

# Functional profile of human influenza virus-specific cytotoxic T lymphocyte activity is influenced by interleukin-2 concentration and epitope specificity

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## Summary

The ability of influenza A virus-specific cytotoxic T lymphocytes (CTL) to degranulate and produce cytokines upon antigenic restimulation was studied in four HLA-A\*0101 and HLA-A\*0201 positive subjects. Peripheral blood mononuclear cells of these subjects were stimulated with influenza A virus in the presence of high or low interleukin (IL)-2 concentrations. CD8<sup>+</sup> T cell populations specific for the HLA-A\*0101 restricted epitope NP<sub>44-52</sub> and the HLA-A\*0201 restricted epitope M1<sub>58-66</sub> were identified by positive staining with tetramers of peptide major histocompatibility complexes (MHC) (NP-Tm and M1-Tm, respectively). Within these populations, the proportion of cells mobilizing CD107a, or expressing interferon (IFN)- $\gamma$  and tumour necrosis factor-(TNF)- $\alpha$  upon short-term peptide restimulation was determined by flow cytometry. Independent of IL-2 concentrations, large subject-dependent differences in the mobilization of CD107a and expression of IFN- $\gamma$  and TNF- $\alpha$  by both NP- and M1-specific T cells were observed. In two of the four subjects, the functional profile of NP-Tm<sup>+</sup> and M1-Tm<sup>+</sup> cells differed considerably. Overall, no difference in the proportion of NP-Tm<sup>+</sup> or M1-Tm<sup>+</sup> cells expressing CD107a was observed. The proportion of M1-Tm<sup>+</sup> cells that produced IFN- $\gamma$  ( $P < 0.05$ ) was larger than for NP-Tm<sup>+</sup> cells, independent of IL-2 concentration. When cultured under IL-2<sub>hi</sub> concentrations higher TNF- $\alpha$  expression was also observed in M1-Tm<sup>+</sup> cells ( $P < 0.05$ ). The IL-2 concentration during expansion of virus-specific cells had a profound effect on the functionality of both M1-Tm<sup>+</sup> and NP-Tm<sup>+</sup> cells.

**Keywords:** CD107a, cytokines, cytotoxic T lymphocytes, epitopes, influenza virus

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## Introduction

Cytotoxic T lymphocytes (CTL) play an important role in the control of virus infections, including those caused by influenza viruses [1,2]. Important effector cell functions include the killing of virus-infected cells through the excretion of perforin and granzyme molecules or induction of apoptosis through Fas/FasL signalling, and the production of cytokines such as interferon (IFN)- $\gamma$  and tumour necrosis factor (TNF)- $\alpha$  [3].

Recent research has identified differential expression of CD45R isoforms, CCR7, CD62L, CD27, CXCR4 and CD28 on functionally different CD8<sup>+</sup> T cell populations [4–12], which have been associated with consecutive stages of human CD8<sup>+</sup> T cell development during and after acute viral

infections. Upon infection naive, effector and memory cells, each with their own characteristics regarding cytokine production, proliferation and effector function, were discriminated. Peptide-HLA class I tetramers (Tm) have also been used to study CTL populations specific for distinct viral epitopes from various viruses (human immunodeficiency virus (HIV), cytomegalovirus (CMV), Epstein–Barr virus (EBV), hepatitis C virus and influenza A virus [13–17], demonstrating differences in perforin and granzyme expression, lytic capacity, cytokine production and cell surface molecule expression between the different epitope-specific CTL populations. In mice, CTL specific for the F- or G-protein of respiratory syncytial virus (RSV) produced less IFN- $\gamma$  than CTL specific for an epitope of influenza A virus [18]. It is unknown, however, whether these functional and

phenotypical differences in CTL are the result of different virus aetiologies or epitope/HLA-specificities or both. Limited data are available on functional heterogeneity in CTL populations specific for various epitopes from a single virus [16,19–22]. Differences in cell surface marker expression and/or cytokine production in human CTL specific for different EBV epitopes and mouse CTL specific for two different influenza virus epitopes have been reported [20,22]. We also demonstrated differences in cytokine expression profiles for influenza virus-specific CTL restricted by different HLA class I molecules. CTL specific for HLA-A1 restricted epitopes produced more TNF- $\alpha$  than IFN- $\gamma$ , while HLA-B8, -B35 and -A2 restricted epitope-specific CTL produced mainly IFN- $\gamma$  and to a lesser extent TNF- $\alpha$  [23].

The roles of microenvironment, T cell receptor (TCR) avidity, peptide concentration, and co-receptor dependence in defining CTL effector functions are still largely unclear. Some studies have demonstrated a positive relationship between TCR avidity and functional profiles of epitope-specific CTL [24], while other studies dispute this relationship and suggest other factors involved in determining the functional profile of CTL [25]. Cytokines such as IL-2 may also be important, as it was shown in mice that the addition of exogenous IL-2 resulted in higher TNF- $\alpha$  expression in CTL [26].

Here the functional profile of polyclonal human CTL populations specific for two different influenza A virus CTL epitopes was investigated in multiple HLA-A\*0101/A\*0201 positive subjects. In addition, the influence of IL-2 concentration during the *in vitro* expansion of these cells on the lytic activity and cytokine expression in these CTL was assessed.

## Materials and methods

### Cells, virus, peptides and tetramers

Four healthy blood donors, between 35 and 50 years of age, were selected according to genetic homology within the A-locus of human leucocyte antigen (HLA) class I molecules and their CTL response to epitopes NP<sub>44–52</sub> and M1<sub>58–66</sub> [23,27]. Genetic subtyping was performed using a commercial typing system (Genovision, Vienna, Austria). Peripheral blood mononuclear cells (PBMC) were isolated from whole blood by Lymphoprep™ (Nycomed, Oslo, Norway) density gradient centrifugation and cryopreserved at –135°C.

Sucrose gradient purified influenza A virus (H3N2) Resvir-9, a reassortant between influenza virus A/Nanchang/933/95 and A/Puerto Rico/8/34, was used for the infection of PBMC. The virus contains both the HLA-A\*0101-restricted epitope NP<sub>44–52</sub> (CTELKLSDY) and the HLA-A\*0201-restricted epitope M1<sub>58–66</sub> (GILGFVFTL).

