD161++IL-18Rα+ CD8+ T cells not only produce Th1 cytokines but also IL-17 in response to mitogen stimulation (9, 10). This discrepancy can possibly be explained by the difference in culture conditions, as we applied a Th1-inducing stimulus. The more stringently regulated activation requirements might prevent excessive tissue damage.

In contrast to HSC, CD161++IL-18Rα+ memory CD8+ T cells were not capable of preserving their telomeres by high expression of telomerase. Interestingly, the CD161++IL-18Rα+ memory CD8+ T cells even had significantly more telomere erosion and less telomerase activity compared with ‘classical’ memory CD8+ T cells. This may mean that CD161++IL-18Rα+ memory CD8+ T cells have a longer replicative history or are further differentiated compared with the ‘classical’ memory CD8+ T cells. Recent data demonstrate that MAIT cells originate from a very small pool of precommitted CD161++CD8αβ+ T cells in cord blood (30), indicating that they indeed may have a long replicative history. Increased telomere erosion has previously been found in the highly differentiated CD28−CD8+ T cells (31, 32) and progressive reduction in telomere length occurs in the differentiation of naïve cells to memory to effector T cells (33). In addition, we showed that CD161++IL-18Rα+ memory CD8+ T cells express KLRG1 and that they express significantly more BLIMP-1 than the ‘classical’ memory CD8+ T cells. KLRG1 is a cell-surface marker known to identify antigen-experienced T cells with reduced proliferative capacity and may play an inhibitory role in human T cells (19). KLRG1-expressing cells are largely CD28− but there is also a small group of CD28−CD8+ T cells that express KLRG1 (18). BLIMP-1 is also preferentially expressed in terminally differentiated effector-type CD8+ T cells and is important for maintenance of homeostasis, preventing the accumulation of activated cells (20). Short telomere length, KLRG1 expression, lack of proliferative capacity and high BLIMP-1 expression are all, possibly related, features of the CD161++IL-18Rα+ CD8+ T cells that coincide with highly differentiated effector-type CD8+ T cells (18, 20, 34). On the contrary, CD161++IL-18Rα+ CD8+ T cells also express co-stimulatory markers such as CD28 and CD27, highly express CD127 and do not contain effector molecules such as granzyme B, which are all phenotypical characteristics of memory CD8+ T cells (22).

ALDH is involved in the detoxification of aldehydes, including xenobiotics. HSC and also cancer stem cells highly express ALDH (24). Cancer stem cells are a subpopulation of tumour cells, which possess stem-cell characteristics and are resistant to chemotherapy (35). We here demonstrate that CD161++CD8+ T cells do not express higher levels of ALDH. Furthermore, we subsequently analysed the expression of CD24, CD44, CD133 and CD166, which are markers used to identify human cancer stem cells (35, 36). Besides a slightly higher expression of CD44 on the CD161++IL-18Rα+CD8+ T cells, we did not find any differences between CD161++IL-18Rα+ and ‘classical’ memory CD8+ T cells (data not shown). These data are yet another argument that CD161++IL-18Rα+ memory CD8+ T cells are not stem cells.

More recently a long-lived memory T-cell subset, within the naïve-like T-cell compartment, has been identified. The cells contained within this subset possess various stem-cell-like properties and may represent true memory stem cells (37). We conclude that except for the expression of the ABC-B1 transporter, CD161++IL-18Rα+ CD8+ T cells do not have any other characteristics marking them as stem cells. ABC-B1 expressing CD8+ T cells may be evolutionary selected by the high exposure to xenobiotics at sites of inflammation and in the liver and gut. At these sites, there is likely to be more exposure to antigens by which they are constantly triggered. This repeated activation and proliferation may explain their highly differentiated phenotype, as suggested by an increase in KLRG1 expression, high BLIMP-1 expression and telomere erosion.

**Supplementary data**

Supplementary data are available at International Immunology Online.

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