hundred microliters of cell suspension was loaded onto the upper wells and placed in a 24-well plate containing 600 μl of media with the indicated doses of CXCL12 (SDF-1α) (PeproTech) or CCL19 (ELC) (R&D Systems, Minneapolis, MN, USA). Cells were incubated at 37°C for 90 min, and cells in the bottom wells were counted using a FACSCalibur.

Activation-induced cell death assay

Sorted $CD4^+$ GFP^+ T cells were cultured at a concentration of 1×10^6 cells ml^{-1} in 96-well plates immobilized with 5 μg ml^{-1} of anti-CD3ε mAb (2C11) (BioLegend, San Diego, CA, USA) in RPMI medium supplemented with 10% FBS. Cells were harvested 2 days later and then re-cultured for 3 days in 96-well plates containing immobilized with anti-CD3 mAb or medium containing 200 U ml^{-1} of human IL-2 (PeproTech). To detect apoptotic cells, a terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling assay was performed using the ApopTag Red In Situ Apoptosis Detection Kit (CHEMICON International Inc., Temecula, CA, USA). Briefly, the cells were collected and deposited on glass slides by cytospin (Shandon, London, UK), fixed with PBS containing 1% PFA for 10 min and the DNA free 3′ OH were enzymatically labeled with digoxigenin-labeled nucleotides, which were detected using rhodamine-labeled anti-digoxigenin polyclonal antibodies according to the manufacturer's instructions. After applying 6 μg ml^{-1} of Hoechst33342 (Invitrogen) for nuclear staining, slides were processed for analysis using an LSM 510 laser-scanning confocal microscope (Carl Zeiss, Jena, Germany). The proportion of apoptotic cells was determined by counting at least 100 cells in the captured images.

T-cell migration in vivo

BALB/c mice were intravenously injected with 2×10^6 of purified $CD4^+$ GFP^+ T cells uninfected or infected with a recombinant adenovirus vector. Twenty-four hours later, the recipient mice were subcutaneously immunized with 0.2 mg of LPS-free OVA in CFA on the back at three sites. The number of $CD4^+KJ1\text{-}26^+$ T cells in the draining lymph nodes was measured by flow cytometry at 5 days after immunization.

Adoptive cell transfer

Transfer of B cells and OVA-specific/$CD4^+$ T cells infected with a recombinant adenovirus vector in adoptive hosts was performed as described previously (14).

Briefly, $CD4^+$ GFP^+ T cells were prepared by FACS sorting from dTg T cells infected with a recombinant adenovirus vector *in vitro*. B cells were negatively selected from the pooled spleens of either naive mice or 4-hydroxyl-3-nitrophenylacetyl-conjugated chicken γ-globulin (NP-CGG)-primed mice using a MACS system and biotinylated anti-CD5 (53–7.3), anti-CD90.2 (53–2.1), anti-Gr1, anti-CD11b (eBioscience), anti-CD43 (57) and anti-CD138 (281-2) (BD PharMingen). The procedure consistently yielded >95% $B220^+$ cells. Purified B cells (5×10^6) together with $CD4^+$ GFP^+ T cells infected with recombinant adenovirus vector (3×10^4) were intravenously injected into CB17-scid mice. One day later, the recipient mice were intraperitoneally challenged with 25 μg of soluble NP-OVA, and the sera were collected from individual mice at day 7 after challenge. Anti-NP serum antibody titers were estimated by ELISA assays using NP_2-BSA and NP_{18}-BSA as coating antigens as described previously (14). The relative affinity of anti-NP antibodies was estimated by calculating the ratio of anti-NP_2/anti-NP_{18} antibody.

Statistics

The results were evaluated statistically by two-tailed Student's *t*-test ($n = 3$) or Mann–Whitney nonparametric test ($n > 4$), with $P < 0.05$ regarded as significant.

Results

Nef impairs T-cell proliferation upon antigen stimulation in vitro

In order to determine the effect of Nef expression in peripheral T cells, we crossed Tg mice that express an OVA-specific T-cell receptor (12) with mice expressing CAR on T cells (11). OVA-specific/$CD4^+$/CAR^+ T cells were purified from the pooled spleens of dTg mice and infected *in vitro* with an adenovirus vector encoding green fluorescence protein (GFP) driven by the CAG promoter with (Ad-nef) or its mutants [Ad-nef (mu)] or without the *nef* gene (Ad) in the presence of IL-7, which supports T-cell survival and promotes progression into the G_{1b} stage of the cell cycle (16, 17). Thereafter, GFP^+ cells were purified by FACS and provided for analysis as below.

Consistent with previous observations in human T-cell lines, Fig. 1(A) shows that CD4 expression on murine peripheral T cells was down-regulated by Nef but not by the Nef mutant carrying amino acids replacements of $^{57}W^{58}L$ to $^{57}A^{58}A$, abrogating the ability to down-regulate CD4 (18). Nef expression had no effect on the expression of CD25, CD28, CD44, CD62L, CD69, TCRβ and MHC class I (data not shown).

To examine the effect of Nef in T-cell response, GFP^+ cells were purified by FACS from $CD4^+$/CAR^+ T cells infected with Ad-nef, Ad-nef (mu) and cultured in the presence of irradiated splenocytes as APCs, which had been pulsed with OVA peptide ($OVA_{323–339}$). Expression of wild-type as well as mutant forms of Nef diminished T-cell proliferation upon stimulations with OVA peptide at a dose of 0.1 μM (Fig. 1B). These Nef proteins also reduced the level of cytokines produced by T cells in response to different doses of OVA peptide (Fig. 1C). These results suggest that Nef prominently affects T-cell proliferation, irrespective of Nef's ability to down-modulate CD4 but not completely abrogate T-cell activation.

Nef-expression diminishes T-cell migration activity in the primary immune response

Chemokines and their receptors play pivotal roles in the initial homing of lymphocytes and their subsequent trafficking during an immune response (6). It has been reported that Nef impairs the migratory capacity of human T-cell lines *in vitro* in response to the chemokine CXCL12, which binds to T-cell receptor, CXCR4, owing to alteration of the signal cascades downstream of chemokine receptors (7, 15). Consistently, the expression of Nef or its mutant in murine $CD4^+$ T cells reduced their migration in response to CXCL12 *in vitro*, without altering the surface receptor expressions (Fig. 2A and B).

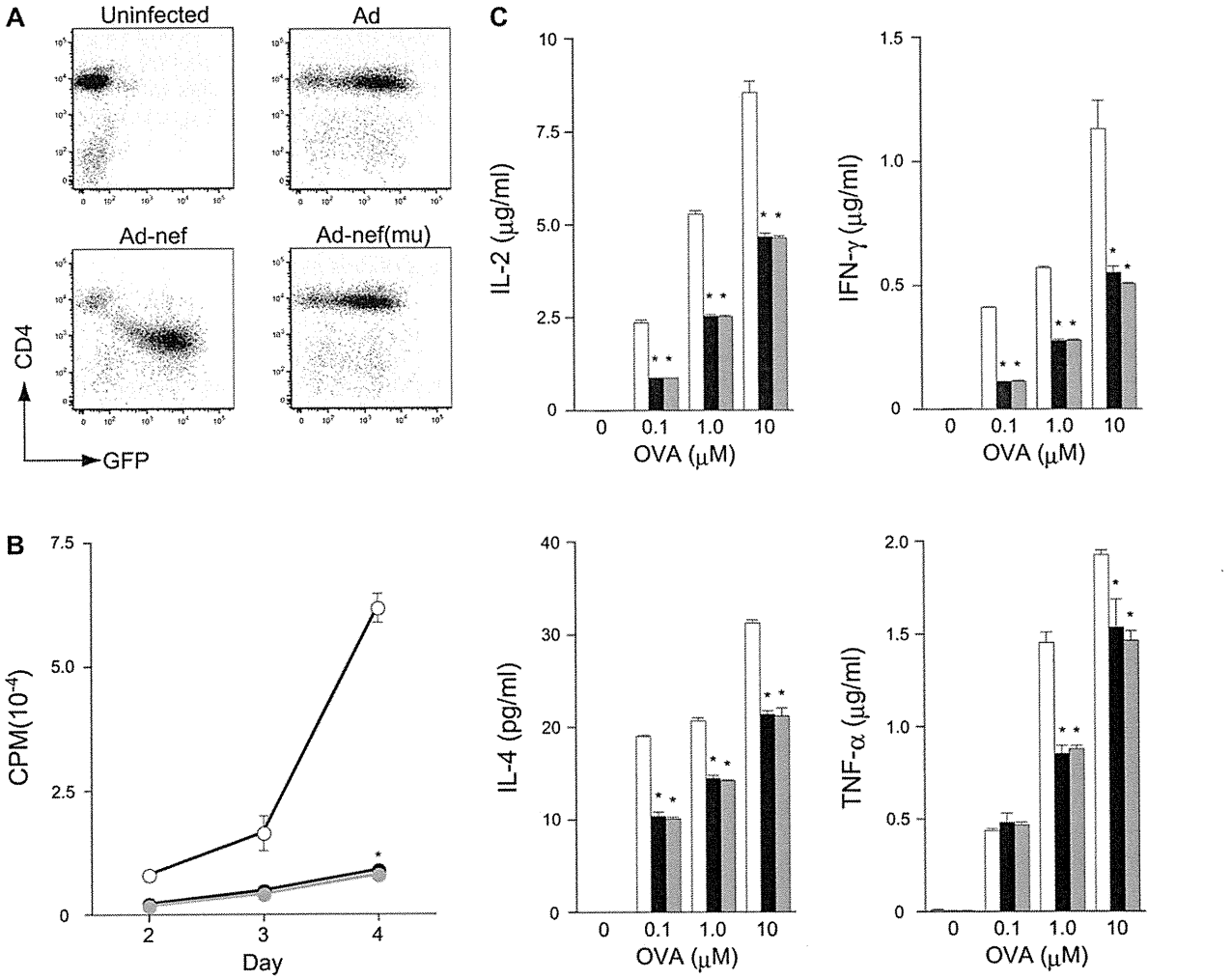

Fig. 1. (A) Characterization of Nef-expressing T cells. A *EGFP* gene-containing adenoviral vector was used to evaluate the efficiency of adenovirus (Ad) infection in DO11.10/CAR$^+$/CD4$^+$ T cells. Naive CD4$^+$ T cells from dTg mice were infected with Ad-nef, Ad-nef (mu) or Ad vector as a control. Two days later, GFP and CD4 expression were assessed by FACS. (B) Nef represses antigen-specific T-cell proliferation. Purified CD4$^+$/GFP$^+$ T cells (5×10^4) infected with Ad-nef (closed), Ad-nef (mu) (gray) and Ad (open) were cultured with T-cell depleted spleen cells as APCs (5×10^5) pulsed with 0.1 μM of $OVA_{323-339}$ peptide. Their DNA synthesis in the triplicate culture was estimated at the indicated periods by the incorporation of [^{3}H] thymidine added 12 h prior to cell harvest. *$P < 0.001$ versus Ad. (C) Purified CD4$^+$ GFP$^+$ T cells and APCs were co-cultured with various concentrations of $OVA_{323-339}$ peptide. Cytokine production in culture supernatant was measured by ELISA on day 3 of culture. *$P < 0.001$ versus Ad. Shown is the representative data from two independent experiments.