Synthetic peptides representing the epitope NP<sub>44–52</sub> and the epitope M1<sub>58–66</sub> were manufactured, high profile liquid chromatography (HPLC) purified and analysed by mass

spectrometry (Eurogentec, Seraing, Belgium). Peptides were dissolved in dimethyl sulphoxide (DMSO) (5.0 mg/ml), diluted in RPMI-1640 medium (Cambrex, East Rutherford, NJ, USA) to 100  $\mu$ M and stored at –20°C until further use.

HLA-A\*0101 and HLA-A\*0201 molecules were complexed with the NP<sub>44–52</sub> and M1<sub>58–66</sub> peptide, respectively, as described previously [28]. Both HLA-A peptide complexes were enzymatically biotinylated, fast protein liquid chromatography (FPLC) purified and tetramerized by addition of phycoerythrin (PE)-conjugated streptavidin (Sanquin Research at CLB, Amsterdam, the Netherlands). The NP<sub>44–52</sub> peptide containing HLA-A\*0101 tetramers are referred to as NP-Tm, while the M1<sub>58–66</sub> peptide containing HLA-A\*0201 tetramers are referred to as M1-Tm.

### *In vitro* stimulation of PBMC with influenza A virus

Stimulation of PBMC with influenza A virus was performed as described previously [27]. Cells were resuspended at 10<sup>6</sup> cells/ml in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) (Greiner Bio-one, Alphen a/d Rijn, the Netherlands), 2 mM L-glutamine, 100  $\mu$ g/ml streptomycin and 100 IU/ml penicillin (Cambrex, East Rutherford, NJ, USA) (R10F) and infected with Resvir-9 at a multiplicity of infection of 3. After 1 h at 37°C, the cells were washed once and resuspended in RPMI-1640 medium supplemented with 10% pooled human AB serum, 2 mM L-glutamine, 100  $\mu$ g/ml streptomycin, 100 IU/ml penicillin and 20  $\mu$ M 2-ME (R10H), and added to uninfected PBMC at a ratio of 1 : 1 in a 25-cm<sup>2</sup> culture flask. After 2 days, 2 U/ml (IL-2<sub>lo</sub>) or 50 U/ml (IL-2<sub>hi</sub>) rIL-2 (Chiron BV Amsterdam, the Netherlands) was added and the cells were incubated for another 6 days at 37°C.

### CD107a mobilization assay

Degranulation by CTL was assessed by mobilization of CD107 [29,30]. Two hundred thousand influenza A virus-stimulated PBMC were incubated in R10F containing Golgistop™ (Monensin) (Becton Dickinson, Alphen a/d Rijn, the Netherlands), in the presence or absence of 10  $\mu$ M synthetic peptide for 5 h at 37°C. During the 5 h incubation, fluorescein isothiocyanate (FITC)-conjugated mouse-antihuman CD107a monoclonal antibody (mAb) (clone H4A3, Becton Dickinson) was added to each well. Next the cells were washed once in phosphate buffered saline (PBS) containing Golgistop and 2% FCS and incubated with NP-Tm or M1-Tm. After 20 min at 20°C, peridinin chlorophyll protein (PerCP)-conjugated mouse-antihuman CD3 mAb (clone SK7, Becton Dickinson) and allophycocyanin (APC)-conjugated mouse-antihuman CD8 mAb (clone DK25, Dako Cytomation, Glostrup, Denmark) were added for 10 min at 4°C. Following one washing step, the cells were acquired and analysed using a FACSCalibur (Becton Dickinson) flow cytometer in combination with CellQuest Pro software (Becton Dickinson). The specific percentage CD107a<sup>+</sup>

cells of the NP-Tm<sup>+</sup> or M1-Tm<sup>+</sup> population was calculated using the following formula:

$$\% \text{ CD107a}^+ \text{ cells}_{(\text{peptide stimulated})} - \% \text{ CD107a}^+ \text{ cells}_{(\text{unstimulated})}$$

### Intracellular cytokine staining

IFN- $\gamma$  and TNF- $\alpha$  expression by influenza A virus-specific CTL was determined using intracellular cytokine staining as described previously [23,31]. Four hundred thousand PBMC were incubated in 200  $\mu$ l R10F containing Golgistop<sup>TM</sup> or Golgiplug<sup>TM</sup> for the IFN- $\gamma$  staining or TNF- $\alpha$  staining, respectively, according to recommendations of the manufacturer. Restimulation of NP<sub>44-52</sub>-specific and M1<sub>58-66</sub>-specific effector cells was achieved by adding synthetic peptides to the wells at a concentration of 10  $\mu$ M. Following 6 h incubation at 37°C, the cells were washed once in PBS with 2% FCS and incubated with NP-Tm and M1-Tm complexes. After 20 min at 20°C, FITC-conjugated mouse-antihuman CD8 mAb (clone DK25, Dako Cytomation) and PerCP-conjugated mouse-antihuman CD3 mAb were added for 10 min at 4°C. Following two washing steps, the cells were fixed (Cytotfix<sup>TM</sup>, Becton Dickinson), permeabilized (Cytoperm<sup>TM</sup>, Becton Dickinson) and incubated with APC-conjugated mouse-antihuman IFN- $\gamma$  mAb (clone 4S.B3, Becton Dickinson) or APC-conjugated mouse-anti-human TNF- $\alpha$  mAb (clone 6401.1111, Becton Dickinson) for 30 min at 4°C. Next the cells were washed once in PBS with 2% FCS and subsequently acquired and analysed using a FACSCalibur (Becton Dickinson) flow cytometer in combination with CellQuest Pro software (Becton Dickinson). At least 50 000 events were acquired. The specific percentage IFN- $\gamma$  or TNF- $\alpha$ <sup>+</sup> cells of the NP-Tm<sup>+</sup> or M1-Tm<sup>+</sup> population was calculated using the following formula: % IFN- $\gamma$ <sup>+</sup> or TNF- $\alpha$ <sup>+</sup> cells<sub>(peptide stimulated)</sub> - % IFN- $\gamma$ <sup>+</sup> or TNF- $\alpha$ <sup>+</sup> cells<sub>(unstimulated)</sub>.

