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Restoring the Association of the T Cell Receptor with CD8 Reverses Anergy in Human Tumor-Infiltrating Lymphocytes

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SUMMARY

For several days after antigenic stimulation, human cytolytic T lymphocyte (CTL) clones exhibit a decrease in their effector activity and in their binding to human leukocyte antigen (HLA)-peptide tetramers. We observed that, when in this state, CTLs lose the colocalization of the T cell receptor (TCR) and CD8. Effector function and TCR-CD8 colocalization were restored with galectin disaccharide ligands, suggesting that the binding of TCR to galectin plays a role in the distancing of TCR from CD8. These findings appear to be applicable in vivo, as TCR was observed to be distant from CD8 on human tumor-infiltrating lymphocytes, which were anergic. These lymphocytes recovered effector functions and TCR-CD8 colocalization after ex vivo treatment with galectin disaccharide ligands. The separation of TCR and CD8 molecules could be one major mechanism of anergy in tumors and other chronic stimulation conditions.

INTRODUCTION

The T cell receptor (TCR) and its coreceptor CD8, expressed on cytolytic T lymphocytes (CTLs), cooperate in the recognition of a complex formed by an antigenic peptide and a class I major histocompatibility complex (MHC) molecule. The TCR$\alpha\beta$ heterodimer interacts with both the peptide and the surrounding groove of the MHC class I molecule with a K_D that is usually higher than 3 μM for syngeneic responses (Arcaro et al., 2001; Holler and Kranz, 2003). In contrast, the heterodimeric coreceptor CD8$\alpha\beta$ contacts the α3 constant region of MHC class I molecules with a K_D of $\sim$100 μM (Arcaro et al., 2001; Holler and Kranz, 2003). Together, the affinity of the TCR-CD8 complexes for MHC-peptide complexes is about 10-fold higher than that of the TCR alone (Holler and Kranz, 2003).

CD8$\alpha\beta$ is located in lipid rafts, where it associates with the intracellular tyrosine kinase $p56^{lck}$ (Arcaro et al., 2000, 2001). TCR$\alpha\beta$, which is associated with the CD3γ, δ, ε, and ζ chains, is not located in rafts, but upon binding of the CTL to the target cell, TCR-CD3 complexes can become associated with raft-resident CD8-$p56^{lck}$ complexes to induce signal transduction (Montixi et al., 1998; Doucey et al., 2001). Soon, additional TCR molecules concentrate into the contact region, called the immunological synapse. This synapse matures to form a distinct pattern, with TCR molecules located in the center being surrounded by LFA-1 adhesion molecules, which are themselves surrounded by CD45 molecules (van der Merwe et al., 2000; Huppa and Davis, 2003). The microtubule organizing center reorients itself toward the synapse and reorganizes the microtubule network to ensure directional release of lytic granules into the target cells (Grakoui et al., 1999; Stinchcombe et al., 2001). Within a few hours, TCR molecules in the synapse start being internalized, resulting in a considerable decrease of TCR molecules at the cell surface. This decrease lasts only 24 hours, as recycled and newly synthesized TCR molecules replenish the TCR surface pool (Valitutti et al., 1995).

CTLs can be maintained in culture for several months by regular stimulation every 7 to 14 days with antigen in the presence of growth factors, but their cytolytic activity sometimes declines or disappears completely. We have observed that some antitumoral human $CD8^+$ CTL clones gradually lose their cytolytic activity if, instead of being stimulated weekly with tumor cells, they are stimulated with peptide-pulsed Epstein-Barr virus-transformed B (EBV-B) cells, which appear to provide a stronger stimulation. For two CTL clones, we have reported that the loss of effector function is always associated with a loss of labeling by human leukocyte antigen (HLA)-peptide tetramers, which are fluorescent tetrameric complexes formed by soluble HLA molecules, β2-microglobulin, and the antigenic peptide (Altman et al., 1996; Demotte et al., 2002). The loss of tetramer binding was not

due to a loss of expression of TCR or CD8 at the cell surface. We report here that, during a substantial part of a stimulation cycle, CTL clones have a strong reduction in their ability to bind HLA-peptide tetramers and to secrete cytokines. We show that this phase is linked to a dissociation between CD8 and TCR molecules on the cell surface. We also show that TCR is distant from CD8 on human tumor-infiltrating lymphocytes (TILs). This type of anergy of TILs appears to be an extension of the decreased activity occurring during the normal stimulation cycle of T lymphocytes.

