Brief Definitive Report

Activity and Phenotype of Natural Killer Cells in Peptide Transporter (TAP)-deficient Patients (Type I Bare Lymphocyte Syndrome)

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Summary

In this paper we describe the function and phenotype of natural killer (NK) lymphocytes from HLA class I–deficient patients. These cells are, as has been previously reported, unable to lyse HLA class I^- K562 cells, but are able to perform antibody-dependent cellular cytotoxicity (ADCC), although with lower efficiency as compared to NK cells from normal individuals. Transporter associated to antigen processing $(TAP)^-$ NK cells proliferate when cultured in the presence of lymphoblastoid B cells (B-LCs) and interleukin 2 and develop a spectrum of cytotoxicity similar to that of activated normal NK cells. Importantly, activation of the TAP^- NK cells induces strong cytotoxicity to autologous B-LCs. Analysis of the phenotype of circulating TAP^- NK lymphocytes showed them to display a normal diverse repertoire of HLA class I–specific NK receptors. These receptors were expressed at normal levels, apart from the CD94–NKG2A complex, which appeared to be overexpressed. This latter finding could reflect an adaptation to the low expression of HLA class I molecules. Finally, functional analyses indicated that the inhibitory receptors in TAP^- individuals can transduce inhibitory signals. Our results suggest that in vivo, the NK cells of TAP^- patients could participate in immune defense, at least through ADCC, but upon activation, may be involved in autoimmune processes.

Type I bare lymphocyte syndrome is a rare disease characterized by a strong reduction in the cell surface expression of HLA class I molecules. The patients are not reported to suffer from severe viral infections, which suggests that cell-mediated cytotoxic immune responses are efficient to some extent. However, chronic lung inflammation develops in late childhood. A few years ago we described two siblings, EMO and EFA, who are homozygous for a stop mutation in the gene encoding the TAP2 subunit of the peptide transporter associated to antigen processing (TAP; 1). As a result of this deficiency, most HLA class I molecules remain peptide-free and cannot reach the cell surface. Thus, the TAP-deficient cells from these patients express $<3\%$ of HLA class I molecules as compared to normal cells. Nevertheless, $CD8^+$ α/β T cells are present among their PBMCs. Recent observations (2) suggest that some of these cells may recognize TAP-independent HLA class I–restricted viral antigens and participate in the development of the immune response, thus explaining the absence of a greater susceptibility to viral infection in these patients.

Immune responses are also controlled by NK cells. These lymphocytes are cytotoxic to certain tumor cells, HLA class I^- cells, and virus-infected cells and mediate antibody-dependent cellular cytotoxicity (ADCC; 3). The importance of these cells in human immune responses is indicated by the association of severe infections with herpesvirus and EBV with an absence of NK cells or with a reduction of their activity (4, 5).

Several molecules expressed at the surface of NK cells are involved in the recognition of target cells and control their cytotoxic activity (6). Some are activating receptors; among these is the type III low affinity receptor for IgG, or CD16, which is involved in ADCC. Other receptors, called killer inhibitory receptors (KIRs), block the cytotoxic process when they interact with the HLA class I molecules expressed on normal cells. In humans, these receptors may be

J. Exp. Med. © The Rockefeller University Press • 0022-1007/98/01/117/06 $2.00
Volume 187, Number 1, January 5, 1998 117–122
http://www.jem.org

classified in two main families. The receptors specific for subsets of the alleles of HLA-C (p58), HLA-B (p70), and HLA-A (p140) belong to the Ig superfamily (Ig-SF; 6). In contrast, the CD94–NKG2A (or -B) receptor complex is composed of proteins homologous to C-type lectins and displays a broader specificity for different HLA class I alleles (7). More recently, receptors have been described that are homologous to KIRs, but activate the cytotoxicity of NK cells (6, 7).

In a previous paper, we reported that NK cells from TAP-deficient patients were unable to lyse HLA class I^- K562 tumor cells, suggesting that these cells were not functional (1). However, since the patients do not seem to suffer from severe herpesvirus or EBV infections, this may indicate that their NK cells possess immunological functions. The cytotoxic activity of NK cells from TAP-deficient patients was therefore reexamined in the present study. In a second step, we investigated whether a defective expression of class I molecules could affect the repertoire of HLA class I–specific receptors on NK cells. Finally, we explored the functionality of the inhibitory receptors of these cells.