Likewise, the Nef proteins, including NL4-3 Nef, did not alter the expression of CXCR4 on human T cells (15, 19), however, there are controversial reports that HIV-1 Nef caused a modest decrease in expression of CXCR4 on human T cells, irrespective of Nef alleles, including NA7 and NL4-3 (7, 20). Further analysis is needed to resolve the discrepancy among these studies.

To examine whether Nef affects T-cell migration *in vivo*, OVA-specific CD4$^+$ T cells were purified from pooled splenocytes of dTg mice and infected with Ad-nef, Ad-nef(mu) or Ad. These cells were transferred into syngeneic recipients, followed by subcutaneous inoculation with OVA in CFA. Five days later, the frequency of OVA-specific (KJ1-26$^+$) CD4$^+$ T cells in the draining lymph node was estimated by FACS. As shown in Fig. 2(C), we observed that Nef impairs the physiological recruitment of T cells into the secondary lymphoid tissues in the immune response. A substantial number of GFP$^+$/OVA-specific/CD4$^+$ T cells infected with Ad accumulated in the draining lymph node after OVA stimulation, however, the number of cells was significantly reduced when the T cells expressed Nef or its mutant. T cells in the draining lymph nodes uniformly expressed high levels of CD44, a marker for activated T cells (21), irrespective of their expression of Nef or Nef mutant (Fig. 2D), suggesting that they were activated, but not involved in functional maturation. These results suggest that Nef affects trafficking of T cells to the regional lymph nodes during an immune response, independently of CD4 down-modulation.

As shown in Fig. 2(E), we examined the possibility that nef expression causes T cells to undergo AICD, which could reduce the number of cells migrating to the regional lymph nodes after stimulation. OVA-specific/CD4$^+$ or

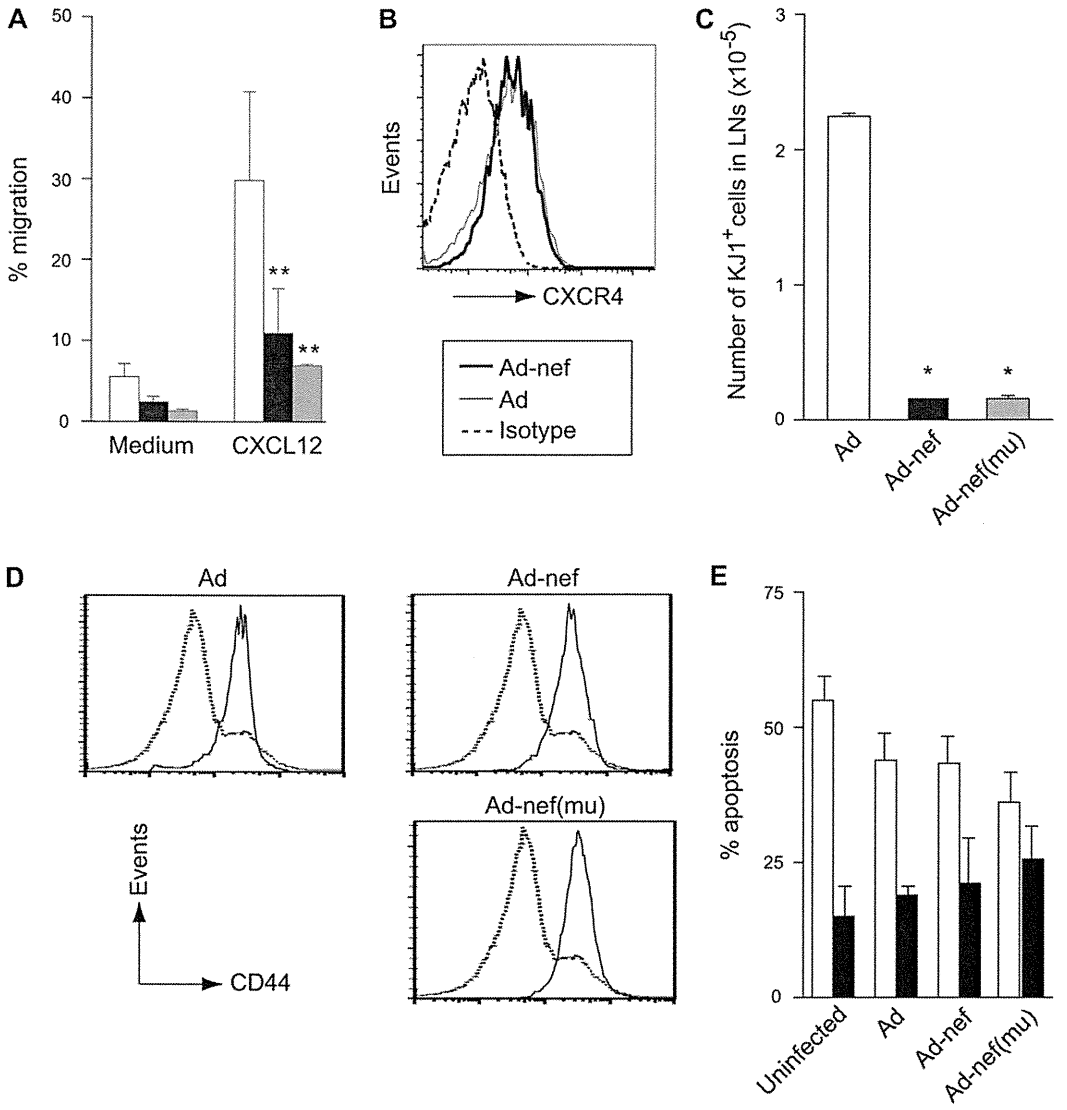

Fig. 2. Nef impairs T-cell migratory activity (A). $CD4^+$ GFP^+ T cells infected with Ad-nef (closed column), Ad-nef (mu) (gray column) and Ad (open column) were used in transwell chemotaxis assays in the presence of CXCL12 (PeproTech). Cells were allowed to migrate in the bottom wells for 90 min, and the proportion of cells that had migrated into the lower wells was determined by flow cytometry. The results are shown as mean ± SD ($n = 3$). *$P < 0.01$ versus Ad. (B) CXCR4 surface staining for $CD4^+$/GFP^+ T cells after infection with Ad-nef (solid line) or Ad (thin line), together with control IgG staining (broken lines). (C and D) $CD4^+$/GFP^+ T cells (2×10^6) infected with Ad-nef (closed column), Ad-nef (mu) (gray column) and Ad (open column) were transferred into BALB/c mice and 24 h later mice were injected subcutaneously with 0.2 mg of LPS-free OVA with CFA on the back in three sites. The cell number (±SD) of $CD4^+$/OVA-specific T cells in the draining lymph nodes (C) and the level of CD44 expression in Ad-infected donor (solid line) and recipient $CD4^+$ T cells (broken line) (D) were measured by flow cytometry using anti-CD4, anti-CD44 and KJ1-26 mAbs on day 5 after OVA injection. *$P < 0.001$ versus Ad. (E) $CD4^+$ T cells (1×10^6) or $CD4^+$/GFP^+ T cells (1×10^6) infected with Ad-nef, Ad-nef (mu) and Ad were stimulated with immobilized anti-CD3ε mAb for 2 days, followed by re-stimulation with anti-CD3 mAb/IL-2 (open column) or IL-2 alone (closed column) for 3 days. Apoptotic cells were analyzed by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling assay. Representative data from two independent experiments in (A), (C) and (D) and from three independent experiments (B) is shown.

OVA-specific/$CD4^+$/GFP^+ T cells were hyperstimulated with immobilized anti-CD3ε mAb at 2-day intervals as previously described (22). The results show that Nef did not enhance the induction of AICD in T cells upon TCR-stimulation *in vitro* nor did it compromise the survival function mediated by IL-2. Therefore, it seems unlikely that Nef causes T-cell death, which could reduce the number of cells migrating to the regional lymph nodes.

Nef expression in T cells affects the primary and memory B-cell responses

To examine T-cell helper activity by Nef, OVA-specific/$CD4^+$ T cells were purified from the pooled spleens of dTg mice, followed by infection with or without Ad-nef, Ad-Nef (mu) or Ad. The GFP^+/$CD4^+$ T cells were purified by FACS (Fig. 3A) and transferred into CB17-scid mice, together with either naive or NP-primed B cells. The recipients were immunized

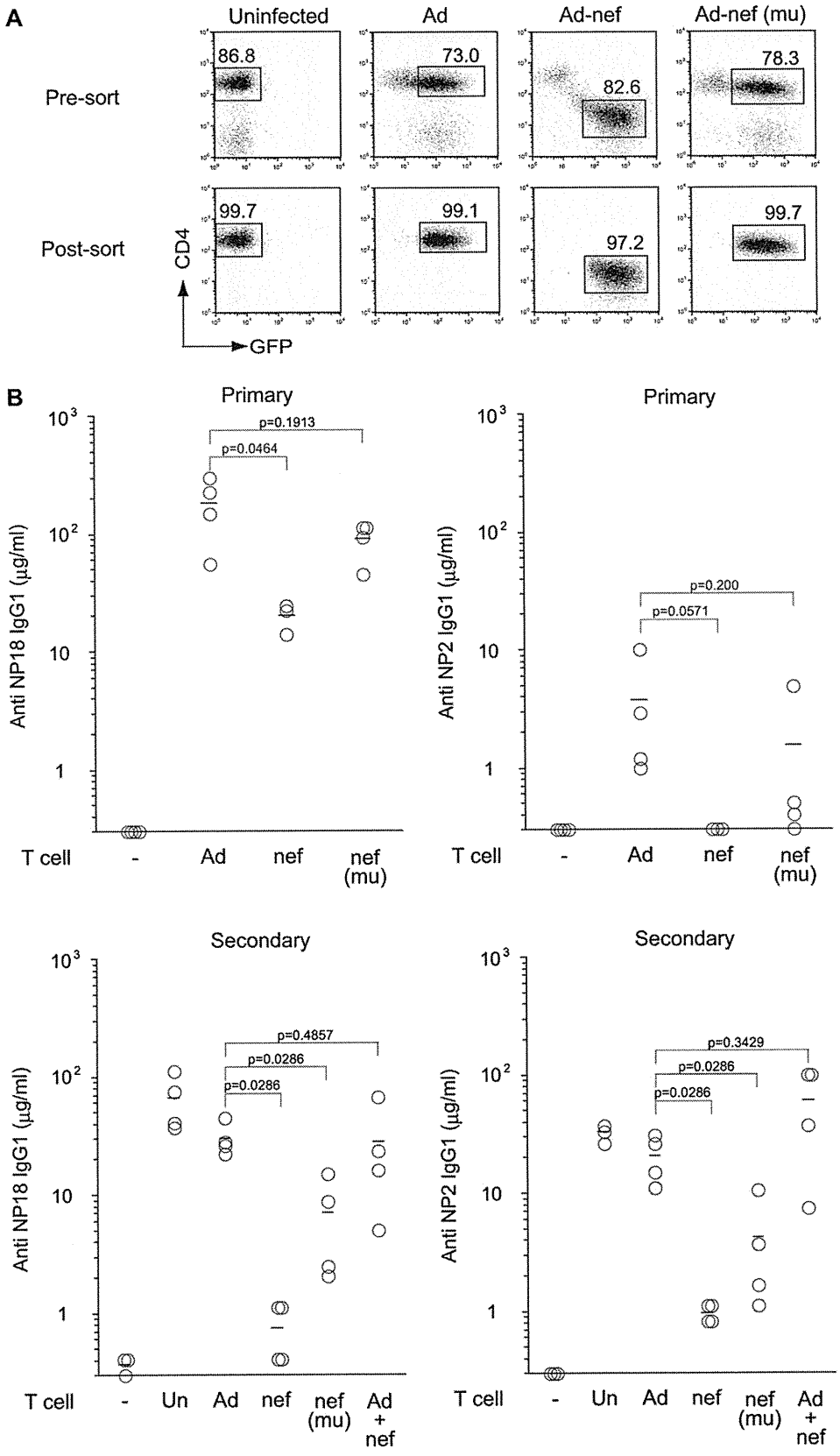

Fig. 3. Nef affects primary and memory B-cell response. (A) OVA-specific/CD4$^+$ T cells were purified from dTg mice and infected with Ad-nef, Ad-nef (mu) and Ad, followed by FACS purification (Post-sort). Numbers in plots indicate percent of GFP$^-$ uninfected cells and GFP$^+$ cells before (Pre-sort) and after purification (Post-sort). (B) Purified GFP$^+$ T cells (3×10^4) were transferred into CB17-scid mice, together with