### Statistical analysis

Mann-Whitney *U*-test was applied to determine statistical significance between NP-Tm<sub>+</sub> or M1-Tm<sup>+</sup> cells of all four subjects or between IL-2<sub>lo</sub> or IL-2<sub>hi</sub> concentrations, using the average frequency of responding cells measured in two or three independently repeated assays. Significant differences within a single subject were determined by comparing the frequencies of both assays. A *P*-value below 0.05 was considered significant.

## Results

### No effect of IL-2 on proportion of NP-Tm<sup>+</sup> and M1-Tm<sup>+</sup> cells

CTL effector cell function was studied in two populations specific for two influenza A virus CTL epitopes following *in vitro* expansion of virus-specific T cells in the presence of 2

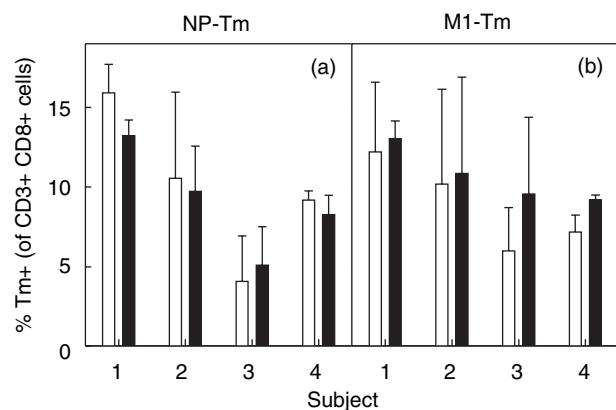
or 50 IU/ml IL-2. The frequency of NP-Tm<sup>+</sup> and M1-Tm<sup>+</sup> cells ranged from 4.5% to 15.3% in the CD3<sup>+</sup>, CD8<sup>+</sup> T cell fraction. In subjects 1, 2 and 4 the frequency of NP-Tm<sup>+</sup> and M1-Tm<sup>+</sup> cells was similar, while in subject 3 the frequency of M1-Tm<sup>+</sup> cells was 1.5-fold higher than that the frequency of NP-Tm<sup>+</sup> cells (Fig. 1). The IL-2 concentration had no effect on the proportion of Tm<sup>+</sup> cells in the *in vitro* expanded virus-specific T cell populations (Fig. 1).

A 1.34-fold increase in cell number was observed following 8 days culture under high IL-2 (IL-2<sub>hi</sub>) conditions. Culturing cells in low IL-2 (IL-2<sub>lo</sub>) concentrations resulted in a 1.05-fold increase in total cell number on day 8 (difference not statistically significant).

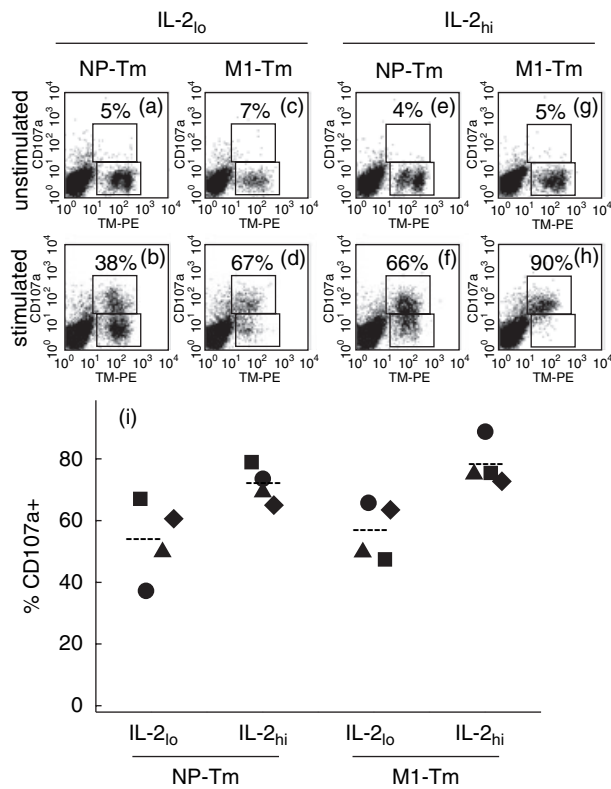
### CD107a mobilization on NP-Tm<sup>+</sup> and M1-Tm<sup>+</sup> cells

The ability of NP-Tm<sup>+</sup> and M1-Tm<sup>+</sup> effector cells to degranulate upon antigenic stimulation was determined by measuring the percentage of Tm<sup>+</sup> cells expressing CD107a on their surface after antigenic restimulation. An example of such an analysis is shown in Fig. 2a-h. Unstimulated polyclonal effector cells specific for NP<sub>44-52</sub> and the M1<sub>58-66</sub> epitopes did not mobilize CD107a. However, after stimulation with the corresponding peptides, expression of CD107a was readily detected in NP-Tm and M1-Tm positive cells. The magnitude of CD107a expression was dependent on the IL-2 concentration added during the expansion of virus-specific CTL. This analysis was performed for all four study subjects (Fig. 2i).

Under IL-2<sub>lo</sub> conditions, the portion of NP-Tm<sup>+</sup> and M1-Tm<sup>+</sup> cells expressing CD107a upon restimulation varied



**Fig. 1.** Proportion of tetramer positive cells in influenza A virus stimulated peripheral blood mononuclear cells cultured under low or high interleukin (IL)-2 conditions. Peripheral blood mononuclear cells of four HLA-A\*0101 and HLA-A\*0201 + subjects were stimulated with influenza A virus for 8 days, in the presence of low (white bars) or high (black bars) IL-2 concentrations. The percentage NP-Tm<sup>+</sup> (a) and M1-Tm<sup>+</sup> (b) cells was determined in the CD8<sup>+</sup> CD3<sup>+</sup> cell fraction as described in the Materials and methods section. Presented are the average percentage Tm<sup>+</sup> cells, plus the standard deviation, of four independently repeated assays.



