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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$^+$ T cell populations specific for the HLA-A*0101 restricted epitope NP$_{44-52}$ and the HLA-A*0201 restricted epitope M1$_{58-66}$ 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)-γ and tumour necrosis factor-(TNF)-α 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-γ and TNF-α by both NP- and M1-specific T cells were observed. In two of the four subjects, the functional profile of NP-Tm$^+$ and M1-Tm$^+$ cells differed considerably. Overall, no difference in the proportion of NP-Tm$^+$ or M1-Tm$^+$ cells expressing CD107a was observed. The proportion of M1-Tm$^+$ cells that produced IFN-γ ($P < 0{\cdot}05$) was larger than for NP-Tm$^+$ cells, independent of IL-2 concentration. When cultured under IL-2$_{hi}$ concentrations higher TNF-α expression was also observed in M1-Tm$^+$ cells ($P < 0{\cdot}05$). The IL-2 concentration during expansion of virus-specific cells had a profound effect on the functionality of both M1-Tm$^+$ and NP-Tm$^+$ 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)-γ and tumour necrosis factor (TNF)-α [3].

Recent research has identified differential expression of CD45R isoforms, CCR7, CD62L, CD27, CXCR4 and CD28 on functionally different CD8$^+$ T cell populations [4–12], which have been associated with consecutive stages of human CD8$^+$ 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 syncitial virus (RSV) produced less IFN-γ 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-α than IFN-γ, while HLA-B8, -B35 and -A2 restricted epitope-specific CTL produced mainly IFN-γ and to a lesser extent TNF-α [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-α 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 $NP_{44–52}$ and $M1_{58–66}$ [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 $NP_{44–52}$ (CTELKLSDY) and the HLA-A*0201-restricted epitope $M1_{58–66}$ (GILGFVFTL).

Synthetic peptides representing the epitope $NP_{44–52}$ and the epitope $M1_{58–66}$ were manufactured, high profile liquid chromatography (HPLC) purified and analysed by mass spectometry (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 μM and stored at −20°C until further use.

HLA-A*0101 and HLA-A*0201 molecules were complexed with the $NP_{44–52}$ and $M1_{58–66}$ 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 $NP_{44–52}$ peptide containing HLA-A*0101 tetramers are referred to as NP-Tm, while the $M1_{5—66}$ 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 10^6 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 μg/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 μg/ml streptomycin, 100 IU/ml penicillin and 20 μM 2-ME (R10H), and added to uninfected PBMC at a ratio of 1 : 1 in a 25-cm^2 culture flask. After 2 days, 2 U/ml ($IL\text{-}2_{lo}$) or 50 U/ml ($IL\text{-}2_{hi}$) rhIL-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 μM synthetic peptide for 5 h at 37°C. During the 5 h incubation, fluorescein isothiocynate (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 $CD107a^+$

cells of the NP-Tm$^+$ or M1-Tm$^+$ population was calculated using the following formula:

% CD107a$^+$ cells$_{(peptide\ stimulated)}$ – % CD107a$^+$ cells$_{(unstimulated)}$.

Intracellular cytokine staining

IFN-γ and TNF-α 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 µl R10F containing Golgistop™ or Golgiplug™ for the IFN-γ staining or TNF-α staining, respectively, according to recommendations of the manufacturer. Restimulation of $NP_{44–52}$-specific and $M1_{58–66}$-specific effector cells was achieved by adding synthetic peptides to the wells at a concentration of 10 µM. 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 (Cytofix™, Becton Dickinson), permeabilized (Cytoperm™, Becton Dickinson) and incubated with APC-conjugated mouse-antihuman IFN-γ mAb (clone 4S.B3, Becton Dickinson) or APC-conjugated mouse-anti-human TNF-α 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-γ or TNF-α$^+$ cells of the NP-Tm$^+$ or M1-Tm$^+$ population was calculated using the following formula: % IFN-γ$^+$ or TNF-α$^+$ cells$_{(peptide\ stimulated)}$ –% IFN-γ$^+$ or TNF-α$^+$ cells$_{(unstimulated)}$.

