Immune responsiveness in immunosuppressed patients

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CHAPTER 1: CD4^{DULL}CD8^{BRIGHT} DOUBLE POSITIVE T-LYMPHOGRANZYMEE B^{POS}CD8^{POS} MEMORY T-LYMPHOGRANZYMEE B^{POS}CD8^{POS} MEMORY T-LYMPHOGRANZYMEE B^{POS}CD8^{POS} MEMORY T-LYMPHOGRANZYMEE B^{POS}CD8^{POS} MEMORY T-LYMPHOGRANZYMEE B^{POS}CD8^{POS} MEMORY T-LYMPHOCYTES

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Abstract

Background: T-lymphocytes that co-express CD4 and CD8 antigens may be found in small percentages in the peripheral blood of healthy individuals, and have a CD4\textsuperscript{bright}CD8\textsuperscript{dull} phenotype. CD4\textsuperscript{dull}CD8\textsuperscript{bright} T-lymphocytes have been found only in temporal association with some viral infections.

Methods: Four-colour flow cytometric analysis of peripheral blood mononuclear cells from a renal transplant recipient with cytomegalovirus infection was performed.

Results: A small but clearly distinguishable subpopulation of CD4\textsuperscript{dull}CD8\textsuperscript{bright} double positive T lymphocytes was detected, that exhibited phenotypic characteristics of cytotoxic T-lymphocytes and were granzyme B positive. Furthermore, no naive cells appeared to be present within this subpopulation.

Conclusions: CD4\textsuperscript{dull}CD8\textsuperscript{bright} double positive T lymphocytes are enriched for memory and effector cytotoxic T cells.
**Introduction**

One of the developmental stages, through which thymocytes go, is marked by the expression of both CD4 and CD8 on their cell surface. When they migrate to the peripheral blood compartment, thymocytes loose expression of one of these two molecules and become either CD4 or CD8 single positive T-lymphocytes [1]. However, CD4CD8 double positive (DP) T-lymphocytes may also be present in the peripheral blood compartment of adult individuals [2]. Concomitant expression of CD4 and CD8 on the cell surface of these T-lymphocytes has been regarded as a representation of immaturity. In an extensive survey on peripheral blood lymphocytes from about 7500 individuals, Ortolani et al. could detect a subpopulation of CD4CD8 DP T-lymphocytes in 52 of them. Based on the phenotype in flow cytometric analysis, they made a distinction between two types of DP T-lymphocytes. In one type, the expression of CD4 was high and identical to that of CD4 single positive T-lymphocytes in the same sample; expression of CD8 was low, defining that subset as CD4\textsuperscript{bright}CD8\textsuperscript{dull} DP cells. In the other type, the situation was the reverse, defining that subset as CD4\textsuperscript{dull}CD8\textsuperscript{bright} DP T-cells. The first type of DP cells was found in apparently healthy individuals. In contrast, CD4\textsuperscript{dull}CD8\textsuperscript{bright} DP T-lymphocytes were found only in association with acute Epstein-Barr virus (EBV) - or cytomegalovirus (CMV) infection and disappeared after resolution of the lymphocytosis [3]. These CD4\textsuperscript{dull}CD8\textsuperscript{bright} DP T-lymphocytes expressed high amounts of HLA-DR - and CD38 activation antigens. Also, virtually all CD4\textsuperscript{dull}CD8\textsuperscript{bright} DP T-lymphocytes were CD45R0\textsuperscript{pos}.

Nowadays, various reports indicate that phenotypic characterisation of memory CD8\textsuperscript{pos} T-lymphocytes should be based on at least two cell surface parameters [4-6]. Accordingly, CD8\textsuperscript{pos} naive T-lymphocytes are defined as CD11a\textsuperscript{dull}CD45RA\textsuperscript{pos} or CD27\textsuperscript{pos}CD45RA\textsuperscript{pos} [4-6]. CD8\textsuperscript{pos} memory cells are defined as CD27\textsuperscript{pos}CD45RA\textsuperscript{neg}, whereas CD8\textsuperscript{pos} effector cells are defined as CD27\textsuperscript{neg}CD45RA\textsuperscript{pos} [4]

Here, we present data on these and other phenotypic characteristics of CD4\textsuperscript{dull}CD8\textsuperscript{bright} DP T-lymphocytes found in a renal transplant recipient, showing that these cells are depleted of naive cells and enriched for memory and effector cytotoxic T-lymphocytes.
Subjects and methods

Patient

The patient presented, forms part of a group of renal transplant recipients involved in a study in which peripheral blood mononuclear cells were collected and frozen (a) three months after transplantation (b) before treatment of an acute rejection episode and (c) during stable transplant function. In this particular patient CMV re-infection appeared to have developed before time point (a).