Materials and Methods

Cells. The human cell lines K562, Daudi, and Raji and the MB40.5 hybridoma were purchased from the American Type Culture Collection (ATCC; Rockville, MD). The EBV-transformed B cell line (B-LC) H0 301 (IHW 9055) was obtained from the XIIth International Histocompatibility Workshop (Saint Malo, France, June 1996), whereas the B-LCs ST-EFA and ST-EMO (HLA-A*0301, -B*1516, -Cw*1402, $TAP2^-$ homozygous), derived from the patients, and ST-EHA (HLA-A*0301/-A30, -B*1516/-B18, -Cw*1402/-Cw5, $TAP2^+/TAP2^-$), derived from their father, have been previously described (1). PBMCs were isolated from citrated venous blood by Ficoll-Hypaque density gradient centrifugation. In some experiments, PBMCs were depleted of monocytes and T cells using immunomagnetic beads (Dynabeads M-450 CD14 and M-450 CD3; Dynal, Oslo, Norway). Activated NK cells were generated by coculture of PBMCs with irradiated B-LCs as previously described (8). After 11 d of culture, T lymphocytes were removed using anti-CD3 immunomagnetic beads and the NK cells were tested for cytotoxicity on day 12.

Antibodies. The murine mAbs GL183 (anti-p58.2), EB6 (anti-p58.1), Z27 (anti-p70/NKB1), Q66 (anti-p70/p140), XA 185 (anti-CD94), Z270 (anti-CD94–NKG2A), FES172 (anti-p50.3), and A6-136 (anti–HLA class I) have been previously described (9). The anti-CD16 mAb was obtained from Serotec (Kidlington, UK). The anti-CD56 mAb was obtained from Becton Dickinson (Mountain View, CA). The FITC-conjugated anti-CD3 and the PE-cyanine 5 (PE-Cy5)-conjugated anti-CD19 mAbs were purchased from Boehringer Ingelheim Bioproducts (Heidelberg, Germany). Unrelated murine IgG1 (Immunotech, Marseille, France), IgG2a-FITC, and IgG1-PE-Cy5 (Boehringer Ingelheim Bioproducts) were used as negative isotype controls. The mAb MB40.5 (anti–HLA class I) was purified from ascites and $F(ab')_2$ fragments of MB40.5 were generated by pepsin digestion and purified by gel filtration on Superdex 200 (Pharmacia, Uppsala, Sweden). Polyclonal anti-Raji antibodies were obtained from rabbits immunized by subcutaneous injection of Raji cells. PE-conjugated goat anti–mouse immunoglobulins (Dako, Glostrup, Denmark) were used as secondary antibodies.

Flow Cytometry. Immunofluorescence analyses were performed using a FACScan® flow cytometer and Cellquest software (Becton Dickinson).

Cytotoxicity Assays. Standard 4-h chromium release assays were performed in V-bottomed 96-well microtiter plates (Costar, Cambridge, MA) as previously described (1). In ADCC assays, anti-Raji antiserum (1 μl/5 × 10^5 cells) was added to Raji cells 30 min before the end of the labeling procedure, whereas in blocking studies, the target cells were incubated with the anti–HLA class I mAb A6-136 (50 μl culture supernatant/well) or MB40.5 (50 μg/ml) just before addition of effector cells. Spontaneous release was determined by incubating the target cells in culture medium alone, and maximum release by adding 100 μl of 1% Triton X-100 (Sigma Chemical Co., St. Louis, MO). The percent specific lysis was calculated according to the relation 100 × (experimental ^{51}Cr release − spontaneous ^{51}Cr release)/(maximum ^{51}Cr release − experimental ^{51}Cr release).