with NP-OVA in alum for the primary response or soluble NP-OVA for the secondary response (Fig. 3B).

The results show that Nef expression in T cells reduced the level of anti-NP IgG1 serum antibodies by ~10-fold (NP_{18}; $P = 0.0464$, NP_2; $P = 0.0571$) in the primary response (Fig. 3B), whereas when the T cells were infected with Ad-Nef (mu), which does not down-regulate CD4 (Fig. 3A), the response was close to the control level (NP_{18}; $P = 0.1913$, NP_2; $P = 0.200$). As shown in Fig. 3(B), the impact of Nef on the secondary response was even more dramatic; there was a 30- to 40-fold reduction in both total and high-affinity anti-NP IgG1 antibodies (NP_{18}; $P = 0.0286$, NP_2; $P = 0.0286$). Reconstitution with equal numbers of non-infected and Nef-expressing OVA-specific $CD4^+$ T cells normalized the secondary adoptive response (NP_{18}; $P = 0.4857$, NP_2; $P = 0.3429$), excluding the possibility that Nef expression was generating suppressor T cells. Expression of the Nef mutant that was unable to down-modulate CD4 also reduced the secondary response (NP_{18}; $P = 0.0286$, NP_2; $P = 0.0286$), although the magnitude of the reduction was less than that induced by expression of wild-type Nef. These results demonstrate that Nef expression in peripheral T cells markedly diminishes their helper activity for the secondary IgG1 response and that this defect was only partially associated with the Nef-induced CD4 down-modulation. By contrast, this CD4 down-regulation appeared to be even more important for the reduced primary IgG1 response. These findings underscore the differential regulation in the primary and memory B-cell response. Thus, Nef affects helper T-cell activities in the primary and secondary response through different processes with different CD4 down-modulation susceptibility.

Discussion

In the present study, we have examined the consequence of Nef expression in primary splenic T cells. In order to avoid complications arising from expression of Nef early in T-cell development, e.g. lymphopenia, we established a double transgenic mouse (dTg), which expresses human CAR adenovirus receptor and an OVA-specific T-cell receptor that recognizes the OVA peptide on APC with high affinity under MHC Class II $I\text{-}A^d$-restriction. OVA-specific/$CD4^+$ T cells were purified from the spleen of dTg mice and infected with a recombinant adenovirus vector encoding Nef and GFP, followed by purification of GFP^+ cells using flow cytometry. To promote efficient introduction of the adenovirus vector into resting T cells, they were cultured for 2 days in the presence of the vector and IL-7, which is known to be important for survival of naive and memory T-cell populations (16). Neither naive nor memory $CD4^+$ T cells proliferate in response to IL-7, but they progress into the G_{1b} stage of the cell cycle (17). Thus, the present system allowed us to study the role of Nef in resting T cells in response to antigen-specific stimulation *in vitro* and *in vivo*.

During HIV-1 infection, the virus enters resting $CD4^+$ T cells and Nef is expressed even before the virus is integrated (1). It has been previously suggested that Nef expression in resting human T cells enhances IL-2 production upon activation by TCR cross-linking (1). This led to the proposal that Nef may enhance TCR signaling pathways that could help virus replication in partially stimulated T cells. In line with this viewpoint, it has been reported that Nef in human leukemic T cell lines and $CD4^+$ T-cell lines established from PBMC enhanced TCR signaling pathways and activated IL-2 production upon stimulation with TCR/CD28 or mitogens (2–5). In addition, Nef affects activation of murine T-cell hybridomas stimulated with anti-CD3 mAb (23), suggesting that the effect of Nef is not species specific.

In striking contrast, the present study demonstrates that Nef significantly reduces OVA-specific T-cell activation *in vitro* as defined by reduced proliferation and cytokine production, including IL-2 and IFNγ, but not completely. Furthermore, we demonstrate for the first time that Nef expression in OVA-specific resting T cells in the periphery reduced their ability to help anti-NP/$IgG1^+$ primary and secondary antibody responses in adoptive hosts after immunization with NP-OVA. In addition, in agreement with a previous *in vitro* analysis (7, 15), our *in vivo* results support the notion that Nef impairs the physiological recruitment of lymphocytes from the blood into the secondary lymphoid tissues after primary immunization, which promotes efficient antigen presentation and immune responses. Thus, Nef expressed in T cells at the early cell cycle stage impairs multiple functions in their subsequent antigen-specific response *in vivo*.

Why is the Nef-associated T-cell hyperresponse previously reported not detected in the present studies? The discrepancy does not reflect the differences in pathogenesis in *Nef* alleles (24) because the previous transgenic mouse models (8–10) and the present studies used the same NL4-3 Nef for characterization of the role of Nef protein in the immune system. Furthermore, the activation phenotype of T cells *in vitro* was induced by Nef proteins, irrespective of their alleles, including NL4-3 Nef (2–5). The discrepancy could be due to the cell state in the previous studies caused by transient over-expression of the protein in either the Jurkat T-cell line or in an activated human $CD4^+$ T-cell line established from PBMC (2–7). Another possible explanation is that previously reported assays utilized different TCR stimuli; the cells were stimulated by strong TCR ligation using immobilized antibodies (2–5). Such strong TCR ligation by antibodies forms stable TCR aggregates associated with the signaling complex (25). However, TCR stimulation with APC-presented antigen peptide forms an immunological synapse (IS) at the

B cells (5×10^6) which were enriched from the pooled spleens of either naive or 4-hydroxyl-3-nitrophenylacetyl-conjugated chicken γ-globulin (NP-CGG)-primed mice using a MACS system, followed by challenge with 100 μg of NP-OVA in alum (primary) or 25 μg of soluble NP-OVA (secondary). Serum anti-NP antibody titers were estimated by ELISA assays at day 7 after challenge using NP_2-BSA and NP_{18}-BSA as coating antigens. The relative affinity of anti-NP antibodies was estimated by calculating the ratio of anti-NP_2/anti-NP_{18} antibody. Representative data from two independent experiments is shown. Bars represent the mean of each group.

T-cell APC interface, facilitating signaling through TCR recognizing the peptide-loaded MHC molecules (26). The formation of IS was impaired *in vitro* by HIV-1 infection in a Nef-dependent manner (27), providing an explanation for the present results that Nef lowers the cognate interaction strength between T cells and APCs in antigen-specific response, thereby denying complete progression and activation of the cell cycle.

Nef affects helper T-cell activities in the primary and secondary response through different processes with different CD4 down-modulation susceptibility. However, the underlying mechanism remains obscure. In the B-cell response, antigen-activated helper T cells form a complex with B cells by interacting with several co-stimulatory molecules as well as with the TCR and peptide-loaded MHC class II molecules on B cells. As a consequence, T cells and B cells are mutually stimulated and T cells produce cytokines promoting B-cell proliferation and differentiation into antibody-forming cell (28). Therefore, it is likely that Nef-induced repression of T-cell helper activity for an antigen-specific B-cell response may also reflect an inefficient cognate interaction between T cells and B cells in the primary and secondary response.

We observed that Nef in resting murine $CD4^+$ T cells down-regulates the expression of CD4 on the cell surface, concordant with the previous results using human and murine T-cell lines (reviewed in ref. 1). It has been previously suggested that CD4 plays an important role in the activation of T cells by increasing the avidity of TCR for the peptide/MHC class II molecule and by transducing signals through the associated tyrosine kinase p56Lck (29). CD4 down-modulation significantly affects T-cell helper activity for the primary antibody response; however, it only partially affects T-cell helper function for the secondary response. On the other hand, Nef-mediated repression of antigen-specific T-cell function for the migratory capacity in the primary immune response is not the result of CD4 down-regulation. Thus, Nef affects multiple antigen-specific T-cell activities in the primary and secondary response through different processes with different CD4 down-modulation susceptibility, probably reflecting the T-cell signature and/or B-cell signature involved in the primary or the secondary antibody response.

CD4 binds to the MHC and boosts the recognition of ligand by the TCR in early T-cell activation, afforded by the IS formation (30) and functions to deliver Lck to the T-cell APC interface (31). However, signaling and co-stimulation later result in the movement of CD4 toward the periphery of the IS (30), suggesting that once Lck has been recruited to the synapse, the function of CD4 may become dispensable, allowing CD4 to leave the synapse, compatible with the idea that initial signal strength for T-cell activation may be crucial for the primary B-cell response. Nef might affect T-cell activity to form the IS with B cells, although, it remains unknown whether primary and memory B-cell responses require the same co-receptor molecules for T-cell interaction or if they need help from the same subset of T cells. Further analysis is needed to clarify how memory and naive B-cell responses are differentially regulated.

In summary, the present results support a model in which Nef expressing HIV-1 infected $CD4^+$ T cells fail to attain multiple functions required for normal immune responses. Thus, these combined effects of Nef may not facilitate extensive HIV-1 productions by increasing the numbers of productively infected cells through T-cell activations in antigen-specific immune response.

What could be the advantages for HIV-1 to inhibit proliferation and multiple T-cell functions required for immune response? Of note, Nef does not completely abrogate T-cell activation upon stimulation, as defined by expression of activated cell surface markers and a low level of cytokine production, which may allow a replicative advantage for the virus (reviewed in ref. 32). In activated $CD4^+$ T cells, viral replication is efficient and cytopathic (reviewed in ref. 32), though rapid death of infected cells may limit the production of the virus. By lowering the T-cell activity, Nef might facilitate a lowered level of viral spread and an increased infected T-cell life span by avoiding viral cytopathic effects. These cells may decay more slowly *in vivo* relative to activated cells, leading to vital consequences for the pathogenic outcome of infection in humans.