Statistical analysis

Mann–Whitney *U*-test was applied to determine statistical significance between NP-Tm$_+$ or M1-Tm$^+$ cells of all four subjects or between IL-2_{lo} or IL-2_{hi} 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$^+$ and M1-Tm$^+$ 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$^+$ and M1-Tm$^+$ cells ranged from 4·5% to 15·3% in the CD3$^+$, CD8$^+$ T cell fraction. In subjects 1, 2 and 4 the frequency of NP-Tm$^+$ and M1-Tm$^+$ cells was similar, while in subject 3 the frequency of M1-Tm$^+$ cells was 1·5-fold higher than that the frequency of NP-Tm$^+$ cells (Fig. 1). The IL-2 concentration had no effect on the proportion of Tm$^+$ 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_{hi}) conditions. Culturing cells in low IL-2 (IL-2_{lo}) concentrations resulted in a 1·05-fold increase in total cell number on day 8 (difference not statistically significant).

CD107a mobilization on NP-Tm$^+$ and M1-Tm$^+$ cells

The ability of NP-Tm$^+$ and M1-Tm$^+$ effector cells to degranulate upon antigenic stimulation was determined by measuring the percentage of Tm$^+$ 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 $NP_{44–52}$ and the $M1_{58–66}$ 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_{lo} conditions, the portion of NP-Tm$^+$ and M1-Tm$^+$ cells expressing CD107a upon restimulation varied

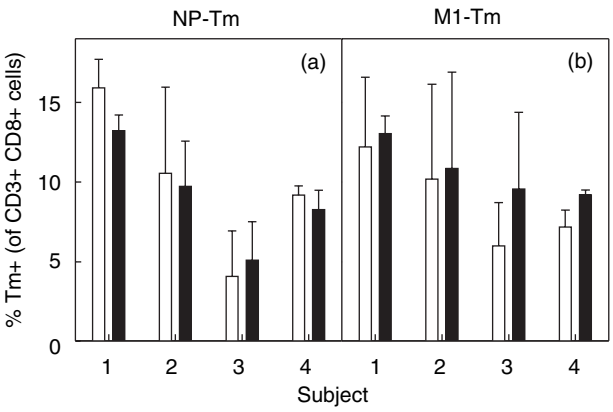

Fig. 1. Proportion of tetramer positive cells in influenza A virus stimulated peripheral blood mononuclear cells cultured under low or high interleukin (IL)-2 conditions. Peripheral blood mononuclear cells of four HLA-A*0101 and HLA-A*0201 + subjects were stimulated with influenza A virus for 8 days,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