Results

Cytotoxicity of TAP^- NK Cells. Numbers of NK cells in the TAP-deficient patients and their father were low but within the normal range (5–10% of PBMCs). First, we investigated the natural cytotoxicity of these cells and whether they could perform ADCC. Freshly isolated PBMCs from one TAP-deficient patient and from a normal donor were depleted of monocytes and T lymphocytes with anti-CD14 and anti-CD3 magnetic beads. The cytotoxic activity of these cells was tested against K562 cells, ST-EMO cells ($TAP2^-/TAP2^-$, derived from the patient), and Raji cells in the presence or absence of a rabbit anti-Raji antiserum (Fig. 1 *A*). Unlike NK cells from the normal donor, NK cells from the TAP-deficient patient were unable to kill K562 cells, thus confirming our previous observations (1). NK cells from the patient were likewise not cytotoxic to

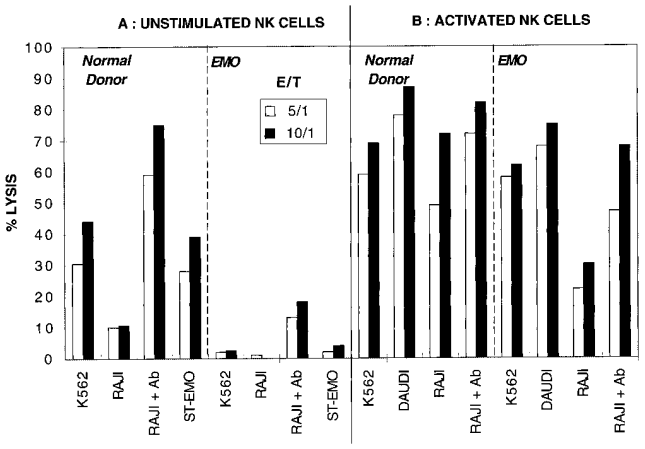

Figure 1. Cytotoxic activity of TAP^- NK cells. (*A*) Unstimulated NK cells. Freshly isolated PBMCs from a normal donor and from a TAP-deficient patient (EMO) were depleted of monocytes and T lymphocytes and tested for cytotoxicity to K562, ST-EMO, and Raji cells preincubated or not with polyclonal rabbit anti-Raji antiserum. (*B*) Activated NK cells. PBMCs from a normal donor and from a TAP-deficient patient (EMO) were cocultured with irradiated ST-EMO B-LCs in the presence of IL-2 for 11 d and depleted of T cells. NK cells were tested on day 12 for cytotoxicity to K562, Daudi, and Raji cells and for ADCC.

autologous ST-EMO B-LCs, although these cells were significantly lysed by normal NK cells. Finally, although normal NK cells efficiently lysed Raji cells preincubated with anti-Raji antibodies, TAP^- NK cells were only weakly cytolytic.

Coculture of PBMCs with irradiated B-LCs in the presence of IL-2 induces the proliferation of NK and T lymphocytes. These proliferating NK cells have higher cytotoxic activity than unstimulated NK cells and kill a wider spectrum of tumor cell lines, including Raji cells and β-2 microglobulin (β2m)-deficient Daudi cells. Proliferation and cytotoxicity of normal and TAP^- NK cells stimulated with IL-2 and ST-EMO^- or unrelated TAP^+ H0 301 B-LCs were thus compared. Both the cell lines induced the proliferation of TAP^- NK and T lymphocytes. However, the growth kinetics of TAP^- cells were slower than those of normal NK cells, with mean proliferation indexes from day 0 to day 10 of 370 and 65 for normal and TAP^- NK cells, respectively. The expansion of TAP^- NK cells was nevertheless adequate to allow use of this culture method to rapidly generate sufficient numbers of activated NK cells.

The cytotoxicity of these cells was tested using K562, Daudi, and Raji cells preincubated or not with rabbit anti-Raji antiserum. PBMCs were stimulated with irradiated ST-EMO or H0 301 cells in the presence of IL-2 for 11 d, depleted of T lymphocytes with anti-CD3 magnetic beads, and tested for cytotoxicity. Similar results were obtained using ST-EMO or H0 301-stimulated NK cells. All the target cells tested were killed by proliferating normal or TAP^- NK cells (Fig. 1 *B*, and data not shown for cells stimulated with H0 301), suggesting that activated TAP^- NK cells develop a spectrum of cytotoxicity comparable to that of activated normal NK cells. Raji cells incubated with anti-Raji antibodies were more susceptible to lysis by activated than by resting NK cells. However, in all experiments, TAP^- NK cells were less cytotoxic than normal NK cells activated under the same conditions. These results, observed using activated NK cells from patient EMO, could be reproduced using equivalent cells from the second patient (EFA, data not shown).