RESULTS

Transient Decrease of Tetramer Binding and Effector Functions of CTL Clones after Stimulation

Having observed that, after several cycles of stimulation, some CTL clones lose their effector functions together with their ability to bind HLA-peptide tetramers (Demotte et al., 2002), we set out to examine the evolution of tetramer binding during one stimulation cycle. CTL clone A10, directed against the MAGE-3 peptide EVDPIGHLY presented by HLA-A1, was maintained by stimulation every 14 days with peptide-pulsed tumor cells. Cells collected 14 days after antigenic stimulation were found to have a strong and homogeneous staining by the relevant tetramer (tetramer^{hi} phenotype). On day 0, these cells were stimulated as usual with peptide-pulsed tumor cells in the presence of feeder cells and IL-2. On day 4, a homogeneous decrease of tetramer staining of more than 95% was observed (tetramer^{lo} phenotype) (Figure 1A). Other experiments with the same clone and with other clones showed that CTLs were already tetramer^{lo} by day 1 after stimulation (Figure 1D; see also Figure S1 available online). By day 7 the CTLs had regained part of their ability to bind the tetramer, and by day 14 they had a tetramer^{hi} phenotype (Figure 1A). A decrease in tetramer binding was observed with tetramer concentrations ranging from 1 to 100 nM (Figure S2). Surface expression of the TCR is known to decrease in the hours after stimulation, but the tetramer^{lo} phenotype observed on day 4 was not due to decreased surface expression of TCRα, TCRβ, CD8α, or CD8β, as the surface expression of these receptor molecules was equal to that observed on day 0 (Figure 1A; Figure S1).

We assessed the effector capability of the CTLs at various moments of the stimulation cycle (Figures 1B and 1C). The CTLs collected on day 0 were capable of producing IFN-γ and IL-2 upon an 18 hr stimulation with peptide-pulsed EBV-B cells. After a 1 hr contact with the antigen, surface TCR molecules were concentrated at the contact site between CTLs and target cells, as expected for functional CTLs. This was also observed for intracellular linker for activation of T cell (LAT) molecules and for CD3ζ molecules (data not shown). In contrast, CTLs collected on day 4 failed to release IFN-γ or IL-2 upon contact with peptide-pulsed cells and did not recruit TCRβ, LAT, or CD3ζ molecules at the contact site. By day 14, the CTLs had recovered their ability to release IFN-γ upon antigen contact. A similar analysis was performed with ten other CTL clones (Figure S3). After stimulation, a transient decrease in tetramer staining and in cytokine production of more than 90% was observed for the clones tested.

The time course of the recovery of tetramer binding of CTL clones was dependent on the conditions of antigenic stimulation. Data obtained with CTL clone 5, another anti-MAGE-3.A1 CTL clone, are shown in Figure 1D. After stimulation with peptide-pulsed irradiated tumor cells, a strong decrease of tetramer binding was observed, and the initial tetramer binding was recovered within 2 weeks. In contrast, after stimulation with peptide-pulsed EBV-B cells, the CTLs showed low tetramer binding for 2 weeks and recovered the initial amount of binding only after 4 weeks (Figure 1D). After phytohemagglutinin (PHA) stimulation, the tetramer^{hi} phenotype was also recovered only after 4 weeks. Moreover, repeating the stimulation with peptide-pulsed cells during the tetramer^{lo} phase appeared to extend this phase indefinitely (Figure 1E). Taken together, these data support the notion that the duration of the tetramer^{lo} phenotype correlates with the strength and duration of antigenic stimulation.