Supplementary data

Supplementary data are available at *International Immunology* Online.

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Differentiation associated regulation of microRNA expression *in vivo* in human CD8$^+$ T cell subsets

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Abstract

Background: The differentiation of CD8$^+$ T lymphocytes following priming of naïve cells is central in the establishment of the adaptive immune response. Yet, the molecular events underlying this process are not fully understood. MicroRNAs have been recently shown to play a key role in the regulation of haematopoiesis in mouse, but their implication in peripheral lymphocyte differentiation in humans remains largely unknown.

Methods: In order to explore the potential implication of microRNAs in CD8$^+$ T cell differentiation in humans, microRNA expression profiles were analysed using microarrays and quantitative PCR in several human CD8$^+$ T cell subsets defining the major steps of the T cell differentiation pathway.

Results: We found expression of a limited set of microRNAs, including the miR-17~92 cluster. Moreover, we reveal the existence of differentiation-associated regulation of specific microRNAs. When compared to naive cells, miR-21 and miR-155 were indeed found upregulated upon differentiation to effector cells, while expression of the miR-17~92 cluster tended to concomitantly decrease.

Conclusions: This study establishes for the first time in a large panel of individuals the existence of differentiation associated regulation of microRNA expression in human CD8$^+$ T lymphocytes *in vivo*, which is likely to impact on specific cellular functions.

Background

CD8$^+$ T cells are major players of the immune response against viruses and cancers. Even though they represent a heterogeneous population, the expression of specific surface molecules characterizes distinct subsets (i.e. central memory, early, intermediate or late effector memory cells), which define the major steps of a process of memory T cell differentiation [1,2]. These multiple subsets present specific transcriptional programs, and therefore distinct range of receptors and intracellular proteins, indicating quite different requirements for stimulation and survival, homing potential and effector functions (reviewed in [3]). For instance, expression of effector molecules such as perforin or granzymes is restricted to the late stages of differentiation [4,5], while central memory cells are known for their superior proliferative capacity and often considered as the memory "precursors". However, the molecular mechanisms controlling peripheral CD8$^+$ T lymphocyte differentiation, and therefore the generation of immunological memory, remain poorly understood in humans.

MicroRNA (miRNA) are 18-22 nucleotide long RNA molecules that regulate gene expression at the post-transcriptional level through base pairing to partially complementary sites in the 3' UTR of the messanger RNA and integration into RNA induced silencing complexes (RISC) (reviewed in [6]). Inhibition of translation or degradation of the miRNA-bound mRNAs modulate protein output, inducing profound physiological effects. MicroRNA, which expression is tightly regulated during lymphopoiesis [7], recently emerged as key regulators of gene expression in the mammalian immune system (reviewed in [8,9]).

Although the biological functions of most miRNAs are not yet fully understood, unequivocal evidence for their

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role in lymphocyte development have been gathered in mouse models in recent years. For instance, the conditional deficiency in mouse lymphocytes of Dicer, the key enzyme in miRNA biogenesis, impaired lymphocytic differentiation [10,11]. Moreover, it was recently shown that abolishing Dicer expression in mature mouse $CD8^+$ T cells strongly impairs their response to pathogens *in vivo* [12], demonstrating that miRNAs are not only important for lymphocytes differentiation, but also for their functions in the periphery. Interestingly, single miRNA such as miR-181 have also been shown to be crucial in mouse haematopoiesis [13]. Thus, mouse models and/or studies on *in vitro* differentiated cells suggested a role for miRNAs in lymphocytes functions [14-16]. However, the modulation of their expression along antigen-driven differentiation of peripheral human $CD8^+$ T lymphocytes *in vivo* has not been studied yet.

We thus addressed this issue using microarrays and quantitative PCR to investigate microRNA expression profiles in different subsets of *ex vivo* sorted human $CD8^+$ T lymphocytes. Our results show the first evidence that these cells express a limited set of microRNAs, some of which displayed differential expression in differentiated $CD8^+$ T cells when compared to naive cells. The microRNA expression regulation uncovered here is likely to strongly influence the gene signature of these different subsets, and therefore to directly impact on their functional properties.

Methods

CD8 T cell subsets purification

Peripheral blood $CD8^+$ T lymphocytes were purified from healthy volunteers in agreement with local ethics committees (Cantonal Commission of Ethics in Research, State of Vaud, Switzerland, authorization #87/06) as described [4]. Briefly, total $CD8^+$ T cell preparations were obtained from leukapheresis by magnetic bead enrichment (Myltenyi Biotech, Bergish Gladbach, Germany) and stained with a combination of antibodies to CD3, CD8, CD45RA, CCR7, CD27 and CD28. All antibodies were from BD Pharmingen (San Diego, CA) except anti-CD45RA purchased from Beckman Coulter (Paris, France). The different subsets were then separated on a FACS Aria device (BD Bioscience, Allschwil, Switzerland) to routinely over 99% purity cell suspensions. Cells were either sorted directly to RNA lysis buffer (RNAlater, Qiagen) and frozen (for single specific qPCR assays) or into cold medium (for TaqMan Low Density Array experiments), before washings in ice-cold PBS and lysis (miRVana kit, Ambion).

MicroRNA expression analysis

The TaqMan® Low-density arrays (TLDA, Applied Biosystems, Foster City, CA) were used following manufacturer's instructions to simultaneously detect expression of 364 individual microRNAs. Briefly, total RNA was extracted with the miRVana kit (Ambion), quantified with a Nanodrop Spectrophotometer, and microRNA mature forms were reverse transcribed (30 min at 16°C, 30 min at 42°C, 5 min at 85°C) with Multiplex RT human primers and 100 mM dNTPs, 50 U MutliScribe reverse transcriptase, 20 U RNase inhibitor, 10 × RT buffer (8 reactions with pools of 48 stem-loop RT primers, 100 ng RNA per reaction). cDNA were then amplified on microfluidic cards in an ABI Prism 7900 HT with single microRNA specific primers (40 cycles of 15 s at 95°C and 1 min at 60°C). The same protocol was applied for single microRNA specific qRT-PCR (miR-17-3p, miR-17-5p, miR-19b, miR-20a, miR-92, miR-21, miR-155, miR-142-3p, miR-142-5p, and RNU44) from 10 ng total RNA, reverse transcribed with TaqMan Reverse transcription MicroRNA kit (Applied Biosystems) and amplified using Universal Fast Start Rox Probe Master Mix (Roche) and microRNA Assay kits in 384 well plates (Applied Biosystems) on a ABI Prism 7900 HT device (Applied Biosystems). Ct (RNU44) was subtracted to Ct (microRNA) to calculate relative expression (ΔCt).

Statistical analysis

Statistical significance was evaluated with the GraphPad Prism software on non normalized expression data using the Friedman test (for non-parametric, paired data set) with Dunn's multiple comparison correction applied. Results were considered significant for p values < 0.05.

Results and Discussion

MicroRNA expression profiling in mature $CD8^+$ T lymphocyte subsets

Different subsets of human $CD8^+$ T lymphocytes can be defined based on the expression of CCR7, CD45RA and CD28 [4]. Naïve cells are $CCR7^+$ (and $CD45RA^+$ $CD28^+$) while the effector memory subsets are $CCR7^-$. Among the latter, the $CD45RA^+$ $CD28^-$ cells representing the end stage of differentiation (late effector memory, L-EM), while $CD45RA^-$ cells can be further divided in two functionally distinct subsets, with $CD28^+$ cells (early effector memory (E-EM) closer to central memory cells) being less differentiated than $CD28^-$ subsets (I-EM), which share features with fully differentiated effectors (L-EM).

These human $CD8^+$ T lymphocyte subsets (naive, E-EM, I-EM and L-EM, ordered from least to most differentiated) were purified from peripheral blood leukocytes from 3 healthy donors as indicated (Figure 1A), and the relative expression of 365 microRNAs was analyzed in these subpopulations using TaqMan Low Density Arrays

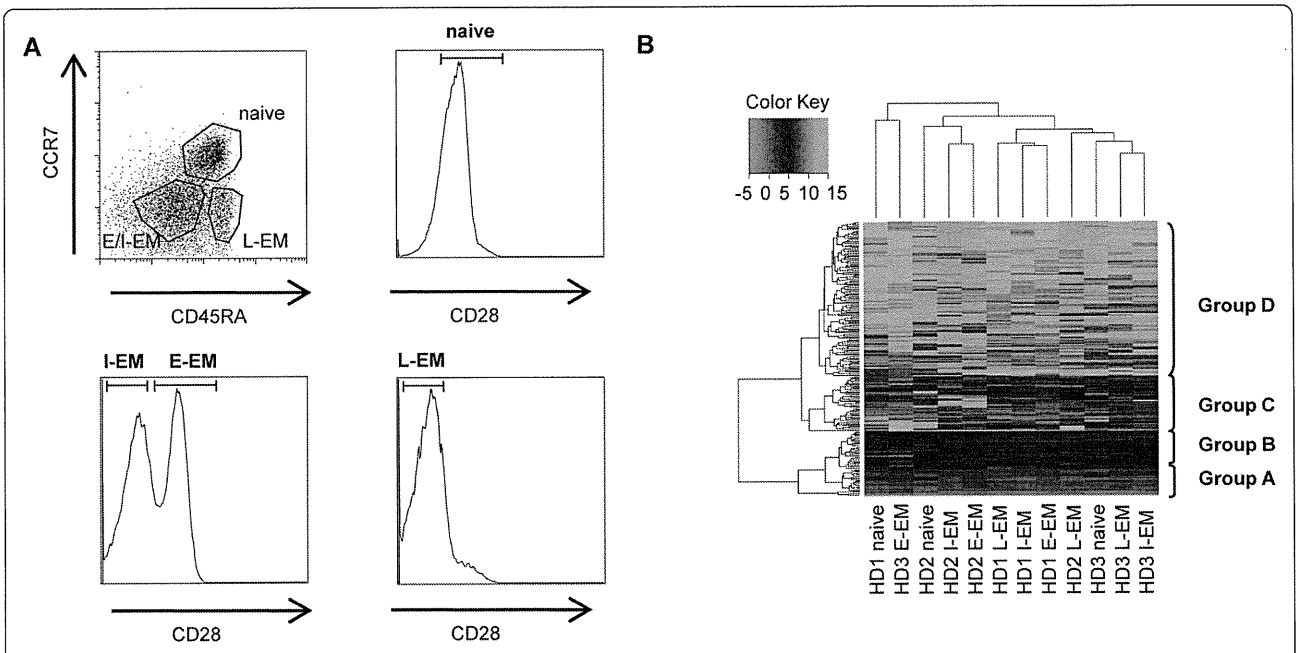

Figure 1 Sorting of human CD8$^+$ T lymphocytes and microRNA large scale expression profiling. a) CD8$^+$ T cells from healthy volunteers were FACS-sorted based on their expression of CD45RA, CCR7 and CD28 to isolate Naive (CD45RA$^+$ CCR7$^+$, CD28$^+$), E-EM (CD45RA$^-$ CCR7$^-$, CD28$^+$), I-EM (CD45RA$^-$ CCR7$^-$, CD28$^-$) and L-EM (CD45RA$^+$ CCR7$^-$, CD28$^-$). Data shown are gated on CD3$^+$ CD8$^+$ cells. b) Unsupervised clustering of the expression levels (ΔCt, normalized to RNU44) of 223 unique microRNAs (detected in at least one sample, i.e. Ct < 36) in CD8$^+$ T lymphocyte subsets from 3 healthy donors (HD1, HD2, HD3) as measured by TLDA (green: low expression/red: high expression).