Activated NK cells from the TAP^- patients, their father, and five healthy unrelated individuals were then analyzed for cytotoxicity to autologous B-LCs. Proliferating NK cells from both patients strongly lysed autologous B-LCs. Conversely, proliferating NK cells from the father and the five other unrelated donors displayed little cytotoxicity to their respective B-LCs (Fig. 2). Other control experiments showed that activated NK cells from the patients did not lyse $TAP2^+/TAP2^-$ hemizygous B-LCs while those from the father and normal donors efficiently killed $TAP2^-$ homozygous cells (data not shown).

Phenotype of TAP-deficient NK Cells. Since TAP^- NK cells were poorly cytotoxic in ADCC assays, we analyzed the expression of CD16 on these cells. PBMCs from the patients, their father, and 10 unrelated normal donors were labeled with anti-CD3, anti-CD16, and anti-CD19 mAbs for triple-color flow cytometry, $CD19^+$ cells being excluded from the analysis. No significant differences in

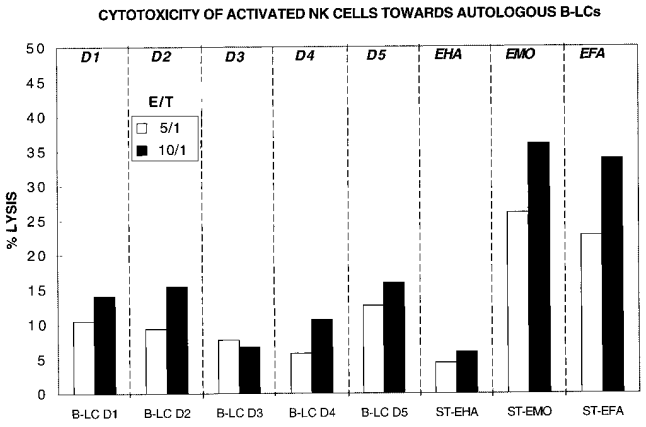

Figure 2. Self-reactivity of activated NK cells. Activated NK cells from the TAP-deficient patients (EMO, EFA), their father (EHA), and five normal donors (*D1–D5*) were tested for cytotoxicity to their respective autologous B-LCs.

numbers of $CD16^+/CD3^-$ cells could be observed between normal donors and TAP-deficient patients (data not shown), and the intensity of fluorescent staining lay within the range for PBMCs from normal blood (data not shown). Similarly, no differences were found between normal donors and patients after staining of PBMCs with an mAb against the CD56 adhesion molecule.

To determine if the low expression of HLA class I molecules could affect the repertoire of HLA class I–specific NK receptors, the phenotype of TAP^- NK cells was further analyzed by flow cytometry using different anti-NK receptor mAbs. PBMCs from the TAP-deficient patients, their father, and the same 10 normal donors (five Bw4-homozygous donors and five Bw6-homozygous donors) were stained with anti-CD3, anti-CD19, and anti-NK receptor mAbs for triple-color flow cytometry, $CD19^+$ cells being excluded from the analysis. The percentages of NK cells ($CD19^-CD3^-$) and T lymphocytes ($CD19^-CD3^+$) expressing these different receptors were determined (Fig. 3 *A*, data not shown for T lymphocytes), as were the mean fluorescence intensities (MFI) of the receptors (Fig. 3 *B*). Experiments showed that the numbers of NK and T lymphocytes expressing these receptors varied among individuals, in agreement with previous reports (10–12). MFI of the different receptors also varied among donors, whereas no significant differences were observed between PBMCs from Bw4 and Bw6 homozygous normal individuals (data not shown). The two TAP-deficient patients expressed the KIRs recognized by the mAbs GL183, EB6, Z27, and Q66 on their NK and T cells. Thus, there was no apparent bias in the repertoire of these different HLA class I–specific KIRs, and no major differences in the MFI of the different receptors could be identified between TAP^- and normal cells. Hence, our results suggest that the TAP deficiency does not affect the repertoire of Ig-SF KIRs. Some of these receptors were expressed at slightly different frequencies on the NK cells of the two HLA-identical patients. Analogous observations have been made by Gumperz et al. (10), who