The patient is a 62-year-old woman with end-stage renal disease caused by adult onset, steroid-resistant minimal change glomerulonephritis. She was admitted to the hospital to receive her first renal transplant. The immunosuppressive regimen post-transplantation consisted of 10 mg prednisolone orally once daily and ciclosporin (Neoral®) orally once daily (dose adjusted to a serum trough level of 100 - 150 µg/L). Before transplantation, IgG anti-CMV, IgG anti-EBV, and IgG anti-HSV antibodies were present. After transplantation, her clinical course was uncomplicated, until an asymptomatic secondary CMV infection was diagnosed 8 weeks after transplantation. The diagnosis was based on a positive urine culture for CMV and, retrospectively, a significant rise in complement binding anti-CMV antibodies in serum. Thirty-eight days later, urine- and blood cultures for CMV were negative, but the complement binding anti-CMV antibody titre remained elevated up to 37 weeks after transplantation. Twenty-nine weeks after transplantation, an acute cellular rejection was diagnosed and confirmed by histology. Rejection treatment, consisting of a three-day course of 1000 mg methylprednisolone daily i.v., was initiated, resulting in a significant decrease in serum creatinine level. After rejection treatment, azathioprine (Imuran®) 100 mg orally once daily was added to her immunosuppressive regimen. Up to now this patient is doing well, with a good functioning kidney graft. During follow-up, no reactivation of either EBV or HSV was diagnosed.

Isolation of PBMC

Peripheral blood samples were drawn in sterile lithium heparin containing tubes at time points indicated above. Heparinized blood was diluted 1:1 in Hank's Balanced Salt Solution, (Bio Whittaker, Verviers, Belgium) supplemented with 0,025 M tris(hydroxymethyl)aminomethane (Merck, Darmstadt, Germany), 100 U/mL sodium penicillin G (Brocades Pharma B.V, Leiderdorp, The Netherlands), 100 µg/mL streptomycin sulphate (Gibco BRL, Paisley, Scotland) (HBSS/TRIS/P/S) in sterile conical polypropylene 50 mL tubes (Falcon, BD Labware, New Jersey, USA). Diluted blood was layered on Lymphoprep (Nycomed, Pharma, Oslo, Norway) for density gradient centrifugation. The PBMC interface was harvested and washed twice in HBSS/TRIS/P/S supplemented with 5%
CD4^{dull}CD8^{bright} double positive T-lymphocytes have a phenotype of Granzyme B^{pos}CD8^{pos} memory T-lymphocytes

foetal calf serum (FCS, Integro, Zaandam, The Netherlands). Subsequently, cells were resuspended in HBSS/TRIS/P/S supplemented with 20% foetal clone serum (FCLS, HyClone Laboratories Inc., Logan, Utah, USA), and frozen in HBSS/TRIS/P/S supplemented with 20% FCS and 10% (v/v) dimethylsulphoxid (DMSO, Merck) in polypropylene biofreeze vials (Costar, Cambridge, UK), until the day of analysis.

Staining of surface molecules
Cells were rapidly thawed, diluted in HBSS/TRIS/P/S supplemented with 20% FCS and incubated for 20 minutes at room temperature to optimally remove DMSO. Subsequently, the cells were washed twice in HBSS/TRIS/P/S supplemented with 5% FCS and resuspended in phosphate buffered saline (PBS), 0.5% (w/v) bovine serum albumin (BSA, Sigma Chemical, St. Louis, USA) and 0.01% (w/v) sodium azide (NaN₃, Merck) (PBS-BSA-NaNa₃) in a concentration of 2 x 10⁶ cells/mL. Next, volumes of 100 µL cell suspension were distributed over polystyrene round bottom tubes (Falcon, BD Labware). Cells were incubated with 10% (v/v) human pool serum (HPS, Bio Whittaker, Walkerville, MD, USA) in PBS-BSA-NaNa₃ for 10 minutes at 4°C, protected from light. Cells were incubated with fluorescent dye conjugated monoclonal antibodies directed against cell surface antigens or appropriate isotype control antibodies for 30 minutes at 4°C, protected from light and washed in PBS-BSA-NaNa₃.