(TLDA). Unsupervised clustering of relative expression levels in all subsets identified 4 main groups of microRNAs (Figure 1B): highly expressed (group A, 22 microRNAs, see Table 1), intermediate (group B, 31 microRNAs, see Table 1), low (group C, 46 microRNAs) and rare/absent (group D, 267 microRNAs). In total, 97 microRNAs were expressed well over detection limit, a figure similar to the 113 microRNAs recently detected in B cells using the same TLDA [17]. This list is likely not comprehensive, since the set of microRNAs detected with TLDA does not cover the whole range of microRNAs cloned in human. However, the low number of microRNAs considered expressed at high levels (group A) is in agreement with previous cloning studies in mouse CD8$^+$ lymphocytes [15,18] showing that a few highly expressed microRNAs prevailed in frequency. Interestingly, these microRNAs include miR16, miR-21, miR-142-3p and miR-142-5p, which were also found in the group A in human CD8$^+$ T lymphocytes, with miR-142-3p showing the highest relative expression levels (Table 1). MiR-26a/b and miR-146a/b also clustered to this group. In addition, 7 microRNAs of the 17~92 and paralog 106b~25 clusters (namely miR-19a, miR-19b, miR-20a, miR-25, miR-92, miR-93 and miR-106b) were identified among the 53 most expressed microRNAs (groups A and B, see Table 1). MiR-17-5p and miR-17-3p are expressed at lower levels (belong to group C), indicating intra-cluster differential expression.

Transgenic overexpression of miR-17~92 cluster in mouse lymphocytes was shown to induce lymphoproliferative disease [16]. High expression levels are therefore likely to be tightly controlled. The high expression levels found for a large set of microRNAs from the 17~92 cluster in primary human CD8$^+$ T lymphocytes had not been reported yet, and strongly suggests a role for this set of microRNAs in the biology of CD8$^+$ T cells.

MiR-21 and miR-155 are upregulated during CD8$^+$ T lymphocyte differentiation

To investigate if defined microRNA profiles can be associated with CD8$^+$ T cell differentiation status, the relative expression levels of the microRNAs from groups A, B and C were calculated for each CD8$^+$ lymphocyte subset relative to those found in the naive cells. Global unsupervised clustering did not identify clear microRNA clusters, and grouped subpopulations from the same donor together, due to strong inter-donor heterogeneity. However, specific patterns could be highlighted when the analysis was focused on a more restricted set of microRNAs (Figure 2A), chosen for their suggested role in immunity. The well expressed miR-21, miR-155 and miR-146a clustered together as consistently upregulated, while the abundant microRNAs of the miR17~92 clusters (miR-19b, miR-20a and miR-92) showed a clear trend towards decreased expression in differentiated cells, as did miR-26a (Figure 2A).

Table 1 Human CD8$^+$ T cells express a limited set of microRNA

Group A		GROUP B	
rank	miR	rank	miR
1	hsa-miR-142-3p	23	hsa-miR-31
2	hsa-miR-26a	24	hsa-miR-125a
3	hsa-miR-16	25	hsa-miR-565
4	hsa-miR-26b	26	hsa-let-7b
5	hsa-miR-146b	27	hsa-let-7a
6	hsa-miR-29a	28	hsa-miR-181b
7	hsa-miR-19b *	29	hsa-miR-30e-3p
8	hsa-miR-92 *	30	hsa-miR-423
9	hsa-miR-342	31	hsa-miR-328
10	hsa-miR-30c	32	hsa-miR-101
11	hsa-miR-20a *	33	hsa-miR-223
12	hsa-let-7g	34	hsa-miR-27a
13	hsa-miR-142-5p	35	hsa-miR-25 *
14	hsa-miR-146a	36	hsa-miR-195
15	hsa-miR-24	37	hsa-miR-425-5p
16	hsa-miR-21	38	hsa-miR-222
17	hsa-miR-29c	39	hsa-miR-197
18	hsa-miR-30b	40	hsa-miR-28
19	hsa-miR-30a-5p	41	hsa-miR-186
20	hsa-miR-191	42	hsa-miR-331
21	hsa-miR-484	43	hsa-miR-192
22	hsa-miR-140	44	hsa-miR-103
		45	hsa-miR-594
		46	hsa-miR-15b
		47	hsa-miR-30e-3p
		48	hsa-miR-155
		49	hsa-miR-374
		50	hsa-miR-93 *
		51	hsa-miR-19a *
		52	hsa-miR-106b *
		53	hsa-miR-30d

MicroRNA ranking according to relative expression in CD8$^+$ T cells. Groups A and B are as identified by unsupervised clustering in Figure 1b. *: microRNA belonging to the miR-17~92 cluster.

MiR-21 has been shown to be upregulated in mouse CD8$^+$ T cells upon *in vitro* activation [15], but its function in these cells remains unknown. MiR-155, encoded by the BIC transcript, is induced upon antigen-receptor triggering in lymphocytes and was shown to be instrumental for the generation of adaptive immunity in mouse [19,20]. Our TLDA analysis suggested that both miR-21 and miR-155 were more expressed in *in vivo* differentiated human primary CD8$^+$ T cells than in their naive counterparts (Figure 2A). These results were confirmed by single specific RT-qPCR on eight additional donors, which all clearly showed higher expression of both miR-21 and miR-155 in antigen-experienced cells (Figure 2B).

Altogether these results demonstrate that an increase of miR-21 and miR-155 expression occurs upon *in vivo* differentiation of human CD8$^+$ T lymphocytes. Dynamic regulation of these microRNAs in mouse CD8$^+$ T cells has been described upon *in vitro* activation [15]. By contrast, the data presented here on *ex vivo* sorted lymphocytes revealed for the first time modulations that occurred during *in vivo* differentiation of human peripheral CD8$^+$ T cell subsets.

The miR-17~92 cluster tends to be downregulated during CD8$^+$ T cell differentiation

Since central memory (CM) CD8$^+$ T cells are present at extremely low frequency in peripheral blood, a new sorting strategy was then designed to include this subset in our analysis. Addition of anti-CD27 to the panel of cell surface molecule specific antibodies allowed the isolation of naïve (CCR7$^+$ CD45RA$^+$ CD27$^+$ CD28$^+$), CM (CCR7$^+$ CD45RA$^-$ CD27$^+$ CD28$^+$), E-EM (CCR7$^-$ CD45RA$^{+/-}$ CD27$^+$ CD28$^+$), I-EM (CCR7$^-$ CD45RA$^{+/-}$ CD27$^+$ CD28$^-$) and L-EM (CCR7$^-$ CD45RA$^{+/-}$ CD27$^-$ CD28$^-$) subsets. Consistent with our previous analysis (Figure 2B), upregulation of miR-155 in antigen experienced cells versus their naïve counterparts was confirmed in the new set of 9 independent donors, with a clear trend toward highest expression levels in the most differentiated CD8$^+$ T cells (L-EM) (Figure 3A). Since the TLDA analysis showed that both miR-142-3p and miR-142-5p were among the most expressed microRNAs in CD8$^+$ T cells (Table 1), their expression levels were assessed in the different subsets. No significant regulation of expression could be found, likely suggesting a constitutive role rather than a differentiation induced function for these microRNAs (Figure 3B).

Seven members of the 17~92 cluster, which recently were shown to be critical regulators of lymphocyte development and proliferation [16], were identified among the 53 most expressed microRNAs in human CD8$^+$ T cells (Table 1), with a trend toward lower expression in more differentiated cells (Figure 2A). Expression levels of miR-17-3p, miR-17-5p, miR-19b, miR-20a and miR-92 were therefore determined by single specific qPCR in differentiated CD8$^+$ T cell subsets, and compared to the levels found in naïve cells. In agreement with the TLDA data, and despite a relatively important inter-donor variability, the expression of some of these miRNAs was found to be preferentially associated with specific subsets of CD8$^+$ T cell differentiation. Interestingly, the expression level of miR-20a was significantly increased in the central memory subset, in contrast to more differentiated subsets. Along the same lines, miR-17-3p expression was significantly

