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*J Immunol* 1999; 162:651-658; ;
http://www.jimmunol.org/content/162/2/651

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CTLA-4 (CD152) Inhibits the Specific Lysis Mediated by Human Cytolytic T Lymphocytes in a Clonally Distributed Fashion

Daniele Saverino,* Claudia Tenca,* Daniela Zarcone,* Andrea Merlo,† Anna M. Megiovanni,‡ Maria T. Valle,‡ Fabrizio Manca,‡ Carlo E. Grossi,*† and Ermanno Ciccone 2†

Since the functional outcome of effector T lymphocytes depends on a balance between activatory and inhibitory receptors, we studied the ability of CTLA-4 (CD152) to inhibit the cytolytic function of CTL. In 22 TCRαβ⁺ CD3⁺8⁺ CTL clones, activation induced by anti-CD3, anti-CD28, or anti-CD2 mAb was inhibited by anti-CD152 mAb in a redirected killing assay. In eight clones inhibition was >40%, in 10 it ranged between 20–40%, and in four it was <20%. This suggests the existence of a clonal heterogeneity as well as for the ability of CTLA-4 to inhibit CD3/TCR-, CD28-, or CD2-mediated CTL activation. To support further this contention, we used an experimental model based upon Ag-specific CTL. Eight Ag-specific T cell clones that lyse autologous EBV-infected B lymphocytes, but are unable to lyse allogeneic EBV-infected B cell lines, were used in a cytolytic assay distributed.

The largest complement of CD152 molecules resides in the post-Golgi/endosomal compartment of activated T lymphocytes (12). When T cells interact with APC presenting a specific Ag or with a cell that has to be killed, CD152 is translocated to the plasma membrane at the time of Ag recognition and coactivatory signals, such as those mediated by CD80/86 molecules, are mandatory for T cell activation (5). In the absence of coactivation, paradoxically, Ag recognition leads to anergy (4). This negative functional outcome could result from a prevalence of inhibitory signals.

At variance from the wide range of activatory molecules, only a few inhibitory molecules are known to be expressed by T lymphocytes, namely the killer cell inhibitory receptors for HLA class I and CD94-NKG2A (both types of receptors are typically found on CD3⁺ NK cells) (6), and CD152 (3, 7), which was originally identified as a molecule present on CTL (8, 9). Killer cell inhibitory receptors and CD94-NKG2A are detected on only a small proportion of normal T cells, whereas CD152 is found in all activated T cells (10, 11).

The mAb used for immunophenotypic analyses were anti-CD2 (Leu 5b), anti-CD3 (Leu 4), anti-CD4 (Leu 3a), anti-CD8 (Leu 2a), anti-TCR-α/β (anti-WT31; Becton Dickinson, San Jose, CA), anti-CD45 (ALB12),

Materials and Methods

Antibodies

Received for publication July 2, 1998. Accepted for publication September 24, 1998.

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Antibodies

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anti-CD45RA (ALB11), anti-CD45RO (UCHL1), anti-CD11a (25.3), anti-CD11b (Becar1), anti-CD18 (TE4; Immunotest, Luminy-Marseille, France), anti-CD80 (BBI/B7-1), anti-CD86 (B70/B7-2), anti-CTLA-4 (anti-CD152; PharMingen, Hamburg, Germany), and anti-HLA class I mAb W6/32 (American Type Culture Collection, Manassas, VA). mAb CD28.2 (specific for CD28) and mAb CD2,1 and CD2,9 (specific for two different CD2 epitopes and able, when used in combination, to activate T cells) were provided by Dr. Daniel Olive (Laboratoire de Immunologie Functionel et Moleculaire, Marseille, France).