Staining of the intracellular antigen granzyme B
After surface staining, cells were fixed in 1% (w/v) paraformaldehyde (PFA, Merck) in PBS for 10 minutes at 4°C, protected from light. Subsequently, cells were permeabilised in PBS-BSA-NaNa₃ supplemented with 0.1% (w/v) saponin (Sigma Chemical) and 50 mmol/L D-glucose (Merck) ("saponin buffer"). Cells were incubated with 10% (v/v) HPS in saponin buffer for 10 minutes at 4°C, protected from light. Phycoerythrin (PE)-conjugated anti-GrB monoclonal antibody or appropriate isotype control antibody was added and cells were incubated for 1 hour at 4°C, protected from light. Next, cells were washed in saponin buffer.

Monoclonal antibodies and second step reagents
The following mouse anti-human monoclonal antibodies, mouse Ig fluorescence control antibodies and second step reagents, purchased from Becton Dickinson Immunocytometry Systems, were used: Fluorescein isothiocyanate (FITC)-conjugated CD2, FITC- and PE-conjugated CD3, FITC- and allophycocyanin (APC)-conjugated CD4, FITC-, PE- and peridinin chlorophyll protein (PerCP)-conjugated CD8, PE-conjugated CD16, FITC-conjugated CD25, PE-conjugated CD38, PE-conjugated CD45R0, FITC-conjugated CD45RA, FITC-conjugated CD57, FITC-conjugated CD69, FITC-conjugated anti-TCRαβ, PE-conjugated anti-TCRγδ, PE-conjugated anti-HLA-DR, FITC-, PE-, PerCP-and APC-
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Conjugated control mouse IgG1, FITC- and PE-conjugated control mouse IgG2a. All control antibodies were raised against keyhole limpet haemocyanin (KLH). Other monoclonal antibodies were purchased as follows. PE-conjugated CD16 (Instruchemie, Hilversum, The Netherlands), PE-conjugated CD1a (Instruchemie), FITC-conjugated CD11a (Dako, Glostrup, Denmark), PE-conjugated CD45RA (Immunotech (Coulter), France). PE-conjugated anti-GrB monoclonal antibody GrB-11 (mouse IgG1) has been described [7].

Flow cytometric analysis
Cells were analysed on a dual laser flow cytometer (FACS® Calibur, Becton Dickinson) equipped with a 488 nm argon ion laser and a 635 nm red diode laser. Photomultiplier tube voltages were adjusted using cells stained only with fluorescent dye-conjugated irrelevant (control) monoclonal antibodies. Electronic compensation was adjusted using cells labelled with a single fluorescent dye-conjugated monoclonal antibody. Cellquest (Becton Dickinson) software was used for data acquisition. List mode data files of ~ 50,000 events were stored containing forward light scatter (FSC), orthogonal light scatter (SSC) and four fluorescence parameters. Data were analysed using Cellquest software. A lymphocyte gate was drawn based on forward light scatter and orthogonal light scatter parameters. Within this lymphocyte gate, cells were selected on CD4 single positivity, CD8bright single positivity or CD4dullCD8bright double positivity. In addition, cells in the lymphocyte gate were simultaneously analysed for one or two other fluorescent parameters.

Virologic evaluation
Virologic evaluation was performed according to standard procedures.
Results

CD4^{dull}CD8^{bright} double positive T-lymphocytes were first detected in an amount of 3.5% of the total lymphocyte population (fig. 1a). This T-lymphocyte subpopulation could not be observed in seven renal transplant recipients suffering from acute CMV infection. A representative CD4 versus CD8 dotplot of one of these patients is shown in figure 1b. The DP population remained detectable for at least 7 months. The CD4^{dull}CD8^{bright} DP T-lymphocytes remained a stable fraction of the total CD8^{bright} lymphocytes, i.e. 8, 10 and 11 percent of all CD8^{bright} (DP included) lymphocytes at three different time points. Just before initiation of rejection treatment the percentage amounted to 5%, and at 37 weeks after transplantation it was 3.5% again. The DP cells lacked CD1a expression. In addition, CD4^{dull}CD8^{bright} T-lymphocytes expressed high frequencies of CD38, CD69 and HLA-DR, markers that are commonly associated with activated cytotoxic T-lymphocytes (table 1).