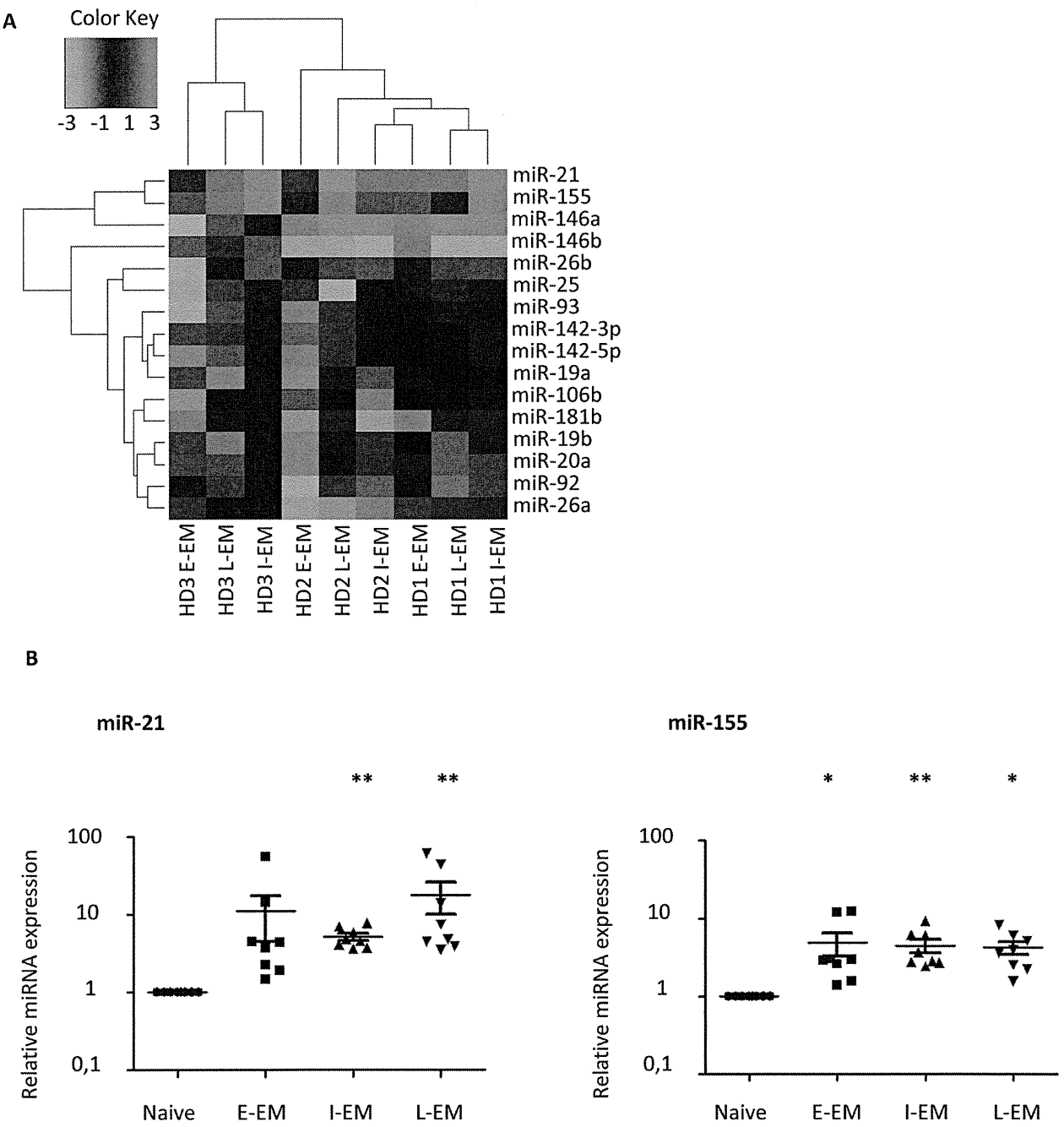

Figure 2 miR-21 and miR-155 are upregulated in differentiated CD8$^+$ lymphocyte subsets. a) Unsupervised heatmap clustering of the expression levels of a selected set of microRNAs in each subset normalized to levels in naive cells (log2 fold change) (green: downregulated/red: upregulated). b) Expression levels of miR-21 and miR-155 in sorted human CD8$^+$ T cell subsets were determined by single specific qPCR performed in triplicates, and are expressed as fold change relatively to levels in naive cells (n = 8; *: $p < 0,05$; **: $p < 0,01$).

decreased in late effector memory cells. There were also non significant trends towards preferential expression of miR-19b and miR-92 in the central memory cells. MiR-17-5p expression showed no association with CD8$^+$ T cell differentiation. On the whole, expression of several members of the miR-17~92 cluster appeared to be found preferentially during early memory differentiation. Interestingly, not all members are regulated concomitantly, indicating a differential intra-cluster regulation in agreement with the TLDA results (Table 1).

Altogether, the data presented here demonstrate for the first time that human CD8$^+$ T cell subsets express a

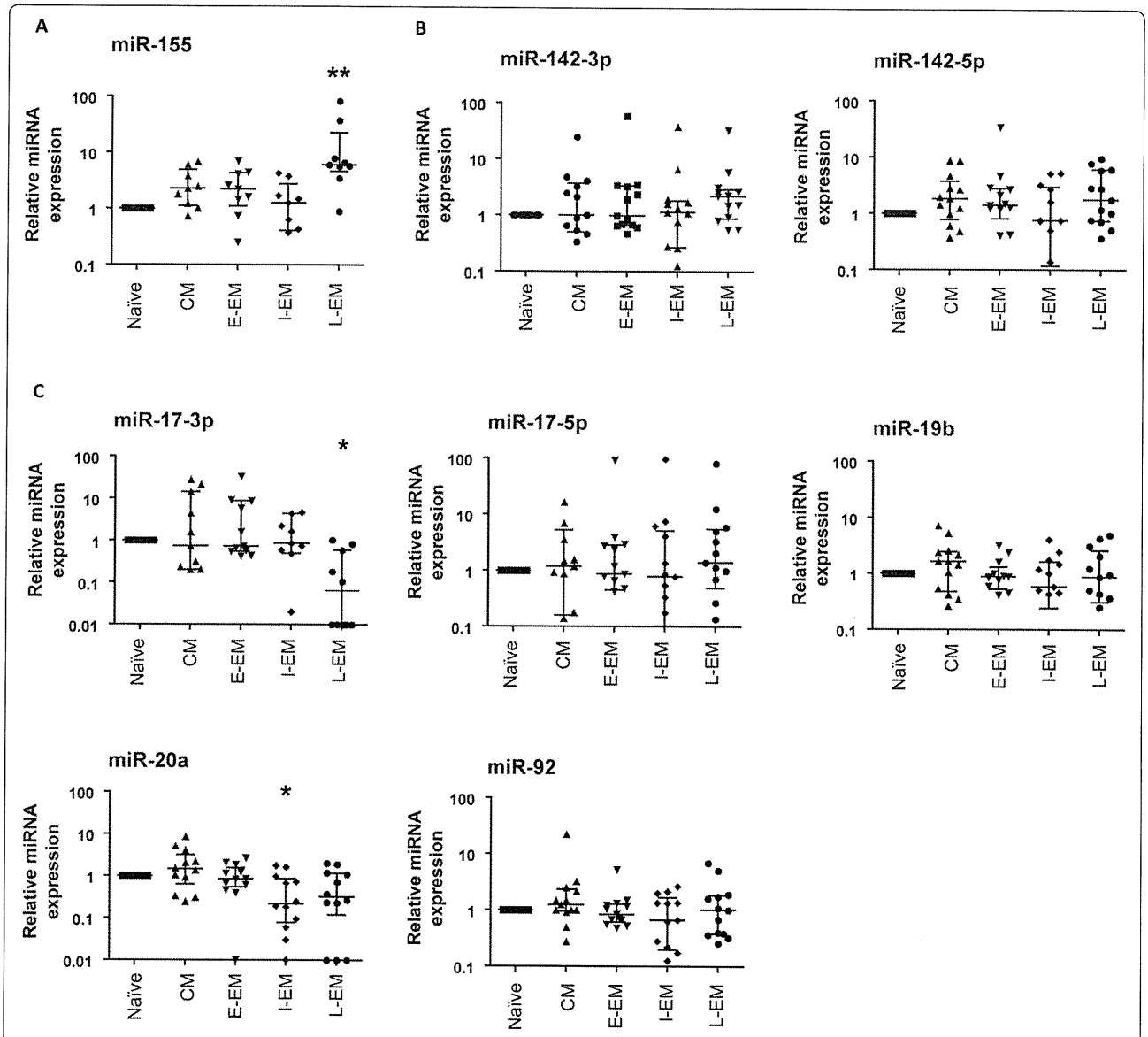

Figure 3 The miR-17~92 cluster is preferentially downregulated in differentiated CD8$^+$ T cell subsets. Expression levels of miR-155 (a), miR-142.3p and miR-142-5p (b) and of members of the miR-17~92 cluster (c) were determined by single specific qPCR in the indicated sorted human CD8$^+$ T cell subsets (a), and are expressed as fold change relatively to levels in naive cells (n = 9; *: $p < 0,05$; **: $p < 0,01$).

limited set of microRNAs, which include several members of the 17~92 and related clusters. Moreover, this study shows that the *in vivo* differentiation from naive cells is associated with a stage-specific regulation of distinct microRNA expression, with miR-21 and miR-155 being upregulated in antigen-experienced subsets, the more differentiated subsets expressing higher levels of these microRNA (Figures 2B and 3A). MiR-155 has been suggested to be part of a negative feedback loop downstream the Toll-like receptor/Interleukin 1 receptor pathway [21]. The results presented here may suggest similar functions in human CD8$^+$ T cells downstream of the TCR. Moreover, miR-155 has been recently shown to target SOCS-1 expression, both in tumor cells [22] and in mouse CD4$^+$ regulatory T cells, in which it modulates the cellular responses to IL-2. Whether miR-155 plays a similar role in CD8$^+$ T cells remains an interesting issue to investigate, as it might shed new light on the relationship between cytokine signaling and lymphocytes differentiation.

In contrast to the clear upregulation observed for miR-155, the expression of the miR-17~92 cluster (especially miR-17-3p and miR-20a) tended to decrease along differentiation. Our results are in line with a recent report by Hackl and colleagues [23] that shows downregulation of this cluster in CD28 negative CD8+ T cells.

Considering the role of the miR-19~92 cluster in lymphocyte development and proliferation, it is tempting to speculate that its expression may be related to the greater proliferative potential and memory precursor-like capacity, characteristic of central memory cells compared to more differentiated subsets (in particular late effector memory cells). Interestingly, expression of this cluster is induced by the transcription factor c-Myc [24], which has been shown in the mouse to be a component of the IL-15-dependent pathway controlling homeostatic proliferation of memory $CD8^+$ T cells [25]. In addition, the 17~92 cluster has been experimentally shown to inhibit Bim expression [16], which plays a key role in activation induced cell death in lymphocytes [26]. Whether 17~92 induced regulation of Bim plays a function in $CD8^+$ T cells survival potential (greater in less differentiated cells) is an interesting issue to be further investigated.

Conclusions

Mouse models have shown that the limited set of microRNAs expressed in mature $CD8^+$ T cells critically support their functions in response to pathogens [12,15]. Similarly, the differential microRNA expression profile observed here for the first time in human $CD8^+$ T cell subsets is likely to have functional relevance in lymphocyte biology, in view of proteomic experiments demonstrating that a single miRNA can directly repress the production of hundreds of proteins in mammalian cells, through both downregulation of mRNA levels and translation inhibition [27]. Further investigation is warranted to dissect the precise role of individual differentially expressed microRNA in the regulation of $CD8^+$ T cell functions. We are currently pursuing analyses on human CD8+ T cell clones and in mouse models to better understand the functional relevance of the results presented here. Understanding this novel aspect of lymphocyte biology will help to better define $CD8^+$ T cell differentiation, and might shed light on the relationship between differentiation and functional properties. In that respect, it might therefore be helpful to elaborate better vaccination strategies for induction of $CD8^+$ T cells with appropriate differentiation and functions.

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Authors' contributions

BS, TY, PM, YTY BA, VA, PR designed the study. BS, TY, BB, RR, HFK, PB, ED, AR, MB generated the data. BS, TY, BB, LB, YTY, DS, VA, PR analyzed the data. BS, VA, PR wrote the paper. All authors have read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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One-step simple assay to determine antigen-specific cytotoxic activities by single-color flow cytometry

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ABSTRACT

Assays for cytotoxicity of CTLs *in vivo* using a fluorescent-based dye, 5- (and 6-) carboxyfluorescein diacetate succinimydyl ester (CFSE), have been established and widely used. On the basis of this experience, we applied it to *in vitro* assay system and established a simpe, highly sensitive flow cytometric assay for CTL activity. In our assay, specific activities of CTLs could be detected by a reduction in sensitive target cell numbers on single-color histogram plot analysis. By using this assay, we could determine the changes in cytotoxic activity by single amino acid substitution within an epitope peptide. Adherent cells were also used as target cells in this assay by treatment with excess EDTA and trypsin reagents after incubation with effector CTLs. Furthermore, when fluorescent calibration beads were used as a control, we could determine the cytotoxicity of CTLs against tumor cells. The results obtained from our assay were almost consistent with those from the conventional ^{51}Cr-release assay. Because our assay uses only a stable non-radioactive reagent, CFSE, this assay is safe, inexpensive and extremely easy. These results indicated that this new assay (FACS-CTL assay) would be sufficiently acceptable alternative to classical ^{51}Cr-release assay.