**Fig. 2.** CD107a staining on NP- and M1-Tm<sup>+</sup> cells cultured under low or high interleukin (IL)-2 conditions. Peripheral blood mononuclear cells of four HLA-A\*0101 and HLA-A\*0201<sup>+</sup> subjects were stimulated with influenza A virus for 8 days, in the presence of low (IL-2<sub>lo</sub>) or high (IL-2<sub>hi</sub>) IL-2 concentrations. CD107a surface expression on NP-Tm<sup>+</sup> and M1-Tm<sup>+</sup> cells was determined after 5 h stimulation with cognate peptides. An example of CD107a surface expression is shown on NP-Tm<sup>+</sup> cells (a, b, e, f) and M1-Tm<sup>+</sup> cells (c, d, g, h) cultured in IL-2<sub>lo</sub> (a–d) and IL-2<sub>hi</sub> (e–h) of subject 2. In (i), the average percentage CD107a<sup>+</sup> cells of the NP-Tm<sup>+</sup> and M1-Tm<sup>+</sup> cells are shown for all four subjects. Subject 1 (■) subject 2 (●), subject 3 (▲), subject 4 (◆). The indicated values in graphs (a–h) represent a single experiment, while graph (i) combines data of three independently repeated experiments.

between 33% and 63% in the four subjects (Fig. 2), demonstrating large subject-dependent differences. The average percentage CD107a<sup>+</sup> cells was similar between NP-Tm<sup>+</sup> (54%) and M1-Tm<sup>+</sup> (56%) cells. In some individuals, however, the CD107a expression on the surface of NP-Tm<sup>+</sup> and M1-Tm<sup>+</sup> cells differed considerably. In subject 1 (squares; Fig. 2i) the CD107a expression on NP-Tm<sup>+</sup> cells (average of 67%) was significantly higher ( $P = 0.05$ ) than the CD107a expression on M1-Tm<sup>+</sup> cells (average of 43%), while in subject 2 (circles; Fig. 2i), the proportion of NP-Tm<sup>+</sup> cells (average 37%) expressing CD107a was lower than that of M1-Tm<sup>+</sup> cells (average 61%) ( $P = 0.121$ ). In the remaining two subjects, no difference (< 10%) in the portion of CD107a<sup>+</sup> cells upon peptide restimulation was measured.

Next, we analysed if higher concentrations of IL-2 (IL-2<sub>hi</sub>), added during the 8-day culture period, had an effect on the

capacity of Tm<sup>+</sup> cells to express CD107a on their surface upon peptide restimulation. In contrast to Tm<sup>+</sup> cells cultured under IL-2<sub>lo</sub> conditions, CD107a expression on NP-Tm<sup>+</sup> and M1-Tm<sup>+</sup> cells of individual subjects did not differ significantly. However, the proportion of Tm<sup>+</sup> cells expressing CD107a following culture under IL-2<sub>hi</sub> conditions was significantly higher (75% versus 55%;  $P < 0.01$ ) than following culture under IL-2<sub>lo</sub> conditions. The same was the case for NP-Tm<sup>+</sup> cells (72% versus 54%;  $P < 0.05$ ) and M1-Tm<sup>+</sup> cells (78% versus 67%;  $P < 0.05$ ).

### IFN- $\gamma$ production by NP-Tm<sup>+</sup> and M1-Tm<sup>+</sup> cells

The ability of NP-Tm<sup>+</sup> and M1-Tm<sup>+</sup> cells from individual study subjects to produce IFN- $\gamma$  was determined. Eight-day-old effector cells, cultured under IL-2<sub>lo</sub> or IL-2<sub>hi</sub> conditions, were restimulated for 6 h with peptides to initiate IFN- $\gamma$  production. As shown in Fig. 3a–d for subject 2, IFN- $\gamma$  production was induced in a proportion of the NP-Tm<sup>+</sup> and M1-Tm<sup>+</sup> cells after stimulation with peptide loaded stimulator cells.

In the four study subjects, IFN- $\gamma$  was produced in 55% (range 33–65%) of the NP-Tm<sup>+</sup> cells cultured in low IL-2 (IL-2<sub>lo</sub>) concentrations (Fig. 3e). In M1-Tm<sup>+</sup> cells this percentage was 73% (range 66–80%), which is significantly higher than the percentage IFN- $\gamma$ <sup>+</sup> cells in the NP-Tm<sup>+</sup> fraction ( $P < 0.05$ , Fig. 3).

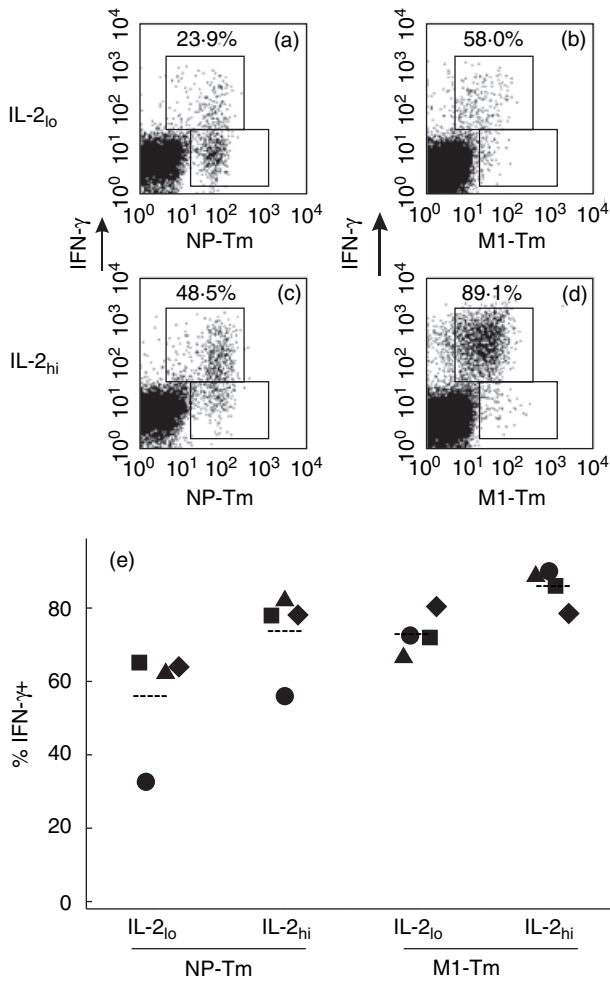
A larger proportion of NP-Tm<sup>+</sup> and M1-Tm<sup>+</sup> cells cultured under IL-2<sub>hi</sub> conditions produced IFN- $\gamma$  upon restimulation. On average 73.5% of the NP-Tm<sup>+</sup> cells (range 56–82%) produced IFN- $\gamma$ , while in M1-Tm<sup>+</sup> cells the average percentage was 85% (range 79–90%) (Fig. 3e). This difference in IFN- $\gamma$  expression by both Tm<sup>+</sup> cell populations was significantly different ( $P < 0.05$ , Fig. 3). The largest difference in proportion of IFN- $\gamma$ <sup>+</sup> cells between NP-Tm<sup>+</sup> (average 56%) and M1-Tm<sup>+</sup> (average 90%) cells was found in subject 2.