**Generation of nonspecific CD8+ T cell clones**

PBMC, separated from heparinized venous blood on Ficoll density gradients, were cultured with PHA (5 μg/ml) in 24-well plates (Costar, Cambridge, MA); human IL-2 (PeproTech, London, U.K.) at a 50 U/ml final concentration was added on days 2 and 4 of culture. After 7 days, cells were collected, run on a Ficoll gradient, depleted of CD4+ T lymphocytes by immunomagnetic negative selection with Dynabeads (Unipath, Milan, Italy), and plated at a limiting dilution of 10/1 cells/well. Cells were re-stimulated every 7–10 days with 1 × 10^5 irradiated allogeneic mononuclear cells/well in medium containing 5 μg/ml PHA and 50 U/ml IL-2. T cell clones were analyzed by immunofluorescence and flow cytometry as described below.

**EBV-transformed B cell lines**

PBMC (2 × 10^6/ml) were incubated for 2 h at 37°C with 2 ml of EBV-containing supernatant from the cell line B95-8 (American Type Culture Collection). Cells were washed twice with PBS and incubated in complete culture medium at 5 × 10^3 cells/ml in the presence of 1 μg/ml cyclosporin in 24-well plates. Cultures were expanded in flasks for 20–30 days.

**Generation of EBV-specific CD8+ T cell clones**

Polyclonal cell lines were generated by stimulating 2 × 10^5 EBV-infected cells with 5 × 10^5 autologous irradiated (6000 rad) B-EBV cells in 24-well plates (24). Responder cells were restimulated in weekly cycles by plating 1 × 10^5 and 3 × 10^5 irradiated B-EBV cells/well in complete medium containing IL-2 at a 50 U/ml final concentration on days 2 and 4 after restimulation. After the third stimulation, the cell lines were enriched for CD8+ T lymphocytes by negative selection with immunomagnetic beads (Unipath). Clones were produced by plating these cells at a limiting dilution of 10/1 cells/well, in the presence of 3 × 10^6 irradiated autologous B-EBV cells.

**Immunophenotyping**

The surface phenotype of T cell clones was determined by indirect immunofluorescence and flow cytometric analysis (FACScalibur, Becton Dickinson, San Jose, CA). The secondary reagent was phycoerythrin- or FITC-labeled goat anti-mouse Ig Ab (Southern Biotechnology Associates, Birmingham, AL). T cells (5 × 10^5) were incubated with specific mAb for 20 min at 4°C. Cells were washed twice with PBS, and the secondary labeled reagent was added. After incubation, cells were washed and fixed with 1% paraformaldehyde. Cell surface markers were stained with the secondary reagent alone.

**B-EBV cells**

B-EBV cells stained with the fusion protein CTLA-4 Ig (a gift from Dr. Antonio Lanzavecchia, Basel Institute for Immunology, Basel, Switzerland) were further incubated with a FITC-labeled polyclonal goat anti-human Fc IgG (Southern Biotechnology Associates) and analyzed by flow cytometry.

**Cytotoxicity assays**

The abilities of various mAb to trigger or to inhibit the cytolytic activity of T cell clones were measured in a conventional 4-h 51Cr release assay. Target cells (the murine mastocytoma cell line P815 or the autologous B-EBV cells) were labeled for 1 h with 51Cr (100 μCi/10^6 cells), washed twice with PBS, resuspended in RPMI with 10% FCS, and plated at 5 × 10^5 cells/well in 96-well U-bottom plates. In the redirected killing assay, effector cells were plated in triplicate at various E:T cell ratios in the presence of one of the following mAb: anti-CD3, anti-CD28, anti-CD2 (CD2.1 and CD2.2), anti-CD4, or anti-CD152. To evaluate a possible inhibitory effect of CD152, effector cells were pretreated for 20 min at 4°C with the anti-CD152 mAb. After preincubation, target cells (P815) were added to each well together with the stimulatory mAb (anti-CD3, anti-CD28, anti-CD2, anti-CD3 and anti-CD28, and anti-CD3 and anti-CD2). An anti-DR mAb (Becton Dickinson) of the same isotype as that of anti-CD152 mAb (i.e., γ2a) was used, in combination with anti-CD1 mAb, as a control to rule out that the inhibitory effect was due to competition of the added mAb with the activatory Ab on the P815 cell line.

mAb anti-CD152 or the fusion protein CTLA-4 Ig was included in the cytolytic test performed with autologous 51Cr-labeled B-EBV to block CD80/CD152 or CD86/CD152 interactions. In this assay, a cross-linking of CD152 was obtained using anti-CD152 mAb in combination with goat antiserum to murine Ig (Southern Biotechnology Associates).