Antigen-specific CD8$^+$ cytotoxic T lymphocytes (CTLs) play an important role in preventing both viral infection and disease progression. Therefore, measurement of cytotoxic activities of these CTLs is needed to analyze the cell-mediated immune responses in viral infection. Generally, the chromium (^{51}Cr)-release assay has been the most popularly used for its high sensitivity and antigenic specificity. However, this assay requires environmental and special techniques due to the use of short-lived radioactive materials. Moreover, it is difficult to handle virus-infected samples such as those with human immunodeficiency virus (HIV). Recently, several alternative non-radioactive assays have been developed to avoid radioisotope use. In particular, highly sensitive flow cytometric assays using various fluorescent-associated dyes such as c'FDA (10), DiO (3), FITC (7), Calcein-AM (11), PKH26 and PKH2 (4) have been reported. Although some of these assays can detect the cytotoxic activities of CTLs and concurrently examine the characterization of cells involved in the cytolytic reaction, they are not suitable to quickly evaluate many samples because of the complicated two-color dot blot analysis on an FACScan analyzer.

Recently, method to determine antigen-specific cytotoxic activities of CTLs *in vivo* have been established by using fluorescent-based dye 5- (and 6-) carboxyfluorescein diacetate succinimydyl ester (CFSE) (1, 2). In this assay, antigen-specific cytotoxic activities in cells from various murine lymphoid tissues can be detected by flow cytometry after injection of control and sensitive target cells labeled with different concentrations of CFSE.

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Therefore, we applied this dye to an *in vitro* assay system and established a simple, highly sensitive flow cytometric assay to detect CTL activity (FACS-CTL assay). In our assay, specific activities of CTLs could be detected as a reduction in sensitive target cell numbers on single-color histogram plot analysis.

Because our assay uses only a stable non-radioactive reagent, CFSE, this assay is safe, inexpensive and extremely easy. The results obtained in this study suggest that this simple assay could be an acceptable alternative to the classical ^{51}Cr-release assay.

MATERIALS AND METHODS

Mice. Female BALB/c (H-2^d) mice were purchased from Charles-River Japan Inc. (Tokyo, Japan). Transgenic mice (Tg-RT1) expressing TCR α and β chains (Vα 42H11 and Vβ8.1) genes of a murine CTL clone RT-1 (13) specific for P18-I10 restricted by a D^d class I MHC molecule (12, 16) and carrying the genetic background of BALB/c were established and bred in our colony (17). All mice used in this study were maintained in a specific pathogen-free microisolator environment. All experiments were performed according to the guidelines of the NIH Guide for the Care and Use of Laboratory Animals.

Recombinant vaccinia virus. vSC-25 (recombinant vaccinia virus expressing the HIV-1, IIIB gp160 envelope glycoprotein) was described previously (12) and was used to infect mice to induce envelope-specific CTL. For infection experiments, mice were intraperitoneally inoculated with 1×10^7 PFU/mouse of vSC-25.

Synthetic peptides and reagents. Peptides used in this study were all purchased from Takara Bio Co. (Tokyo, Japan). Peptide P18IIIB (aa: RIQRGPGRAF VTIGK) is the immunodominant CTL epitope presented by the murine class I MHC molecule H-2D^d in the V3 loop of the HIV-1 gp160 glycoprotein found in strain IIIB (12) and peptide P18-I10 (aa: RGPGRAFVTI) is the minimal active 10-mer peptide within P18IIIB (16). Control peptide P18MN (aa: RIHIGPGRAFYTTKN), P18RF (aa: SITKGPG RVIYATGQ) and P18MN substituted peptides (See Fig. 3A) were also used.

A Cell Trace™ CFSE cell proliferation Kit [Flourescein-based dye 5- (and 6-) carboxyfluorescein diacetate succinimydyl ester; CFSE] was purchased from Life Technology Corporation (Carlsbad, CA, USA) and dissolved in dimethylsulfoxide at a concentration of 5 mM according to the manufacturer's instructions. This stock solution was kept at −20°C until experiments. Calibration beads were purchased from Polysciences Inc. (Warrington, PA, USA).

Cell lines. P815 cells (a DBA/2-derived mastocytoma cell line; H-2^d) were used as target cells. BALB/c.3T3 (H-2^d) fibroblast transfectants expressing HIV-1 gp160 of the IIIB isolate (15-12 cells) and control transfectants with only the selectable marker genes (Neo) were described previously (12) and were also used as target cells. These lines were used with more than ninety percent viability. The CTL line (LINE-IIIB) specific for the HIV-1 envelope protein of the IIIB-strain has been described previously (12, 13). This CTL line was restricted by the D^d class I MHC molecule and is specific for an immunodominant epitope of HIV-1 gp160, P18IIIB (12).

Cytotoxicity assay using the CFSE fluorescent-based dye. On the day of the assay, peptide P18-I10-pulsed P815 cells or unpulsed control P815 target cells were labeled with CFSE as follows: Cells were suspended in phosphate-buffered saline (PBS) containing 1% FCS and adjusted to 1×10^6/mL. For sensitive targets, 0.5 μL of CFSE stock solution (5 mM) was added to 1 mL of cell suspension and incubated for 4 min at room temperature under shading. For control targets, 0.5 μL of diluted CFSE solution (100 μM) was used for labeling. After incubation, 9 mL of PBS containing 5% FCS (FCS-PBS) was added to stop the labeling reaction. Then, cells were washed once with FCS-PBS and resuspended with complete T cell medium (CTM) (12). Effector cells were washed once and resuspended in CTM to a final concentration of $1.25–5 \times 10^6$/mL. For sensitive reactions, 5×10^4 of peptide P18-I10-pulsed P815 target cells and various numbers of effector cells were added to round-bottom polystyrene tubes (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) at a final volume of 500 μL to yield various effector/target (E/T) ratios. Control tubes containing 5×10^4 of unpulsed target cells and the same effector cells were also separately prepared. Tubes were gently mixed, centrifuged at $200 \times g$ for 1 min, and then incubated at 37°C for 4 h. After incubation, sensitive target cells were mixed with control target cells in one tube with PBS containing 1% FCS and 0.1% sodium azide (FACS buffer) at the same E/T ratio and mixed cells were washed once with FACS buffer and resuspended with 4% paraformaldehyde (PFA; Sigma)-containing PBS (PFA-PBS).

Flow cytometric acquisition and calculation. Acquisition was performed using a FACScan analyzer (Beckton Dickinson Immunocheminal Systems, Mountain View, CA) with Cell Quest software (Beckton Dickinson). A detailed description of the sample acquisition and analysis is shown in results. All samples were assayed in duplicate and the mean percentage of specific lysis was calculated as follows: % specific lysis = [(number of sensitive target cells in control sample − number of sensitive target cells in test sample / number of sensitive target cells in control sample)] × 100. In this formula, number of cells refers to the event count on Cell Quest software. Control sample indicates target cells incubated without added effector cells and test sample shows target cells incubated with added effector cells.

^{51}Cr release assay. A ^{51}Cr-release assay was performed as previously described (12).

RESULTS

Detection of antigen-specific cytotoxicity of CTLs by FACS-CTL assay

We have already established a $CD8^+$, H-$2D^d$ class I MHC molecule-restricted, murine cytotoxic T lymphocyte (CTL) line, LINE-IIIB, specific for the envelope glycoprotein 160 (gp160) of the IIIB strain of HIV-1 (12). Furthermore, we identified an immunodominant epitope within gp160 as a 15-mer peptide, P18IIIB (aa: RIQRGPGRAFVTIGK), as well as the minimal active 10-mer peptide, P18-I10 (aa: RGPGRAFVTI) within P18IIIB (16). By using this CTL line and P815 cells as effector and target cells, respectively, we examined the basic conditions for our FACS-CTL assay. Peptide pulsed-P815 cells were labeled with a high concentration of CFSE and used for sensitive target cells, while unpulsed P815 cells were labeled with a low concentration of CFSE and were used as control target cells. After labeling with CFSE reagent, these target cells were separately incubated with various numbers of LINE-IIIB for 4 h at 37°C. After incubation, sensitive targets were mixed with control targets in one tube with FACS buffer at the same E/T ratio. Mixed target cells were washed once with FACS buffer and resuspended with PFA-PBS to stop any additional reaction. In acquisition of the cells by the FACScan analyzer, five thousand cells which were contained in the gate consisted of P815 cells (region 1; R1) and two CFSE peaks on a histogram plot (region 2; R2) were acquired (see Fig. 1).

First, to see whether antigenic specificities could

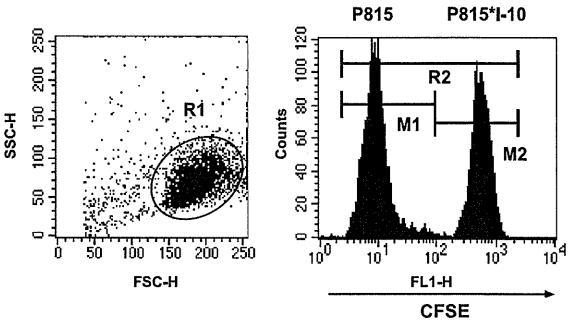


Fig. 1 Acquisition on flow cytometry. Left: Target cells containing 2.5 μM of CFSE-labeled, peptide P18-I10-pulsed P815 cells and 50 nM of CFSE-labeled, unpulsed P815 cells appeared in a gated region (R1) on dot blot analysis by FACScan analyzer. Right: Histogram profile of the cells in the gated region (R1) is shown. For acquisition, 5×10^3 cells which were contained in both regions, R1 and R2, were collected.

be detected by FACS-CTL assay, cytotoxic activities of LINE-IIIB against an epitope peptide, P18-I10-pulsed P815 cells, or a control peptide, P18RF-pulsed P815 cells, were determined, respectively. As shown in Fig. 2, after 4 h incubation the number of P18-I10-pulsed P815 cells was significantly reduced in proportion to the number of added effector cells. In contrast, when LINE-IIIB was incubated with P18RF-pulsed or unpulsed P815 cells, no reduction in fluorescence was observed. These results clearly indicated that antigen-specific cytotoxic activities could be detected by this FACS-CTL assay.

In previous studies, we demonstrated that the amino acid at position 325 plays a critical role in the specific recognition of LINE-IIIB to epitope peptide P18IIIB (14, 15). Although this CTL line could not recognize peptide P18MN, the same immunodominant site from another strain of HIV-1, it could cross-recognize the substituted peptide P18MN(Y-I) at which position 325(Y) was replaced by isoleucine (I) (15). As shown in Fig. 3, fluorescence of target cells sensitized with P18IIIB and P18MN(Y-I) was significantly reduced after incubation with LINE-IIIB. In contrast, the number of target cells pulsed with peptide P18MN or P18MN(Y-E), which is not recognized by LINE-IIIB, was unchanged. These results indicate that changes in cytotoxic activity due to single amino acid substitution within an epitope peptide could be detected by the FACS-CTL assay.