Finally, IFN- $\gamma$  production by Tm<sup>+</sup> cells cultured under IL-2<sub>hi</sub> and IL-2<sub>lo</sub> conditions was compared. Of the Tm<sup>+</sup> cells cultured under IL-2<sub>hi</sub> conditions 80% produced IFN- $\gamma$  upon peptide stimulation, while only 64% of the Tm<sup>+</sup> cells, cultured under IL-2<sub>lo</sub> conditions were IFN- $\gamma$ <sup>+</sup>. This difference in IFN- $\gamma$  production between both IL-2 conditions was statistically significant for M1-Tm<sup>+</sup> cells and all Tm<sup>+</sup> cells ( $P < 0.05$ ).

### TNF- $\alpha$ production by NP-Tm<sup>+</sup> and M1-Tm<sup>+</sup> cells

In addition to IFN- $\gamma$ , the production of a second proinflammatory cytokine, TNF- $\alpha$ , was investigated. The TNF- $\alpha$  expression by NP-Tm<sup>+</sup> and M1-Tm<sup>+</sup> cells of subject 2 is shown in Fig. 4a–d. After expansion under IL-2<sub>lo</sub> conditions 26% of the NP-Tm<sup>+</sup> cells and 46% of the M1-Tm<sup>+</sup> cells produced TNF- $\alpha$  upon restimulation (Fig. 4e). After expansion under IL-2<sub>hi</sub> conditions, these numbers increased. On





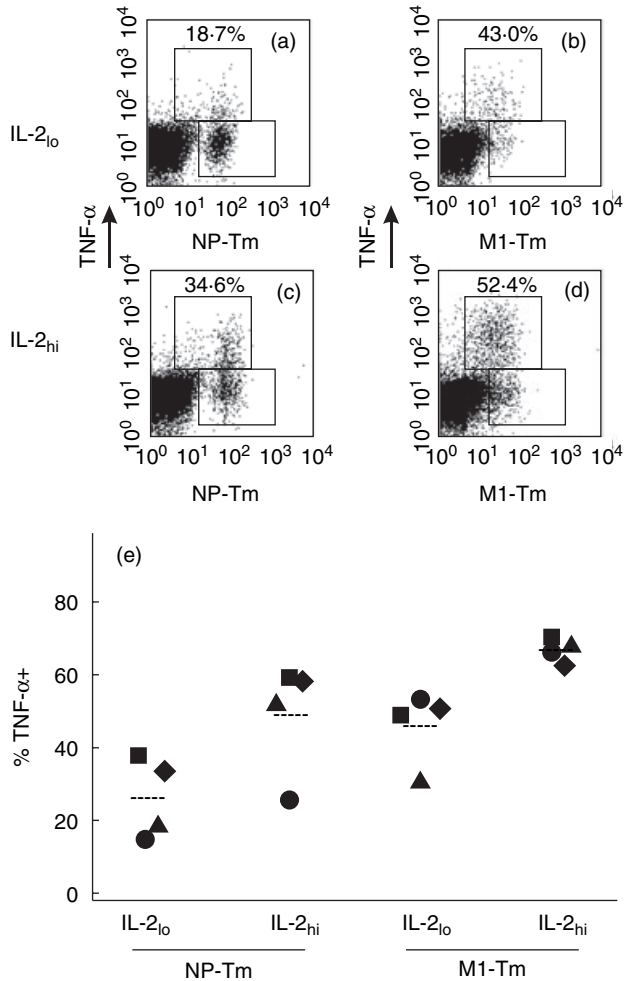
**Fig. 3.** Intracellular interferon (IFN)- $\gamma$  staining of NP-Tm<sup>+</sup> and M1-Tm<sup>+</sup> cells cultured under low or high interleukin (IL)-2 conditions. Peripheral blood mononuclear cells of four HLA-A\*0101 and HLA-A\*0201<sup>+</sup> subjects were stimulated with influenza A virus for 8 days, in the presence of low (IL-2<sub>lo</sub>) or high (IL-2<sub>hi</sub>) IL-2 concentrations. Intracellular IFN- $\gamma$  staining was performed as described in Materials and methods following peptide restimulation of NP-Tm<sup>+</sup> and M1-Tm<sup>+</sup> cells. IFN- $\gamma$  staining in NP-Tm<sup>+</sup> cells (a and c) and M1-Tm<sup>+</sup> cells (b and d) cultured in IL-2<sub>lo</sub> (a and b) and IL-2<sub>hi</sub> (c and d) of subject 2 are shown as an example. In (e), the percentage IFN- $\gamma$ <sup>+</sup> cells of the NP-Tm<sup>+</sup> and M1-Tm<sup>+</sup> cells are presented for all four subjects. Subject 1 (■) subject 2 (●), subject 3 (▲), subject 4 (◆). The indicated values in graphs (a–d) represent a single experiment, while graph (e) combines data of two independently repeated experiments.

average 48% of the NP-Tm<sup>+</sup> cells produced TNF- $\alpha$ , which was significantly ( $P < 0.05$ ) lower than the average percentage of TNF- $\alpha$ <sup>+</sup> M1-Tm<sup>+</sup> cells (67%) (Fig. 4).