After a 4-h culture period, 100 μl of supernatant was collected from each well and analyzed in a gamma counter for 51Cr release. The percent specific lysis was calculated as: [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100.

**Results**

**Selection of cytolytic T cell clones**

To ascertain the regulatory role of CD152 in the activity of CTL, 22 cytolytic T cell clones were derived from PBL of normal donors. Fourteen CTL clones obtained after PHA stimulation were thus characterized by a random range of specificities. The remaining eight CTL clones were Ag specific and were selected on the basis of their ability to lyse autologous, but not allogeneic, B-EBV. All these clones displayed a homogeneous surface phenotype and expressed CD3, CD8, TCRβ, and CD28, but CD152 was expressed at extremely low levels.

**After cross-linking with appropriate mAb, CD152 inhibits CTL activation induced via CD3 in a redirected killing assay**

We used the murine P815 cell line as the target cell in a redirected cytotoxicity assay for two reasons. 1) the presence of Fc receptors for γ1, γ2a, and γ2b murine Ig isotypes on these cells provides a useful model for a killing assay that makes use of mouse mAb against human lymphocyte receptors. In addition, in this assay, receptors are cross-linked and thus mimic their function. 2) Since P815 cells are of murine origin, ligands expressed by these cells do not react with human receptors and therefore do not interfere in the cytolytic assay (see Fig. 1, left panel).

All the CTL clones lysed P815 target cells poorly at an E:T cell ratio of 2:1. In contrast, addition of anti-CD3 mAb in the assay resulted in a substantial lysis of the target (Table I). The presence of mAb specific for CD152 in combination with the activatory anti-CD3 mAb yielded inhibition of target cell lysis in the majority of the clones. According to the level of inhibition, three groups of CTL clones could be distinguished. In eight clones inhibition was >40%, in 10 it ranged between 20–40%, and in four it was negligible (<20%; Table I). Six representative clones from the three different groups are shown in Fig. 2. In clones 25A and Ge3, anti-CD152 mAb yielded a reduction of the cytolytic activity, obtained by addition of anti-CD3 mAb, from 88 to 39% and from 62 to 29%, respectively. In clones Co3 and 10A the reduction of the specific lysis was calculated as: [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100.
in Table II, the inhibitory property of anti-CD152 mAb is consistently maintained in time, and therefore, all CTL clones belong stably to the respective arbitrary group.

Despite the different levels of inhibition obtained using the various clones, surface expression of CD152 remained low in every case (data not shown).