Transient Decrease of Tetramer Binding of Anti-EBV T Cells Stimulated Ex Vivo

We wondered whether the reduced tetramer binding observed after stimulation of long-term CTL clones could also be observed with blood T cells stimulated ex vivo. We identified an individual with a high percentage (1.7%) of CD8$^+$ blood T cells that were stained with an HLA-A2 tetramer bound to an EBV peptide encoded by *BMLF1*. When freshly collected blood T cells were stimulated with the BMLF1 peptide, almost no tetramer^{hi} cells were detected for 1 week (Table 1). On day 20, 15% of the CD8$^+$ cells were stained with the tetramer, indicating not only a recovery of the tetramer^{hi} phenotype but also a selective proliferation of the anti-BMLF1 cells. A similar decrease in tetramer binding, followed by recovery, was observed upon stimulation with PHA. Stimulation with an irrelevant peptide did not result in a tetramer^{lo} phase. We conclude that the transient loss of tetramer staining observed during the stimulation cycle of functional T cell clones is also observed in freshly collected lymphocytes.

TCR and CD8 Colocalize on Tetramer^{hi} but Not Tetramer^{lo} CTLs

To understand why cells that expressed both CD8 and TCR molecules at their surface in normal amounts failed to bind to HLA-peptide tetramers, the surface distribution of CD8 and TCR molecules was compared in tetramer^{lo} and tetramer^{hi} subpopulations of clone A10. Cells were double labeled with TCRβ and CD8α fluorescent antibodies and analyzed by confocal microscopy (Figure 2A; Table S1). On most cells of the tetramer^{hi} population, CD8 and TCR were colocalized, appearing as a continuous yellow ring on the equatorial sections. In contrast, on most cells of the tetramer^{lo} population, separation of TCR and CD8 was obvious, with TCR (red) appearing as an inner ring and CD8 (green) appearing as an outer ring. As expected for colocalizing molecules, TCR and CD8 showed a uniform cocapping on tetramer^{hi} CTLs and did not cocap on tetramer^{lo} cells (Figure 2B).

The ultrastructural correlate of the two rings in the tetramer^{lo} state remains unclear because the antibodies used for confocal microscopy have failed thus far to produce sufficient immunogold labeling for electron microscopy. One hypothesis that would be compatible with the distance observed between the inner TCR ring and the outer CD8 ring is a localization of CD8