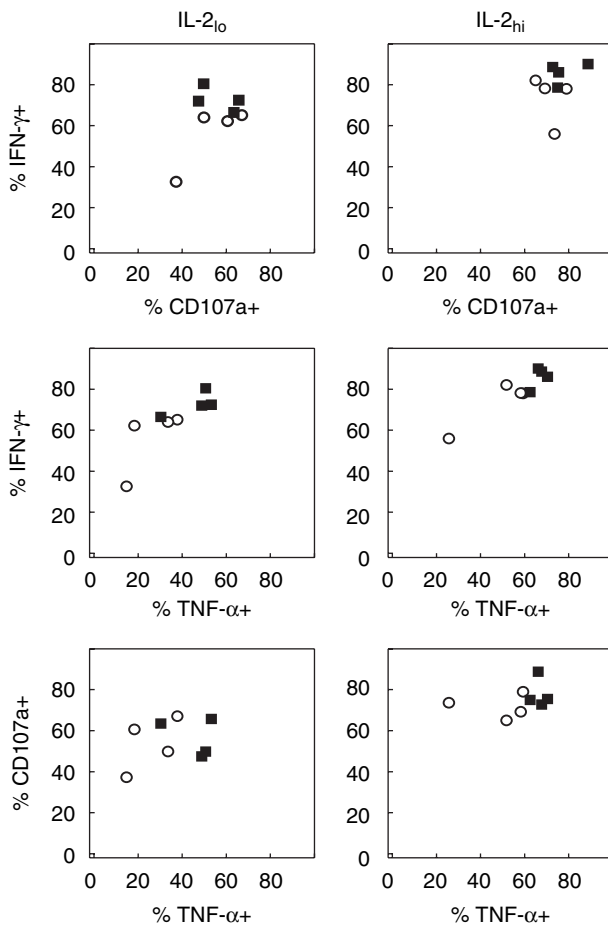
Overall, the proportion of Tm<sup>+</sup> cells producing TNF- $\alpha$  was higher after expansion under IL-2<sub>hi</sub> conditions than after expansion under IL-2<sub>lo</sub> conditions (Fig. 4e). These differences were statistically significant for M1-Tm<sup>+</sup> and all Tm<sup>+</sup> cells ( $P < 0.05$ ) but not for NP-Tm<sup>+</sup> cells ( $P = 0.083$ ).

**Discussion**

The functional properties of polyclonal CTL populations specific for two influenza A virus epitopes were studied in four HLA-A\*0101, HLA-A\*0201 positive study subjects using a combination of tetramer-staining and cell-surface CD107a or intracellular cytokine staining. Epitope-dependent as well as subject-dependent differences in the expression of these effector cell molecules were identified.



**Fig. 4.** Intracellular tumour necrosis factor (TNF)- $\alpha$  staining of NP-Tm<sup>+</sup> and M1-Tm<sup>+</sup> cells cultured under low or high interleukin (IL)-2 conditions. Peripheral blood mononuclear cells of four HLA-A\*0101 and HLA-A\*0201<sup>+</sup> subjects were stimulated with influenza A virus for 8 days, in the presence of low (IL-2<sub>lo</sub>) or high (IL-2<sub>hi</sub>) IL-2 concentrations. Intracellular TNF- $\alpha$  staining was performed as described in Materials and methods, following peptide restimulation of NP-Tm<sup>+</sup> and M1-Tm<sup>+</sup> cells. TNF- $\alpha$  staining in NP-Tm<sup>+</sup> cells (a and c) and M1-Tm<sup>+</sup> cells (b and d) cultured in IL-2<sub>lo</sub> (a and b) and IL-2<sub>hi</sub> (c and d) of subject 2 are shown as an example. In (e), the percentages TNF- $\alpha$ <sup>+</sup> cells of the NP-Tm<sup>+</sup> and M1-Tm<sup>+</sup> cells are presented for all four subjects. Subject 1 (■) subject 2 (●), subject 3 (▲), subject 4 (◆). The indicated values in graphs (a–d) represent a single experiment, while graph (e) combines data of two independently repeated experiments.



**Fig. 5.** Functional comparison of NP-Tm<sup>+</sup> and M1-Tm<sup>+</sup> cells cultured under interleukin (IL)-2<sub>lo</sub> and IL-2<sub>hi</sub> conditions. The proportion of NP-Tm<sup>+</sup> (white circles) and M1-Tm<sup>+</sup> cells (black squares) mobilizing CD107a or expressing interferon (IFN)- $\gamma$  or tumour necrosis factor (TNF)- $\alpha$  were plotted against each other for both IL-2<sub>lo</sub> (a, c, e) and IL-2<sub>hi</sub> conditions (b, d, f). The data were obtained from Figs 2–4.

Also, IL-2 concentration influenced the functional profile of these polyclonal CTL populations.

Mobilization of CD107a (LAMP-1) and CD107b (LAMP-2) to the cell surface of CTL has been identified recently as a marker of degranulation of perforin and granzyme [29], and therefore as a surrogate marker of target cell lysis. Thus, in combination with tetramer-staining, CD107a staining allows the investigation of the cytolytic capacity of polyclonal CTL populations specific for single epitopes. In addition to subject-dependent differences in the lytic capacity of CTL, epitope-dependent differences were also identified in two of four subjects studied. These differences were profound in cells cultured in IL-2<sub>lo</sub> conditions. A fraction of the polyclonal Tm<sup>+</sup> population did not express CD107a upon stimulation, a phenomenon reported previously by others [32]. It is unlikely, however, that these CD107a negative cells form a subset of the polyclonal Tm<sup>+</sup> population, with a different TCR and CDR3 profile [32].

For cytokine expression profiles of the Tm<sup>+</sup> cells populations, subject- and epitope-dependent differences were also observed (Fig. 5). In particular, NP-Tm<sup>+</sup> cells of subject 2 were consistently less functional than the M1-Tm<sup>+</sup> cells of the same subject or NP-Tm<sup>+</sup> cells of other subjects. TCR avidity analysis of NP-Tm<sup>+</sup> cells of these four subjects demonstrated that the avidity of NP-Tm<sup>+</sup> cells of subject 2 is significantly lower than the TCR avidity of the NP-Tm<sup>+</sup> cells of the other three subjects (data not shown). Although a correlation between TCR avidity and the functional profile of epitope-specific CTL has been described previously [25], it is of interest that epitope-specific T cell repertoires in subjects differ regarding their functional avidity. The underlying mechanism for these differences remains to be elucidated.