**Table I. Different degree of inhibition of CD3-induced lysis by cross-linking of CD152 molecules**

<table>
<thead>
<tr>
<th>Clone</th>
<th>P815*</th>
<th>P815 + αCD3</th>
<th>P815 + αCD3 + αCD152b</th>
<th>P815 + αCD152c</th>
<th>P815 + αDRd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ge3</td>
<td>22a</td>
<td>62</td>
<td>29 (82)</td>
<td>22</td>
<td>60</td>
</tr>
<tr>
<td>25A</td>
<td>10</td>
<td>88</td>
<td>39 (63)</td>
<td>21</td>
<td>91</td>
</tr>
<tr>
<td>99</td>
<td>7</td>
<td>95</td>
<td>51 (50)</td>
<td>9</td>
<td>98</td>
</tr>
<tr>
<td>Co4</td>
<td>12</td>
<td>95</td>
<td>56 (47)</td>
<td>20</td>
<td>89</td>
</tr>
<tr>
<td>G2A</td>
<td>3</td>
<td>50</td>
<td>29 (45)</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>Co1</td>
<td>4</td>
<td>100</td>
<td>59 (44)</td>
<td>8</td>
<td>90</td>
</tr>
<tr>
<td>Ge2</td>
<td>2</td>
<td>99</td>
<td>59 (41)</td>
<td>1</td>
<td>91</td>
</tr>
<tr>
<td>RO-CD8-2</td>
<td>11</td>
<td>90</td>
<td>58 (41)</td>
<td>17</td>
<td>ND</td>
</tr>
<tr>
<td>Co3</td>
<td>10</td>
<td>90</td>
<td>58 (40)</td>
<td>22</td>
<td>98</td>
</tr>
<tr>
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<td>29</td>
<td>82</td>
<td>60 (39)</td>
<td>20</td>
<td>79</td>
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<td>28C</td>
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<td>54 (36)</td>
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<td>ND</td>
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<td>36 (35)</td>
<td>4</td>
<td>51</td>
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<tr>
<td>G4A</td>
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<td>70 (30)</td>
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<td>ND</td>
</tr>
<tr>
<td>Co2</td>
<td>18</td>
<td>91</td>
<td>70 (29)</td>
<td>22</td>
<td>88</td>
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<tr>
<td>AK</td>
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<td>G3e</td>
<td>8</td>
<td>94</td>
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<td>58 (18)</td>
<td>9</td>
<td>66</td>
</tr>
<tr>
<td>G1B</td>
<td>0</td>
<td>58</td>
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<td>8</td>
<td>62</td>
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<tr>
<td>GR-CD8</td>
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<td>40 (14)</td>
<td>1</td>
<td>44</td>
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<tr>
<td>31A</td>
<td>9</td>
<td>66</td>
<td>60 (11)</td>
<td>22</td>
<td>72</td>
</tr>
</tbody>
</table>

*a The E:T ratio was 2:1.
*b Percent of lysis inhibition (reported in parentheses) was calculated using the following formula:
\[
\frac{[\% \text{ lymphocytes with } \alpha\text{CD3}] - [\% \text{ lymphocytes with } \alpha\text{CD3} + \alpha\text{CTLA-4}]}{[\% \text{ lymphocytes with } \alpha\text{CD3}]} \times 100.
\]

c Positive control using a mAb specific for HLA-DR (isotype IgG2a); lysis inhibition is null.

d Percent of specific lysis.

**Inhibition of CD2- or CD28-induced activation by cross-linked CD152**

Since CD152 inhibits CTL activation via CD3/TCR, we next investigated whether this molecule is able to inhibit other activatory pathways, such as those mediated by CD2 and CD28. Addition of anti-CD28 mAb or of mAb CD2.1 and CD2.9 (specific for two different epitopes of CD2 and able, when used in combination, to activate T cells) in the redirected killing assay, significantly increased lysis of P815 target cells. Anti-CD152 mAb, included in the cytolytic assay in association with these activatory mAb, yielded variable levels of target cell lysis inhibition. The level of inhibition was comparable to that provided by anti-CD2 in association with anti-CD3 mAb (see Fig. 2). In addition, CD152 mAb inhibit lysis when anti-CD2 or anti-CD28 mAb are used together with anti-CD3 mAb. These data indicate that CD152 also inhibits activatory pathways other than those mediated by CD3/TCR and, again, that this property is clonally distributed.

**Definition of a role for CD152 in the CTL-specific target cell lysis**

Eight Ag-specific T cell clones that lyse autologous Ag-presenting B-EBV lymphocytes, but not unrelated allogeneic B-EBV cells
were used in a cytolytic assay. mAb specific for CD152, capable of blocking the receptor/ligand interaction (rather than cross-linking the receptor), were added at the onset of the test (see Fig. 1, right panel). In all these clones, lysis of specific targets was inhibited by addition of anti-CD3 or anti-HLA class I mAb (not shown). When anti-CD152 mAb was included in the assay, an