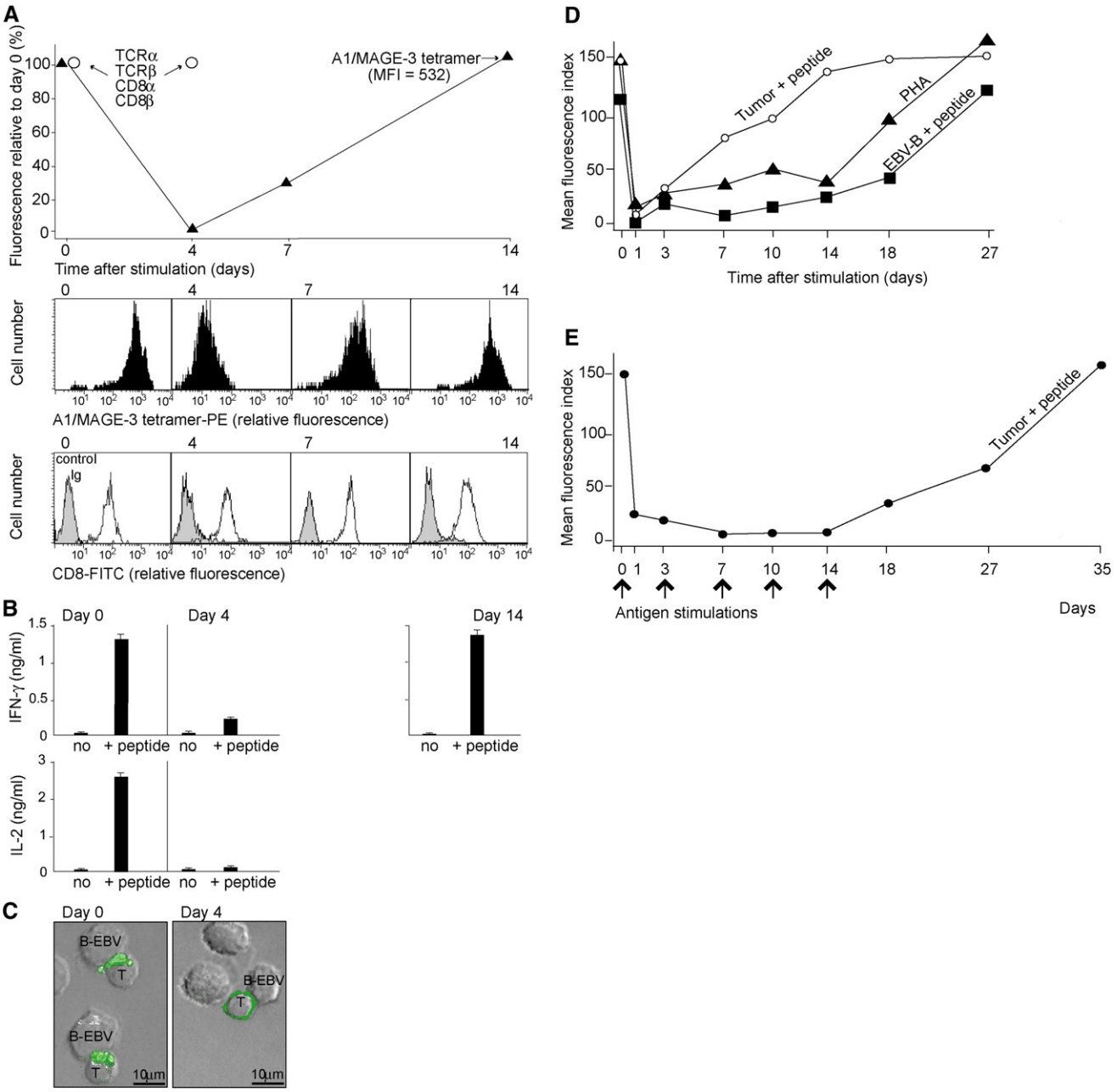

Figure 1. Time Course of Tetramer Binding and Effector Functions of Cytolytic T Lymphocytes

(A–C) Cytolytic T lymphocyte (CTL) clone A10 was stimulated on day 0 with peptide-pulsed tumor cells in the presence of feeder cells and IL-2.

(A) To assess tetramer binding, staining with an A1/MAGE-3 PE-labeled tetramer (5 nM) was performed at room temperature (RT). Staining with anti-TCRα, anti-TCRβ, anti-CD8α, and anti-CD8β was performed at 4°C. The mean fluorescence index (MFI) is the geometric mean of the fluorescence as measured by flow cytometry for $CD3^+$ cells. Results shown are representative of more than 20 experiments with more than 10 CTL clones.

(B) To assess effector functions, CTLs (5,000) collected at the indicated time were cocultured overnight with HLA-A1 EBV-B cells pulsed with 1 μM peptide or not pulsed. IFN-γ and IL-2 were then measured in triplicate in the supernatant of the coculture by ELISA and cytokine bead array (CBA) analysis, respectively. Results shown are representative of five experiments. Error bars represent standard deviation of triplicate measurements.

(C) To assess the ability to form synapses, peptide-pulsed EBV-B cells (1.5×10^5) were attached to coverslips and cocultured for 1 hr with 3×10^5 CTLs. Cells were fixed, labeled with an anti-TCRβ primary antibody and a secondary antibody coupled to Alexa 488 (green), and examined by confocal microscopy. The same experiment was performed with CTL clone F3.2, with very similar results.

(D) CTL clone 5 was stimulated on day 0 with peptide-pulsed tumor cells, peptide-pulsed EBV-B cells, or phytohemagglutinin (PHA) and feeder cells. Tetramer labeling was performed at RT with an A1/MAGE-3 PE-labeled tetramer (5 nM).

(E) Cells of CTL clone 5 were repeatedly stimulated on the days indicated (arrows) with peptide-pulsed tumor cells. The same experiment was performed twice with CTL clone 5 and twice with another CTL clone, with very similar results.