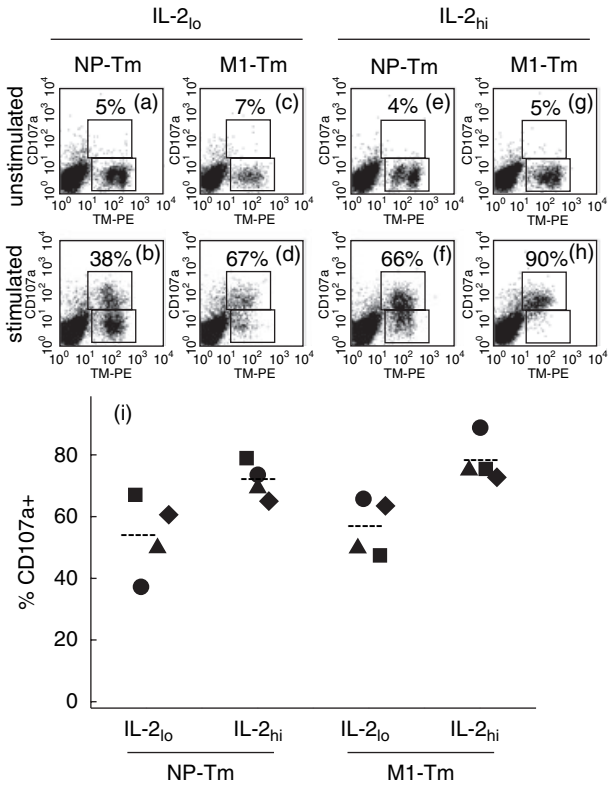

Fig. 2. CD107a staining on NP- and M1-Tm$^+$ cells cultured under low or high interleukin (IL)-2 conditions. Peripheral blood mononuclear cells of four HLA-A*0101 and HLA-A*0201$^+$ subjects were stimulated with influenza A virus for 8 days, in the presence of low (IL-2$_{lo}$) or high (IL-2$_{hi}$) IL-2 concentrations. CD107a surface expression on NP-Tm$^+$ and M1-Tm$^+$ cells was determined after 5 h stimulation with cognate peptides. An example of CD107a surface expression is shown on NP-Tm$^+$ cells (a, b, e, f) and M1-Tm$^+$ cells (c, d, g, h) cultured in IL-2$_{lo}$ (a→d) and IL-2$_{hi}$ (e→h) of subject 2. In (i), the average percentage CD107a$^+$ cells of the NP-Tm$^+$ and M1-Tm$^+$ 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$^+$ cells was similar between NP-Tm$^+$ (54%) and M1-Tm$^+$ (56%) cells. In some individuals, however, the CD107a expression on the surface of NP-Tm$^+$ and M1-Tm$^+$ cells differed considerably. In subject 1 (squares; Fig. 2i) the CD107a expression on NP-Tm$^+$ cells (average of 67%) was significantly higher ($P = 0{\cdot}05$) than the CD107a expression on M1-Tm$^+$ cells (average of 43%), while in subject 2 (circles; Fig. 2i), the proportion of NP-Tm+ cells (average 37%) expressing CD107a was lower than that of M1-Tm$^+$ cells (average 61%) ($P = 0{\cdot}121$). In the remaining two subjects, no difference (< 10%) in the portion of CD107a$^+$ cells upon peptide restimulation was measured.

Next, we analysed if higher concentrations of IL-2 (IL-2$_{hi}$), added during the 8-day culture period, had an effect on the capacity of Tm$^+$ cells to express CD107a on their surface upon peptide restimulation. In contrast to Tm$^+$ cells cultured under IL-2$_{lo}$ conditions, CD107a expression on NP-Tm$^+$ and M1-Tm$^+$ cells of individual subjects did not differ significantly. However, the proportion of Tm$^+$ cells expressing CD107a following culture under IL-2$_{hi}$ conditions was significantly higher (75% *versus* 55%; $P < 0{\cdot}01$) than following culture under IL-2$_{lo}$ conditions. The same was the case for NP-Tm$^+$ cells (72% *versus* 54%; $P < 0{\cdot}05$) and M1-Tm$^+$ cells (78% *versus* 67%; $P < 0{\cdot}05$).

IFN-γ production by NP-Tm$^+$ and M1-Tm$^+$ cells

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

In the four study subjects, IFN-γ was produced in 55% (range 33–65%) of the NP-Tm$^+$ cells cultured in low IL-2 (IL-2$_{lo}$) concentrations (Fig. 3e). In M1-Tm$^+$ cells this percentage was 73% (range 66–80%), which is significantly higher than the percentage IFN-γ$^+$ cells in the NP-Tm$^+$ fraction ($P < 0{\cdot}05$, Fig. 3).

A larger proportion of NP-Tm$^+$ and M1-Tm$^+$ cells cultured under IL-2$_{hi}$ conditions produced IFN-γ upon restimulation. On average 73·5% of the NP-Tm$^+$ cells (range 56–82%) produced IFN-γ, while in M1-Tm$^+$ cells the average percentage was 85% (range 79–90%) (Fig. 3e). This difference in IFN-γ expression by both Tm$^+$ cell populations was significantly different ($P < 0{\cdot}05$, Fig. 3). The largest difference in proportion of IFN-γ$^+$ cells between NP-Tm$^+$ (average 56%) and M1-Tm$^+$ (average 90%) cells was found in subject 2.