![FIGURE 2. Different levels of inhibition obtained by the use of mAb specific for CD152 in the redirected killing assay. Six T cell clones with the same surface phenotype (TCRαβ; CD28; CD3; CD8αβ) were used in this experiment. To obtain activation, the following mAb were added to the assay: Leu 4 (anti-CD3), CD28.2 (anti-CD28), CD2.1, and CD2.9 (specific for two different epitopes of CD2 and able, when used in combination, to activate T cells). Addition to the activatory mAb of anti-CD152 mAb BNI3.1 leads to different levels of inhibition. In clones 25A and Ge3 (upper row) inhibition of CD3-induced activation was >40%, in clones Co3 and 10A (middle row) it ranged between 20–40%, and in clones 28A and G1B (lower row) it was <20%. The target was the P815 cell line, and the E:T cell ratio in this experiment was 2:1.](http://www.jimmunol.org/)
increment of >40% of the lysis was achieved in three of eight clones. The cytolytic activities of these three clones are shown in Fig. 3. A control of the cross-linking capability of anti-CD152 mAb was included in the cytolytic assay. This molecule competes with CD152 expressed by CTL clones for the binding to CD80/CD86 present on B-EBV cell targets. Staining of B-EBV cell lines by CTLA-4 Ig is significantly reduced by the simultaneous addition of anti-CD80 and anti-CD86 mAb (see Fig. 4A).

CTLA-4 Ig added to the cytolytic test produced a significant increase in the specific target cell lysis in a dose-dependent fashion (Fig. 4B). CTL clones used in this assay were the same as those that showed an increase in specific lysis when anti-CD152 mAb were added to the cytolytic test.

Identical results were obtained when anti-CD80 (IgM isotype) and anti-CD86 (IgG2b isotype) mAb were added to the cytolytic assay in combination (Fig. 4C). Addition of CTLA-4 Ig, anti-CD80, or anti-CD86 to CTL clones that had shown a minimal increment of specific lysis induced by anti-CD152 mAb produced a very weak effect (not shown).

In conclusion, blocking CD152 ligands expressed by target cells leads to an increase in target cell lysis. This suggests that under physiologic conditions also, CD80 and CD86 could protect target cells from the lysis mediated by CTL clones in which an inhibitory function of CD152 is effective.

Discussion

The functional outcome of effector T lymphocytes depends on a balance between opposite effects mediated by activatory and inhibitory receptors. Therefore, a prevalence of activation should render T cells responsive to Ags, whereas a prevalence of inhibition should lead to T cell anergy.

The majority of the data on the inhibitory function exerted by CD152 have been gathered by studies of proliferation or cytokine production of naive T lymphocytes during T cell priming. In our study we have investigated the role of CD152 during effector T cell functions. Namely, we assessed the ability of CD152 to inhibit the cytolysis exerted by CTL clones derived following PHA stimulation or by Ag selection. It should be noted that these CTL clones were used several weeks after Ag or mitogen stimulation.

In a redirected killing assay, CD152 cross-linking resulted in inhibition of the activation mediated by CD3, CD2, and CD28, also when these pathways were triggered in combination. It is of note that although some reports suggest a possible activatory role of CTLA-4 (25–27), in the redirected killing assay mAb specific for CTLA-4 failed to induce activation. A possible explanation for this apparently contradictory phenomenon is that the addition of anti-CD152 mAb or soluble CTLA-4 ligand, which are able to interfere with receptor/ligand interaction in the experimental models, leads to an activation resulting from a loss of inhibition. The ability of this inhibitory molecule to negatively regulate activatory pathways distinct from CD3 suggests that CD152 could inhibit the CTL activation that follows interaction between effector and target cells. In fact, this is a complex event that requires the involvement of several and distinct activatory molecules. To kill their target, T cells must recognize Ags specifically, making use of their CD3/TCR receptors. However, this is not sufficient to activate the effector cells, and the involvement of CD2, LFA-1, CD40 ligand, and other activatory molecules is required. Thus, the ability of
FIGURE 4. Soluble recombinant CTLA-4 Ig and anti-CD80/CD86 mAb react with ligands expressed by target cells and increase specific lysis. A, CTLA-4 Ig stains B-EBV cells (right peak); addition of anti-CD80 and anti-CD86 mAb in combination with CTLA-4 Ig reduces the mean fluorescence intensity (arbitrary units) from 94 to 22 (middle peak). The basal fluorescence obtained with the use of the second reagent alone (FITC-labeled polyclonal goat anti-human Fc IgG) is indicated by the left peak. B and C, CTL clones Co1, Co3, and Co4 were used in a specific cytolytic assay. The targets were autologous B-EBV cell lines, and the E:T cell ratio in this experiment was 5:1. Addition to the test of the fusion protein CTLA-4 Ig yielded an enhancement of specific lysis in a dose-dependent fashion (B). Addition to the test of mAb anti-CD80 and anti-CD86, used in combination, yielded an enhancement of specific lysis in a dose-dependent fashion (C).
CD152 to inhibit distinct activatory pathways suggests an involvement of this molecule in vivo during Ag recognition by effector CTL.