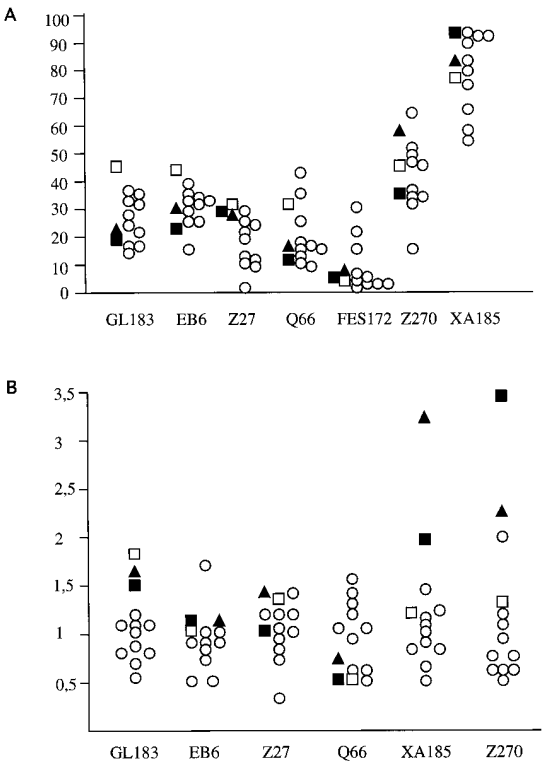

Figure 3. (*A*) Repertoire of HLA class I–specific receptors on NK cells. PBMCs from the TAP-deficient patients (*filled squares and triangles*), their father (*open squares*), and 10 normal donors (*open circles*) were first incubated with anti-NK receptor mAbs, stained with a PE-conjugated goat anti–mouse secondary antibody, and then labeled with FITC-conjugated anti-CD3 and PE-Cy5–conjugated anti-CD19 mAbs. $CD19^-$ lymphocytes were analyzed by flow cytometry and results were expressed as the percentage of positive cells among NK cells. (*B*) Comparison of the expression of NK receptors. The mean fluorescence intensities (MFI) of NK cell subsets labeled with anti-NK receptor mAbs were determined by flow cytometry for each donor. Mean values of MFI were calculated for each marker and the ratio of the MFI to its corresponding mean value was determined for each marker and individual. In the case of EB6, Z27, and XA185, results were calculated for the bright subsets.

reported that the frequency of NK cells expressing the p70 receptor appeared to be controlled by genetic loci not linked to HLA.

Staining of PBMCs from the patients with the mAb XA185 revealed $CD94^{bright}$ and $CD94^{dim}$ subsets with the $CD94^{dim}$ subset being very small (data not shown). Interestingly, the MFI of $CD94^{bright}$ NK cells from TAP-deficient patients was higher than the MFI of $CD94^{bright}$ NK cells from normal individuals (Fig. 3 *B*). This increase in the expression of CD94 on NK cells was nevertheless not observed in the $CD94^{dim}$ population (data not shown). The percentage of TAP^- NK cells expressing the inhibitory CD94–NKG2A complex, recognized by the mAb Z270, was comparable to that of normal cells (Fig. 3 *A*). The MFI of $Z270^+$ cells was also significantly higher for TAP^- NK cells than for normal NK cells (Fig. 3 *B*) and, to a lesser extent, also for TAP^- as compared to normal T lymphocytes (data not shown). However, although Z270 revealed a greater increase in MFI on the NK cells of patient EFA, XA185 showed a stronger increase in MFI on the NK cells of patient EMO. This pattern may reflect differences in the $Z270^-$ $CD94^{bright}$ subsets (13) of the two patients, the percentages of $XA185^+$ and $Z270^+$ NK cells in EFA and EMO being 91 and 82% (XA185) and 34 and 58% (Z270), respectively. Since numbers of CD94–NKG2A molecules appeared to be increased on TAP^- NK cells, an additional panel of 50 other unrelated healthy donors was investigated. FACS® analysis of NK cells stained with XA185 or Z270 confirmed that NK cells from the two TAP-deficient patients expressed higher levels of CD94–NKG2A complexes (data not shown).