Finally, IFN-γ production by Tm$^+$ cells cultured under IL-2$_{hi}$ and IL-2$_{lo}$ conditions was compared. Of the Tm$^+$ cells cultured under IL-2$_{hi}$ conditions 80% produced IFN-γ upon peptide stimulation, while only 64% of the Tm$^+$ cells, cultured under IL-2$_{lo}$ conditions were IFN-γ$^+$. This difference in IFN-γ production between both IL-2 conditions was statistically significant for M1-Tm$^+$ cells and all Tm$^+$ cells ($P < 0{\cdot}05$).

TNF-α production by NP-Tm$^+$ and M1-Tm$^+$ cells

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

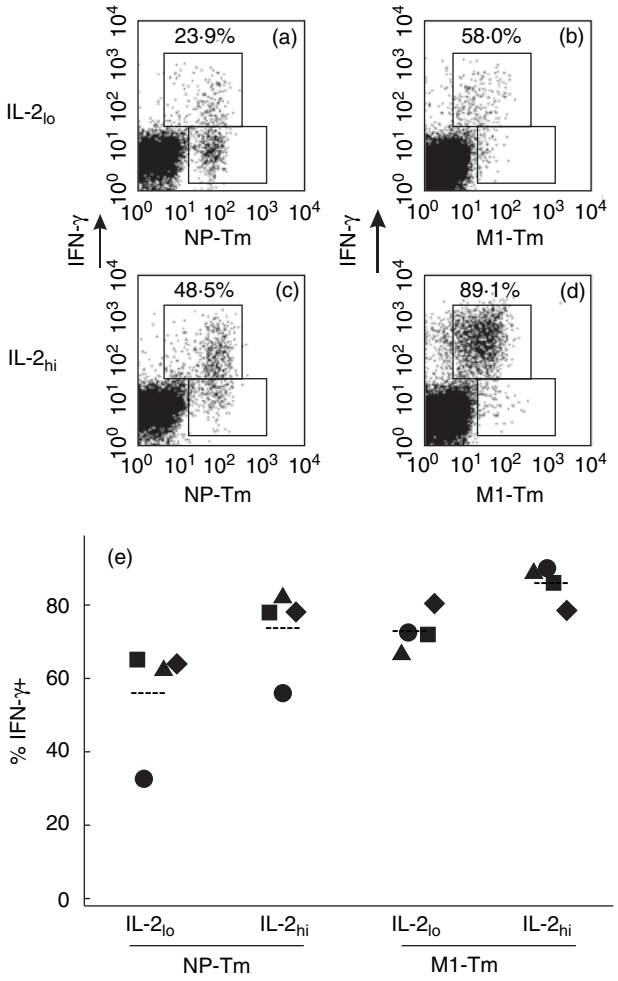

Fig. 3. Intracellular interferon (IFN)-γ staining of NP-Tm$^+$ and M1-Tm$^+$ cells cultured under low or high interleukin (IL)-2 conditions. Peripheral blood mononuclear cells of four HLA-A*0101 and HLA-A*0201$^+$ subjects were stimulated with influenza A virus for 8 days, in the presence of low (IL-2$_{lo}$) or high (IL-2$_{hi}$) IL-2 concentrations. Intracellular IFN-γ staining was performed as described in Materials and methods following peptide restimulation of NP-Tm+ and M1-Tm$^+$ cells. IFN-γ staining in NP-Tm$^+$ cells (a and c) and M1-Tm$^+$ cells (b and d) cultured in IL-2$_{lo}$ (a and b) and IL-2$_{hi}$ (c and d) of subject 2 are shown as an example. In (e), the percentage IFN-γ$^+$ cells of the NP-Tm$^+$ and M1-Tm$^+$ 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$^+$ cells produced TNF-α, which was significantly ($P < 0{\cdot}05$) lower than the average percentage of TNF-α$^+$ M1-Tm$^+$ cells (67%) (Fig. 4).