Comparison of average frequencies of CD107a, IFN- $\gamma$  and TNF- $\alpha$  positive cells within the Tm<sup>+</sup> cell population (Fig. 5) showed that the proportion of epitope-specific CTL mobilizing CD107a and producing IFN- $\gamma$  is similar and larger than the proportion of epitope-specific T cells producing TNF- $\alpha$ . These data indicate that the hierarchy in effector CTL function is CD107a = IFN- $\gamma$  > TNF- $\alpha$ , confirming and extending the previously reported cytokine production hierarchy of influenza virus-specific CTL [33].

In a previous study it was demonstrated that HLA-A\*0101 restricted influenza A virus-specific CTL were more prone to produce TNF- $\alpha$  than IFN- $\gamma$ , while influenza A virus-specific CTL restricted by HLA-A\*0201 and -B\*3501 produced more IFN- $\gamma$  than TNF- $\alpha$  [23]. In the present study, this was not the case for the HLA-A\*0101 restricted NP-Tm<sup>+</sup> cells that produced mainly IFN- $\gamma$ . CTL responses to other HLA-A1 restricted epitopes may have been responsible for the predominant TNF- $\alpha$  expression observed previously.

We also noted a significant effect of IL-2 on the functionality of polyclonal epitope-specific CTL. High IL-2 concentrations during effector cell differentiation resulted in highly efficient CTL, while effector cell differentiation in IL-2<sub>lo</sub> conditions resulted in CTL less efficient in mobilizing CD107a and producing IFN- $\gamma$  and TNF- $\alpha$ . The effect of IL-2 on effector cell function of CTL has been reported previously for T cells from humans, monkeys and mice [26,34–38]. In humans, only CTL clones or T cell lines, passaged multiple times *in vitro*, have been studied. Increased IFN- $\gamma$  production and lysis by human dengue virus-specific CTL clones cultured under IL-2<sub>hi</sub> conditions were observed [37]. Also, it has been found more recently that IL-2 had a positive effect on influenza A virus epitope (M1<sub>58–66</sub>)-specific CTL that had been stimulated three times with peptide-pulsed APC before their functional properties were investigated [36]. Finally, addition of exogenous IL-2 following RSV challenge of mice resulted in increased effector cell function of RSV-specific CTL [26]. The mechanism of the stimulatory effect of IL-2 on effector cell function is as yet unknown. Recent literature suggests a role for regulatory T-cells which may inhibit the effector cell function of CTL, that is inhibited in turn by IL-2 [39,40]. Increased effector cell function as a result of IL-2

was also found for influenza virus stimulated natural killer (NK)-cells [41], the vital role of which was demonstrated previously in mice in the induction of an influenza virus-specific CTL response [42]. Our data also have important implications for the development of future vaccines, aiming at the induction of CTL-mediated immunity. These vaccines should also induce strong IL-2-producing T helper cell responses, which facilitate the induction of functional antigen-specific CTL.

Collectively, the data presented here demonstrate that there is functional heterogeneity among influenza virus-specific CD8<sup>+</sup> T cells. Functional properties of CTL depend on the epitope that is recognized, but also Tm<sup>+</sup> cell populations displayed functional differences depending on the subject from which they were obtained. In addition, the concentration of IL-2 used during expansion or virus-specific T lymphocytes has a major influence on the functionality of these cells. These variables should be taken into account when interpreting CTL responses induced after infection or vaccination.