![Figure 1](image-url)

Panel a: CD4^{dull}CD8^{bright} DP T-lymphocyte population at time point (b) (see text). Dot plot of lymphocytes with respect to CD4 (X-axis) and CD8 (Y-axis) fluorescence (arbitrary units, log scale). Cells are gated based on these parameters in CD8^{bright} single positive (upper left, R2), CD4^{dull}CD8^{bright} DP (upper right, R3) and CD4 single positive (lower right, R4) lymphocytes. Panel b: Dot plot of lymphocytes from one of seven renal transplant recipients with respect to CD4 (X-axis) and CD8 (Y-axis) fluorescence (arbitrary units, log scale). No DP cells were present in these patients (suffering from acute CMV infections).
Table 1
Expression of surface markers and GrB within CD4<sub>dull</sub>CD8<sub>bright</sub> DP CD8<sub>bright</sub> single positive, and CD4 single positive lymphocytes, at several time points

<table>
<thead>
<tr>
<th></th>
<th>CD4&lt;sub&gt;dull&lt;/sub&gt;CD8&lt;sub&gt;bright&lt;/sub&gt;</th>
<th>CD8&lt;sub&gt;bright&lt;/sub&gt;</th>
<th>CD4&lt;sub&gt;pos&lt;/sub&gt;</th>
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<tbody>
<tr>
<td>CD1a</td>
<td>0%*</td>
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<tr>
<td>CD2</td>
<td>100%</td>
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<tr>
<td>CD3</td>
<td>100%</td>
<td>100%</td>
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<tr>
<td>TCRαβ</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>CD25</td>
<td>1-4%**</td>
<td>0-2%</td>
<td>1-43%</td>
</tr>
<tr>
<td>CD38</td>
<td>62-87%</td>
<td>64-84%</td>
<td>37-79%</td>
</tr>
<tr>
<td>CD69</td>
<td>1-17%</td>
<td>1-17%</td>
<td>1-2%</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>31-63%</td>
<td>12-66%</td>
<td>4-18%</td>
</tr>
<tr>
<td>GrB</td>
<td>70-92%</td>
<td>61-92%</td>
<td>6-19%</td>
</tr>
<tr>
<td>CD56</td>
<td>7-13%</td>
<td>4-7%</td>
<td>0%</td>
</tr>
<tr>
<td>CD57</td>
<td>64-83%</td>
<td>67-78%</td>
<td>4-13%</td>
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</table>

The numbers represent percentages of the designated markers within the CD4<sub>dull</sub>CD8<sub>bright</sub> DP, CD8<sub>bright</sub> single positive and CD4 single positive cells respectively. * Mean; ** range of the measurements at week 14, 28 and 37 after transplantation; GrB: granzyme B.

Figure 2 shows that the CD4<sub>dull</sub>CD8<sub>bright</sub> T-lymphocytes express granzyme B and CD57 in percentages comparable to CD8<sub>pos</sub> T lymphocytes.
CD4\textsuperscript{dull}CD8\textsuperscript{bright} double positive T-lymphocytes have a phenotype of Granzyme B\textsuperscript{pos}CD8\textsuperscript{pos} memory T-lymphocytes.

Figure 2
Expression of CD57, GrB and CD11a versus CD45RA by CD8\textsuperscript{bright} single positive, CD4\textsuperscript{dull}CD8\textsuperscript{bright} DP and CD4 single positive lymphocytes at time point (b) (see text). Based on fluorescence parameters, cells were gated in CD8\textsuperscript{bright} single positive, CD4\textsuperscript{dull}CD8\textsuperscript{bright} DP and CD4 single positive lymphocytes (R2, R3 and R4 refer to gates set like in fig. 1). Histograms are shown of CD57 fluorescence (A) and GrB fluorescence (B) (X axis, arbitrary units, log scales) versus cell number (Y axis) within the respective gates. Negative control was added before permeabilisation and used for instrument setup. Control antibody for intracellular staining is shown in (B). Dot plots of CD45RA fluorescence (X axis, arbitrary units, log scale) versus CD11a fluorescence (Y axis, arbitrary units, log scale) of cells within the respective gates (C). The subpopulation of CD45RA\textsuperscript{bright}CD11a\textsuperscript{dull} naive cells (indicated with an arrow) is typically present within CD4 and CD8\textsuperscript{bright} single positive T-lymphocytes but virtually absent in the DP population.