Overall, the proportion of Tm$^+$ cells producing TNF-α was higher after expansion under IL-2$_{hi}$ conditions than after expansion under IL-2$_{lo}$ conditions (Fig. 4e). These differences were statistically significant for M1-Tm$^+$ and all Tm$^+$ cells ($P < 0{\cdot}05$) but not for NP-Tm$^+$ cells ($P = 0{\cdot}083$).

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.

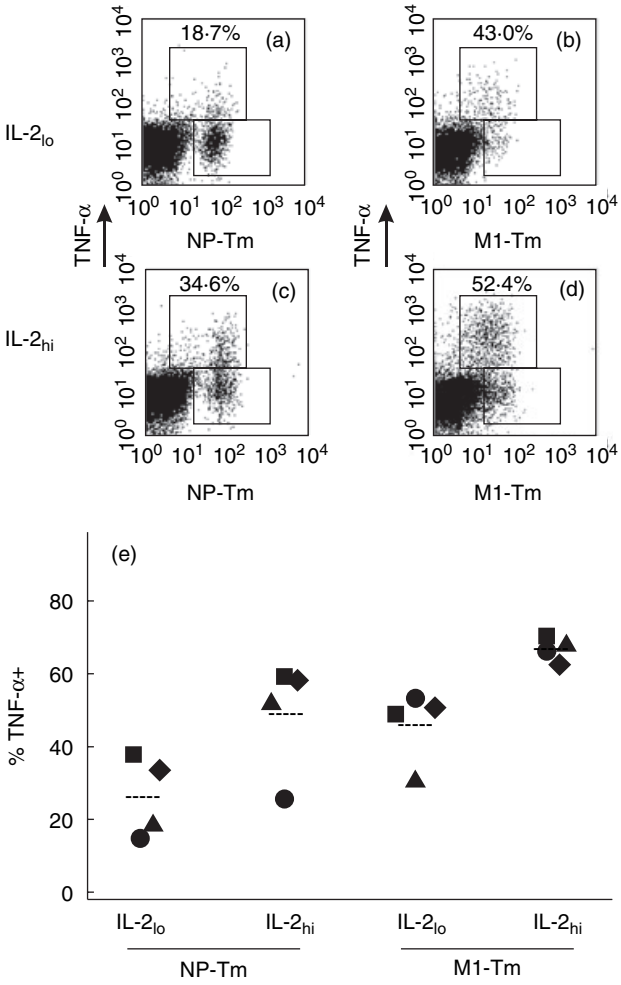

Fig. 4. Intracellular tumour necrosis factor (TNF)-α staining of NP-Tm$^+$ and M1-Tm$^+$ cells cultured under low or high interleukin (IL)-2 conditions. Peripheral blood mononuclear cells of four HLA-A*0101 and HLA-A*0201$^+$ subjects were stimulated with influenza A virus for 8 days, in the presence of low (IL-2$_{lo}$) or high (IL-2$_{hi}$) IL-2 concentrations. Intracellular TNF-α staining was performed as described in Materials and methods, following peptide restimulation of NP-Tm$^+$ and M1-Tm$^+$ cells. TNF-α staining in NP-Tm$^+$ cells (a and c) and M1-Tm$^+$ cells (b and d) cultured in IL-2$_{lo}$ (a and b) and IL-2$_{hi}$ (c and d) of subject 2 are shown as an example. In (e), the percentages TNF-α$^+$ cells of the NP-Tm$^+$ and M1-Tm$^+$ 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.