Functionality of Inhibitory NK Receptors. Since the reactivity of NK cells towards autologous cells is controlled by inhibitory NK receptors, the patterns of reactivity of TAP^+ and TAP^- NK cells can be explained by the inhibitory activity of their NK receptors. Cytotoxicity assays were therefore performed using anti–HLA class I mAbs A6-136 (IgM) or $F(ab')_2$ fragments of MB40.5 (IgG1) which block the recognition of HLA class I molecules by inhibitory NK receptors without interacting with CD16 on NK cells (data not shown). The cytotoxicity of normal activated NK cells to autologous B-LCs was increased in the presence of anti-HLA class I mAbs, thus confirming the functionality of HLA class I–specific inhibitory receptors on TAP^+ NK cells. Activated NK cells from the TAP-deficient patient did not lyse the hemizygous ST-EHA B-LCs expressing normal levels of HLA class I molecules. However, in the presence of anti-HLA class I mAbs, activated TAP^- NK cells were highly cytotoxic to these cells, presumably due to the absence of engagement of their inhibitory receptors by the masked HLA class I molecules on the target cells. Hence, these experiments demonstrated the functionality of the NK inhibitory receptors on TAP^- NK cells.

Discussion

Freshly isolated unstimulated NK cells from TAP-deficient patients were as previously reported unable to lyse HLA class I^- K562 cells and furthermore did not kill autologous B-LCs, which were targets of normal NK cells. This would suggest that TAP^- NK cells are not completely mature, and consequently avoid autoreactivity. In contrast, these cells could lyse antibody-coated cells, although they were less cytotoxic than NK cells from normal individuals. Such findings are reminiscent of observations in TAP or β2m knockout transgenic mice (14–16), despite a present lack of reports concerning the mediation of ADCC activity by NK cells from these class I^- animals.

When PBMCs were cocultured with irradiated autologous or allogeneic B-LCs in the presence of IL-2, TAP^- NK cells proliferated, as is observed with normal NK cells. Moreover, these cells became cytotoxic to a series of targets known to be killed by normal activated NK cells, including Raji cells and HLA class I^- K562 and Daudi cells. The cytotoxic activity of TAP^- NK cells was nevertheless always lower than that of normal NK cells activated under the same experimental conditions, suggesting a partial defect in

signal transduction or a lower expression of the molecules essential for cytolytic processes. These in vitro results further suggest that in TAP$^-$ patients, NK cells could be activated in vivo by cytokines such as IL-2 and IFN-α released in the course of immune responses to viral infections. Indeed, it has been shown in β2m-deficient mice that NK cells can be activated in vivo as a result of viral infection (17–19) in an IL-2–dependent process (17). Preliminary experiments in our laboratory likewise showed that (*a*) TAP$^-$ NK cells became cytotoxic to K562 cells after a short period of in vitro activation in the presence of exogenous IL-2 alone and (*b*) develop higher cytolytic activity in ADCC assays.

Interestingly, unlike NK cells from control donors, TAP$^-$ NK cells cultured in the presence of B-LCs and IL-2 became strongly cytotoxic towards autologous B-LCs. The fact that activated TAP$^-$ NK cells are not cytotoxic to hemizygous B-LCs, except in the presence of anti–HLA class I mAbs, raises the question as to whether the mechanism of cytotoxicity to EBV B-LCs is different in TAP-deficient patients and normal individuals. It is most likely that the autoreactivity of activated TAP$^-$ NK cells simply reflects the ability of these cells to kill HLA class I$^-$ cells, as do normal activated NK cells. Alternatively, in TAP$^-$ individuals the calibration of signal transduction by KIRs might be different in TAP$^-$ as compared to normal individuals. This last hypothesis would, however, seem unlikely since preliminary experiments showed that the cytotoxicity of activated TAP$^-$ NK cells to autologous B-LCs was not enhanced in the presence of anti–HLA class I mAbs. Another question is whether self-tolerance of TAP$^-$ NK cells can be broken by activation. In this regard, it should be noted that in all cytotoxicity experiments using conventional targets (Daudi, Raji, and ADCC assays), activated TAP$^-$ NK cells were less cytotoxic than activated normal NK cells, whereas the cytotoxicity of TAP$^-$ NK cells to autologous B-LCs was significantly greater than that of normal NK cells towards autologous B-LCs.