Using CD45RA\textsuperscript{bright}CD11a\textsuperscript{dull} expression as marker for naive T-lymphocytes, we could not detect naive cells in the CD4\textsuperscript{dull}CD8\textsuperscript{bright} DP subpopulation as opposed to their presence in the CD4 and CD8\textsuperscript{bright} single positive T-lymphocytes (fig. 2). Accordingly, as compared to CD8 positive T-lymphocytes, CD4\textsuperscript{dull}CD8\textsuperscript{bright} DP T-lymphocytes were impoverished for CD27\textsuperscript{pos}CD45RA\textsuperscript{pos} (16-25%, respectively 8-13%), and enriched for memory (8-15%)
respectively 10-19%) and effector cells (50-62% respectively 57-70%).

**Discussion**

In this study, we provide data indicating that CD4\textsuperscript{dull}CD8\textsuperscript{bright} DP T-lymphocytes may be present in the peripheral blood compartment, that are not immature, but - on the contrary - bear the characteristics of activated cytotoxic T-lymphocytes with a memory phenotype. These cells lack CD1a expression, a characteristic of late developmental stages of thymocytes. Moreover, these cells are enriched for memory and effector lymphocytes expressing high levels of cytotoxic cell granule constituent granzyme B [7]. We therefore hypothesise that these cells are CD8\textsuperscript{pos} cytotoxic T-lymphocytes that acquired CD4 on their cell surface.

The presence of CD4\textsuperscript{dull}CD8\textsuperscript{bright} DP T-lymphocytes in peripheral blood in vivo has been described before, but their functional characteristics were not examined. They appeared strongly associated with acute EBV or CMV infection [3]. The latter association could not be confirmed in our patient. However, she underwent a secondary CMV infection in the month before testing. Thus, though not proven, the presence of DP cells in her circulation may be related to previous CMV infection.

In vitro, CMV can neither infect CD8\textsuperscript{pos} T-lymphocytes completely and productively, nor induce CD4 expression in these cells [8]. In contrast, many other stimuli do induce CD4 expression on CD8\textsuperscript{pos} T-lymphocytes in vitro. Among these are T-cell receptor (TCR) triggering of CD8\textsuperscript{pos} T-lymphocytes by superantigens, by CD3 or PHA [9]. Furthermore, human herpes virus-6 (HHV-6) and human T cell leukaemia virus type-1 (HTLV-1) infection induce expression of CD4 in CD8\textsuperscript{pos} T-lymphocytes in vitro [8,10]. We have no information regarding our patient’s HHV-6 and HTLV-1 serology. These in vitro findings, however, do not satisfactorily explain the presence of the DP T-lymphocytes in our patient. For, many TCR’s are triggered in systemic infections. Still, peripheral blood derived CD4\textsuperscript{dull}CD8\textsuperscript{bright} DP T-lymphocytes remain a rarely encountered observation. In our population of renal transplant recipients we did not observe this population in 7 other patients who underwent primary or secondary CMV infection. Second, if HHV-6 or HTLV-1 causes the appearance of CD4\textsuperscript{dull}CD8\textsuperscript{bright} DP T-lymphocytes in vivo, it does not explain the previously described strong association between the appearance of CD4\textsuperscript{dull}CD8\textsuperscript{bright} DP T-lymphocytes and just two other viral infections, EBV- and CMV- infection.

In our patient, the CD4\textsuperscript{dull}CD8\textsuperscript{bright} DP T-lymphocytes may have arisen during primary CMV infection and subsequently became an undetectable subpopulation of CD8\textsuperscript{pos} T-lymphocytes. On CMV reactivation, the DP cells may have become detectable again because of expansion of CD8\textsuperscript{pos} lymphocytes accompanying such an infection. This is in accordance with the fact that in all tested peripheral blood samples the DP cells remained a relatively stable fraction of
CD4^{dull}CD8^{bright} \text{ double positive T-lymphocytes have a phenotype of Granzyme B^{pos}CD8^{pos}} \text{ memory T-lymphocytes}

the total CD8^{bright} T-lymphocyte subset. Recently, studies using labelled MHC class I-peptide tetrameres showed that during viral infections the majority of the expanded CD8^{pos} T-lymphocytes are antigen-specific [11]. Analogously, CD4^{dull}CD8^{bright} DP T-lymphocytes, rising and decreasing as part of the CD8 lymphocytosis may contain virus-specific T-lymphocytes. This fits with the present finding that CD4^{dull}CD8^{bright} DP T-lymphocytes are activated, non-naive, granzyme B^{pos} T-lymphocytes.

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References


Chapter 1