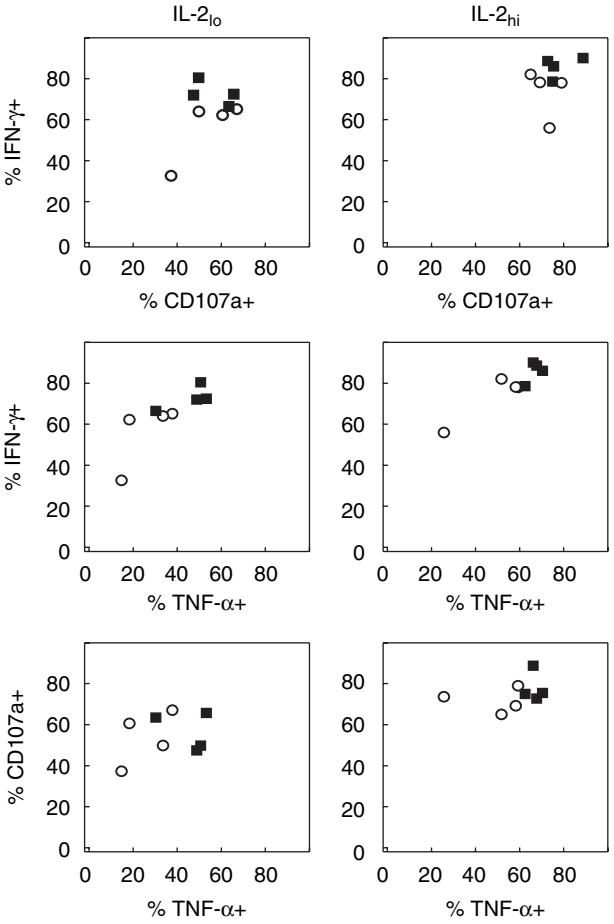

Fig. 5. Functional comparison of NP-Tm$^+$ and M1-Tm$^+$ cells cultured under interleukin (IL)-2$_{lo}$ and IL-2$_{hi}$ conditions. The proportion of NP-Tm$^+$ (white circles) and M1-Tm$^+$ cells (black squares) mobilizing CD107a or expressing interferon (IFN)-γ or tumour necrosis factor (TNF)-α were plotted against each other for both IL-2$_{lo}$ (a, c, e) and IL-2$_{hi}$ 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$_{lo}$ conditions. A fraction of the polyclonal Tm$^+$ 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$^+$ population, with a different TCR and CDR3 profile [32].

For cytokine expression profiles of the Tm$^+$ cells populations, subject- and epitope-dependent differences were also observed (Fig. 5). In particular, NP-Tm$^+$ cells of subject 2 were consistently less functional than the M1-Tm+ cells of the same subject or NP-Tm$^+$ cells of other subjects. TCR avidity analysis of NP-Tm$^+$ cells of these four subjects demonstrated that the avidity of NP-Tm$^+$ cells of subject 2 is significantly lower than the TCR avidity of the NP-Tm$^+$ 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-γ and TNF-α positive cells within the Tm$^+$ cell population (Fig. 5) showed that the proportion of epitope-specific CTL mobilizing CD107a and producing IFN-γ is similar and larger than the proportion of epitope-specific T cells producing TNF-α. These data indicate that the hierarchy in effector CTL function is CD107a = IFN-γ > TNF-α, 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-α than IFN-γ, while influenza A virus-specific CTL restricted by HLA-A*0201 and -B*3501 produced more IFN-γ than TNF-α [23]. In the present study, this was not the case for the HLA-A*0101 restricted NP-Tm$^+$ cells that produced mainly IFN-γ. CTL responses to other HLA-A1 restricted epitopes may have been responsible for the predominant TNF-α 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$_{lo}$ conditions resulted in CTL less efficient in mobilizing CD107a and producing IFN-γ and TNF-α. 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-γ production and lysis by human dengue virus-specific CTL clones cultured under IL-2$_{hi}$ conditions were observed [37]. Also, it has been found more recently that IL-2 had a positive effect on influenza A virus epitope (M1$_{58–66}$)-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 $CD8^+$ T cells. Functional properties of CTL depend on the epitope that is recognized, but also Tm^+ 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 of vaccination.

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