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## References

- Bender BS, Croghan T, Zhang L *et al.* Transgenic mice lacking class I major histocompatibility complex-restricted T cells have delayed viral clearance and increased mortality after influenza virus challenge. *J Exp Med* 1992; **175**:1143–5.
- McMichael AJ, Gotch FM, Noble GR *et al.* Cytotoxic T-cell immunity to influenza. *N Engl J Med* 1983; **309**:13–7.
- Barry M, Bleackley RC. Cytotoxic T lymphocytes: all roads lead to death. *Nat Rev Immunol* 2002; **2**:401–9.
- Geginat J, Lanzavecchia A, Sallusto F. Proliferation and differentiation potential of human CD8<sup>+</sup> memory T-cell subsets in response to antigen or homeostatic cytokines. *Blood* 2003; **101**:4260–6.
- Hamann D, Baars PA, Rep MH *et al.* Phenotypic and functional separation of memory and effector human CD8<sup>+</sup> T cells. *J Exp Med* 1997; **186**:1407–18.
- Rufer N, Zippelius A, Batard P *et al.* *Ex vivo* characterization of human CD8<sup>+</sup> T subsets with distinct replicative history and partial effector functions. *Blood* 2003; **102**:1779–87.
- Tussey L, Speller S, Gallimore A *et al.* Functionally distinct CD8<sup>+</sup> memory T cell subsets in persistent EBV infection are differentiated by migratory receptor expression. *Eur J Immunol* 2000; **30**:1823–9.
- Sallusto F, Langenkamp A, Geginat J *et al.* Functional subsets of memory T cells identified by CCR7 expression. *Curr Top Microbiol Immunol* 2000; **251**:167–71.
- Sallusto F, Mackay CR, Lanzavecchia A. The role of chemokine receptors in primary, effector, and memory immune responses. *Annu Rev Immunol* 2000; **18**:593–620.
- Sallusto F, Lenig D, Forster R *et al.* Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 1999; **401**:708–12.
- Aandahl EM, Sandberg JK, Beckerman KP *et al.* CD7 is a differentiation marker that identifies multiple CD8 T cell effector subsets. *J Immunol* 2003; **170**:2349–55.
- van Lier RA, ten Berge IJ, Gamadia LE. Human CD8(+) T-cell differentiation in response to viruses. *Nat Rev Immunol* 2003; **3**:931–9.
- Appay V, Nixon DF, Donahoe SM *et al.* HIV-specific CD8(+) T cells produce antiviral cytokines but are impaired in cytolytic function. *J Exp Med* 2000; **192**:63–75.
- Appay V, Dunbar PR, Callan M *et al.* Memory CD8<sup>+</sup> T cells vary in differentiation phenotype in different persistent virus infections. *Nat Med* 2002; **8**:379–85.
- Sandberg JK, Fast NM, Nixon DF. Functional heterogeneity of cytokines and cytolytic effector molecules in human CD8<sup>+</sup> T lymphocytes. *J Immunol* 2001; **167**:181–7.
- Tussey LG, Nair US, Bachinsky M *et al.* Antigen burden is major determinant of human immunodeficiency virus-specific CD8<sup>+</sup> T cell maturation state: potential implications for therapeutic immunization. *J Infect Dis* 2003; **187**:364–74.
- He XS, Mahmood K, Maecker HT *et al.* Analysis of the frequencies and of the memory T cell phenotypes of human CD8<sup>+</sup> T cells specific for influenza A viruses. *J Infect Dis* 2003; **187**:1075–84.
- Chang J, Braciale TJ. Respiratory syncytial virus infection suppresses lung CD8<sup>+</sup> T-cell effector activity and peripheral CD8<sup>+</sup> T-cell memory in the respiratory tract. *Nat Med* 2002; **8**:54–60.
- Ravkov EV, Myrick CM, Altman JD. Immediate early effector functions of virus-specific CD8<sup>+</sup>CCR7<sup>+</sup> memory cells in humans defined by HLA and CC chemokine ligand 19 tetramers. *J Immunol* 2003; **170**:2461–8.
- Belz GT, Xie W, Doherty PC. Diversity of epitope and cytokine profiles for primary and secondary influenza A virus-specific CD8<sup>+</sup> T cell responses. *J Immunol* 2001; **166**:4627–33.
- Catalina MD, Sullivan JL, Brody RM *et al.* Phenotypic and functional heterogeneity of EBV epitope-specific CD8<sup>+</sup> T cells. *J Immunol* 2002; **168**:4184–91.
- Hislop AD, Gudgeon NH, Callan MF *et al.* EBV-specific CD8<sup>+</sup> T cell memory: relationships between epitope specificity, cell phenotype, and immediate effector function. *J Immunol* 2001; **167**:2019–29.
- Boon AC, de Mutsert G, Graus YM *et al.* Preferred HLA usage and HLA dependent differential cytokine expression in the influenza virus-specific cytotoxic T lymphocyte response. *J Immunol* 2004; **172**:4435–43.
- Hofmann M, Radsak M, Rechtsteiner G *et al.* T cell avidity determines the level of CTL activation. *Eur J Immunol* 2004; **34**:1798–806.
- Kristensen NN, Madsen AN, Thomsen AR *et al.* Cytokine production by virus-specific CD8(+) T cells varies with activation state and localization, but not with TCR avidity. *J General Virol* 2004; **85**:1703–12.
- Chang J, Choi SY, Jin HT *et al.* Improved effector activity and memory CD8 T cell development by IL-2 expression during experimental respiratory syncytial virus infection. *J Immunol* 2004; **172**:503–8.
- Boon AC, de Mutsert G, Graus YM *et al.* The magnitude and specificity of influenza A virus-specific cytotoxic T-lymphocyte

- responses in humans is related to HLA-A and -B phenotype. *J Virol* 2002; **76**:582–90.
- 28 Altman JD, Moss PA, Goulder PJ *et al.* Phenotypic analysis of antigen-specific T lymphocytes. *Science* 1996; **274**:94–6.
- 29 Betts MR, Brenchley JM, Price DA *et al.* Sensitive and viable identification of antigen-specific CD8<sup>+</sup> T cells by a flow cytometric assay for degranulation. *J Immunol Meth* 2003; **281**:65–78.
- 30 Rubio V, Stuge TB, Singh N *et al.* *Ex vivo* identification, isolation and analysis of tumor-cytolytic T cells. *Nat Med* 2003; **9**:1377–82.
- 31 Boon AC, Fringuelli E, Graus YM *et al.* Influenza A virus specific T cell immunity in humans during aging. *Virology* 2002; **299**:100–8.
- 32 Betts MR, Price DA, Brenchley JM *et al.* The functional profile of primary human antiviral CD8<sup>+</sup> T cell effector activity is dictated by cognate peptide concentration. *J Immunol* 2004; **172**:6407–17.
- 33 La Gruta NL, Turner SJ, Doherty PC. Hierarchies in cytokine expression profiles for acute and resolving influenza virus-specific CD8<sup>+</sup> T cell responses: correlation of cytokine profile and TCR avidity. *J Immunol* 2004; **172**:5553–60.
- 34 Xiong Y, Luscher MA, Altman JD *et al.* Simian immunodeficiency virus (SIV) infection of a rhesus macaque induces SIV-specific CD8(+) T cells with a defect in effector function that is reversible on extended interleukin-2 incubation. *J Virol* 2001; **75**:3028–33.
- 35 Manjunath N, Shankar P, Wan J *et al.* Effector differentiation is not prerequisite for generation of memory cytotoxic T lymphocytes. *J Clin Invest* 2001; **108**:871–8.
- 36 Lyngstrand ST, Wurtzen PA, Odum N *et al.* IL-15 induces unspecific effector functions in human peptide-specific CD8<sup>+</sup> T-cell cultures. *Scand J Immunol* 2002; **56**:602–10.
- 37 Livingston PG, Toomey S, Kurane I *et al.* Modulation of the functions of dengue virus-specific human CD8<sup>+</sup> cytotoxic T cell clone by IL-2, IL-7 and IFN gamma. *Immunol Invest* 1995; **24**:619–29.
- 38 Cousens LP, Orange JS, Biron CA. Endogenous IL-2 contributes to T cell expansion and IFN-gamma production during lymphocytic choriomeningitis virus infection. *J Immunol* 1995; **155**:5690–9.
- 39 Nelson BH. IL-2, regulatory T cells, and tolerance. *J Immunol* 2004; **172**:3983–8.
- 40 Bach JF. Regulatory T cells under scrutiny. *Nat Rev Immunol* 2003; **3**:189–98.
- 41 He XS, Draghi M, Mahmood K *et al.* T cell-dependent production of IFN-gamma by NK cells in response to influenza A virus. *J Clin Invest* 2004; **114**:1812–9.
- 42 Kos FJ, Engleman EG. Role of natural killer cells in the generation of influenza virus-specific cytotoxic T cells. *Cell Immunol* 1996; **173**:1–6.