The reactivity of NK lymphocytes from normal individuals to autologous cells is negatively controlled by inhibitory NK receptors (6). Different NK clones may express different receptors, whereas the percentages of NK cells expressing these KIRs vary among individuals (6, 10, 11). Our patients are HLA-A*0301, -B*1516, and -C*1402 homozygous and their HLA-B and -C subtypes belong to the Bw4 and HLA-C "group 2" subsets, respectively. The TAP deficiency could therefore have an incidence on the frequency of NK receptors recognized by the mAbs GL183, Z27, and Q66. Our results nevertheless showed that the low expression of HLA class I molecules (<3%, as compared to normal individuals) does not significantly alter the repertoire of these receptors on NK cells. Moreover, the percentages of NK cells expressing a given receptor were somewhat different in the two HLA-identical patients, in agreement with the hypothesis that the NK receptor repertoire may be shaped by genetic loci other than HLA (10). Finally, Ig-SF KIRs were expressed at similar levels in TAP-deficient patients and normal donors. Thus, the TAP deficiency has no apparent incidence on the repertoire of these KIRs, at least those for which mAbs are available. In contrast, the inhibitory receptor complex CD94–NKG2A was expressed at a higher density on TAP$^-$ NK cells as compared to normal NK cells. Since this receptor should recognize HLA-A3 and HLA-C self-molecules (13), which are weakly expressed by TAP-deficient patients, its overexpression may indicate a compensatory mechanism.

The finding that activated TAP$^-$ NK cells lyse hemizygous ST-EHA (TAP2$^+$/TAP2$^-$) cells only when these cells have been incubated with mAbs blocking the interaction between KIRs and HLA class I molecules demonstrates the functionality of the inhibitory receptors on activated TAP$^-$ NK lymphocytes. However, the KIRs on activated TAP$^-$ NK cells do not appear to be able to transduce inhibitory signals triggered by the low levels of HLA class I molecules expressed on autologous cells. This would also imply that the stronger expression of CD94 may not be sufficient to ensure full protection against autoreactivity when these NK cells are activated in vitro.

Our results show that NK cells in TAP-deficient patients and normal individuals are phenotypically very similar, apart from the overexpression of CD94–NKG2A in TAP$^-$ individuals. Functionally, the greatest differences were observed in resting NK cells. Thus, unlike normal NK cells, TAP$^-$ lymphocytes were unable to lyse HLA class I$^-$ cells. When normal and TAP$^-$ NK cells were activated in vitro, they became cytotoxic towards the same panel of NK-susceptible targets, although TAP$^-$ NK cells displayed lower cytolytic activity. Importantly, stimulated TAP$^-$ NK cells were cytotoxic to autologous B-LCs. If these in vitro findings can be considered as representative of events occuring in vivo, our data suggest that upon activation TAP$^-$ NK cells acquire functional capacities similar to those of normal NK cells. On this basis, due to the low levels of cell surface HLA class I expression, it is possible that in TAP$^-$ individuals NK cell activation may be at the origin of autoimmune responses.

We thank H. Bausinger for expert technical assistance.

J. Zimmer was the recipient of a grant (BFR 96/003) from the Ministère de l'Education Nationale et de la Formation Professionnelle, Luxembourg. This work was supported by the Etablissement de Transfusion Sanguine de Strasbourg (Strasbourg, France), and by Institut National de la Santé et de la Recherche Médicale (CJF 94-03).

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Received for publication 16 May 1997 and in revised form 1 October 1997.

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