To lend support to this hypothesis, experiments with specific CTL were performed since this model mimics a physiologic condition. It should be noted that cross-linking of CD152 obtained by the addition of a goat antiserum to murine Ig to anti-CD152 mAb leads to inhibition of the specific lysis. On the contrary, addition to the cytolytic test of a mAb specific for the inhibitory receptor blocks the receptor-ligand interaction and enhances specific target cell lysis. These data were also supported by experiments performed with soluble CTLA4 Ig and with anti-CD80 and anti-CD86 mAb in which an increment of specific lysis was also observed. Thus, in our experimental model also, CD152 delivers inhibitory signals able to downregulate the cytolytic activity of CTL clones. It should be noted that for three specific clones in which CD152-mediated inhibition prevails, the fusion protein CTLA4-Ig increased lysis. This strongly suggests that, at least for these three clones, the fusion protein competes only with CD152 expressed by effector T cells and not with CD28. At variance, data in the literature indicate that during Ag priming of virgin T cells, CTLA4-Ig induces anergy by subtraction of ligands to CD28 (17, 28). Thus, it is possible to foresee a different role played by CD28 during Ag priming of virgin T cells and during lysis of Ag-presenting target cells by effector CTL.

The ability of CD152 to inhibit cytolytic functions could render tumor-specific T lymphocytes unable to recognize tumor cells, thus providing a mechanism of escape that could allow tumor growth in cancer patients. The expression of ligands (i.e., CD80/CD86) on target cells is crucial for the protection from lysis mediated by CTL clones. Since the expression of these Ags was recently demonstrated on tumor cells, such as malignant B lymphocytes (29), the possibility of blocking interactions between CD80/CD86 and CD152 could provide a mechanism to overcome anergy of tumor Ag-specific CTL.

Inhibition of the cytolytic function of effector CTL by CD152 is not equally shared by all clones. In some clones, inhibition is prevalent; in others, activation predominates. This suggests the existence of a clonal heterogeneity. The mechanisms that regulate inhibition are presently unknown. The level of CD152 expression is equally low in all tested clones. The molecule is largely confined to the endosomal cell compartment. It is suggested that at the time of effector-target conjugate formation, endosomes fuse with the cell membrane in the area of cell-to-cell contact, and the molecule is therefore translocated into the plasma membrane (7, 13). A quantitative regulation of this mechanism could responsible for the different levels of inhibition.

The following questions arise from our observations. Is only a proportion of T lymphocytes rendered anergic by this mechanism or, on the contrary, do all T cells, sooner or later, become anergic following chronic antigenic challenge? Although we cannot provide an answer to these questions, this type of regulation could play a crucial role in autoimmunity (21, 30) and in the prevention of uncontrolled proliferation of T lymphocytes, as it occurs in CD152 knockout mice (31).

The lack, in our experimental system, of CTL clones showing complete inhibition, is probably due to the fact that CTL characterized by such an efficient CD152 activity are unable to proliferate and, thus, to undergo clonal expansion. Cell cycle and apoptosis analyses could answer this question. Presently, there is evidence that CD152 is capable of blocking the cell cycle in G1 (32, 33) or, alternatively, in a different model, that it leads to apoptosis of effector cells (34, 35).

Acknowledgments

We thank Silvia Bruno for helpful discussions. We also thank Riccardo Gavioli (University of Ferrara, Ferrara, Italy) for help with the generation of B-EBV-specific CTL.

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