Usage of adherent cells as target cells on FACS-CTL assay

In some experiments, we used adherent cells such as BALB/c.3T3-derived fibroblast cells as target

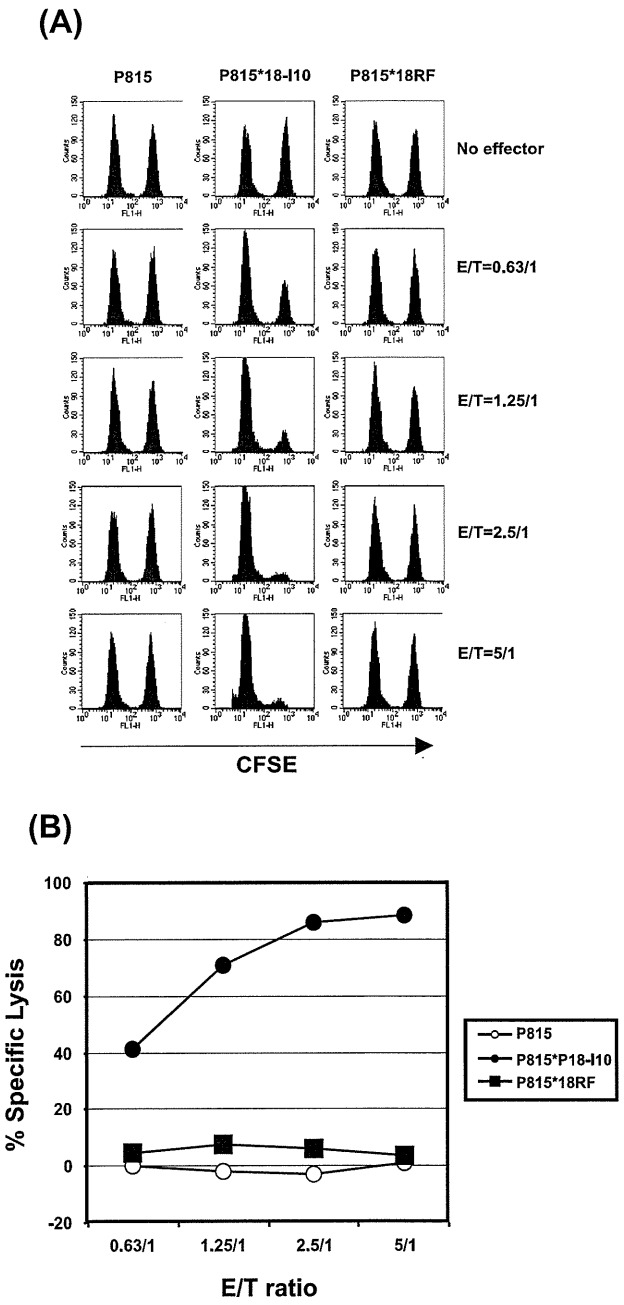
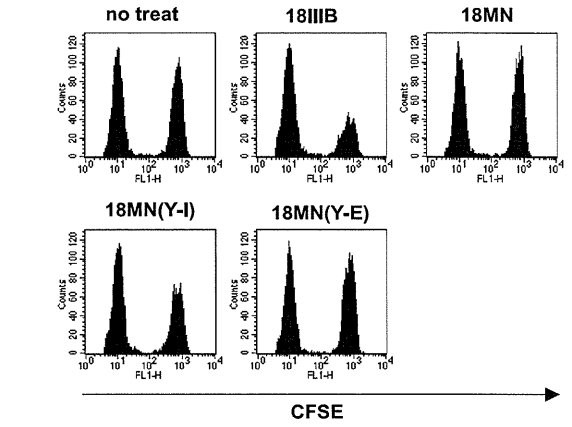


Fig. 2 Detection of antigen-specific cytotoxicity by FACS-CTL assay. (A) For sensitive targets, epitope peptide P18-I10-pulsed, control peptide P18RF-pulsed or unpulsed P815 cells were labeled with 2.5 μM of CFSE, respectively. For control targets for acquisition, unpulsed P815 cells were labeled with 50 nM of CFSE. Each labeled target cells (5×10^4) were co-incubated with effector CTLs (LINE-IIIB) at various effector/target (E/T) ratios in 5 mL polystyrene-round bottom tubes in a 500 μL final volume for 4 h at 37°C. After incubation, sensitive targets were mixed with control targets in one tube at the same E/T ratio and mixed cells were washed once with FACS buffer and resuspended with PFA-PBS. Histogram profiles of each sample are shown. (B) Percent specific lysis calculated by a formula is shown (see Materials and Methods).

(A)

Peptides	Sequences
18IIIB	RIQRGPGRAFVTIGK
18MN	RIHIGPGRAFYTTKN
18MN (Y-I)	RIHIGPGRAFITTKN
18MN (Y-E)	RIHIGPGRAFETTKN



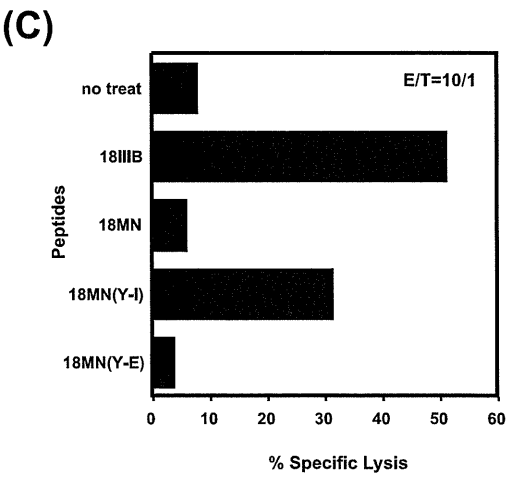


Fig. 3 Detection of fine antigenic specificity by FACS-CTL assay. For sensitive targets, epitope peptide P18IIIB-pulsed, control peptide P18MN-pulsed or P18MN substituted peptides-pulsed P815 cells were labeled with 2.5 μM of CFSE, respectively. For control targets for acquisition, unpulsed P815 cells were labeled with 50 nM of CFSE. Assays were done the same as in Fig. 2. Histogram profile of each sample and percent specific lysis calculated by a formula (E/T ratio = 10/1) are shown.

cells in the ^{51}Cr-release assay. However, when CTLs were incubated with adherent target cells for 4 h in the FACS-CTL assay, most target cells adhered to inside of the assay tubes, and so we could not collect the target cells after incubation. Therefore, we tried to collect target cells by treatment with high concentrations of EDTA and trypsin solutions. After 4 h incubation, a 100 µL aliquot of 2% EDTA solution and a 100 µL aliquot of 5% trypsin solution were added to each assay tube and further incubated for 10 min at 37°C. After incubation, cells were stirred for 5 s and then sensitive and control target cells were mixed with FACS buffer in one tube for FACS analysis. As shown in Fig. 4, most adherent target cells such as 15-12 cells and Neo were harvested with this treatment and the cytotoxic activities of LINE-IIIB against these adherent target cells could be detected by the FACS-CTL assay.

The accuracy of FACS-CTL assay and comparison with standard ^{51}Cr-release assay

To confirm the accuracy of our FACS-CTL assay, we next assessed the same samples in quadruplicate

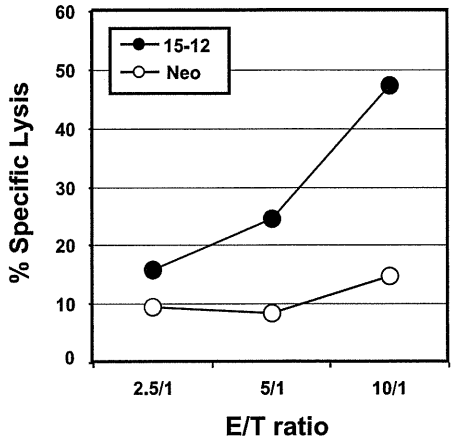


Fig. 4 Cytotoxic activities of LINE-IIIB were determined by FACS-CTL assay using HIV-1 gp160 gene-transfected BALB/c.3T3 cells (15-12) and control BALB/c.3T3 cells (Neo) as target cells. For sensitive targets, 15-12 or Neo was labeled with 2.5 µM of CFSE, respectively, and for control targets, Neo was labeled with 50 nM of CFSE. Each sample of labeled target cells was incubated with LINE-IIIB at various E/T ratios for 4 h at 37°C. After incubation, 100 µL of 2% EDTA solution and 100 µL of 5% trypsin solution were added to each sample and further incubated for 10 min at 37°C. After incubation, cells were stirred by a voltex mixer three times. Sensitive and control target cells were mixed in one tube at each E/T ratio and mixed cells were washed once with FACS buffer and resuspended with PFA-PBS for FACS analysis. Open symbols, both sensitive and control targets were Neo; closed symbols, the sensitive target was 15-12 and control target was Neo.

at each E/T ratio. The results clearly indicated that the FACS-CTL assay was very accurate because little standard error was detected at all E/T ratios (Fig. 5A).

The ^{51}Cr-release assay has been widely used to examine the cytotoxic activities of CTLs against various virus-infected cells or tumor cells. Therefore, next we examined the cytotoxicities of LINE-IIIB using the FACS-CTL assay and compared the results with those obtained by a ^{51}Cr-release assay. A direct comparison of the two assays was performed concurrently using identical effectors, target cells and E/T ratios. In these experiments, two LINE-IIIB cells with different activities were used

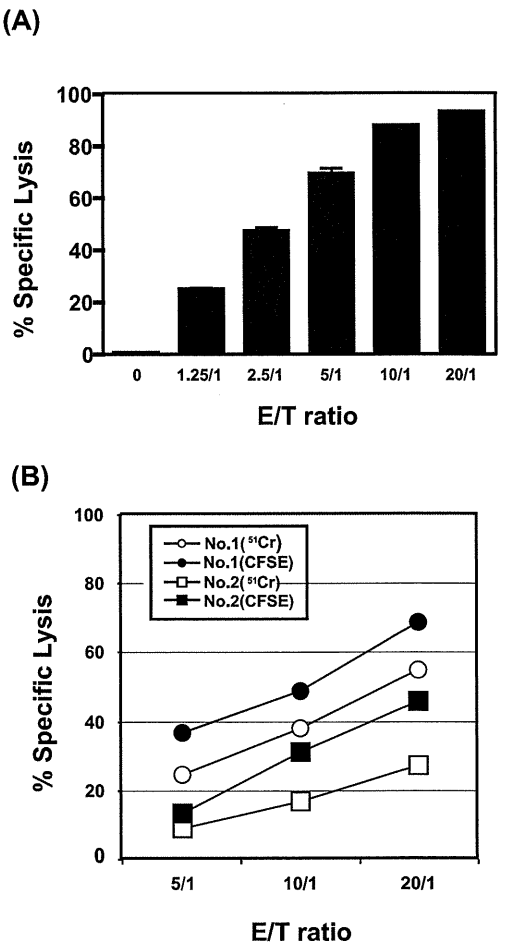


Fig. 5 The accuracy of FACS-CTL assay and comparison with standard ^{51}Cr-release assay. (A) The same samples were determined in quadruplicate at each E/T ratios on FACS-CTL assay. LINE-IIIB cells and P18-I10-pulsed and unpulsed P815 cells were used as effector and target cells, respectively. (B) Cytotoxic activities of two LINE-IIIB cells with different activity were determined by ^{51}Cr-release assay (open symbols) and FACS-CTL assay (closed symbols) using P18-I10-pulsed and unpulsed P815 cells as target cells.