Table 1. Loss of Tetramer Staining of Anti-EBV T Cells Analyzed Ex Vivo

	$CD8^+$ $Tetramer^{hi}$ Cells (%)			
Stimulation	Day 0	Day 4	Day 8	Day 20
peptide BMLF1.A2	1.7	<0.1	<0.1	15.0
irrelevant peptide	1.7	1.5	1.4	1.4
PHA	1.7	<0.1	0.2	1.5

Freshly collected blood mononuclear cells were stimulated with either the BMLF1 peptide GLCTLVAML or the irrelevant peptide $MelanA_{26–35}$ at 5 μM or with phytohemagglutinin (PHA; 0.5 μg/ml) and cultured in medium with 10% HS and IL-2 (50 U/ml). Cells were stained for tetramer binding, before stimulation and at days 4, 8, and 20 after stimulation, with an A2-BMLF1 PE-labeled tetramer (5 nM) and a CD8 antibody. Data are given as percentages of $tetramer^{hi}$ cells among the $CD8^+$ cells.

molecules on microvilli and a localization of the TCR molecules at the bottom of microvilli or in intermicrovillar membrane domains, as previously reported (Singer et al., 2001). CTLs, which have a cell diameter of 10 μm, are known to bear abundant microvilli of about 0.5 μm in length (Majstoravich et al., 2004). This is also the case for our CTLs (Figure 2C).

To examine whether TCR and CD8 molecules that appeared to be colocalized on $tetramer^{hi}$ CTLs are close enough to cooperate in binding to HLA-peptide tetramers, fluorescence resonance energy transfer (FRET) can be used. Upon excitation at the donor fluorochrome wavelength, transfer of energy from the donor to the acceptor fluorochrome is evaluated by measuring the acceptor fluorescence emission. Transfer occurs provided that the distance between the two molecules is shorter than 10 nm. On a structural basis, a proximity closer than 10 nm is compatible with joint binding of a TCR and a CD8 molecule to the same MHC-peptide complex. $Tetramer^{lo}$ and $tetramer^{hi}$ populations were labeled with a TCRβ antibody coupled to phycoerythrin (PE) (donor) and CD8α antibody coupled to Alexa 647 (acceptor). A substantial transfer of energy was observed only with the cells of the $tetramer^{hi}$ population, indicating that the TCR and CD8 molecules were very close to each other in that population, and not in the $tetramer^{lo}$ population (Figure 2D). Similar results were obtained using a microscopy-based FRET approach, namely donor recovery after acceptor photobleaching (Figure S5). Thus, our data suggest that TCR and CD8 are constitutively associated on the surface of functional CTLs. The coreceptor CD8 has a critical role in TCR binding and activation, and our data suggest that the mere separation of TCR and CD8 is sufficient to explain the functional impairment of the $tetramer^{lo}$ CTLs. In line with these results, we observed that, 4 days after stimulation, the tetramer binding of a CD8-independent CTL decreased only slightly, but this did not result in a decreased ability to release cytokines upon stimulation (Figure S6).

Recovery of Tetramer Binding and Effector Function by Restoring Proximity of TCR and CD8

Because TCR and CD8 coreceptors are separated on $tetramer^{lo}$ cells, we considered the possibility that some molecules anchor them in different locations on the cell membrane. It has been shown that $p56^{lck}$ anchors CD4 in lipid rafts localized on microvilli (Foti et al., 2002). $p56^{lck}$ is also known to bind to CD8 by a zinc-dependent process (Barber et al., 1989; Lin et al., 1998). If $p56^{lck}$ acts as the anchoring molecule that keeps CD8 at a distance from TCR in $tetramer^{lo}$ cells, one might expect that a zinc chelator could release CD8 and therefore restore the proximity of CD8 to TCR. $Tetramer^{lo}$ cells were treated for 2 hr with the zinc chelator *o*-phenanthroline, after which they partially regained tetramer binding as well as FRET between TCR and CD8 (Figure 3). These cells were then washed and incubated overnight with antigen-presenting cells, after which they were found to have recovered the ability to release IFN-γ. (The cells were incubated overnight in the absence of *o*-phenanthroline because $p56^{lck}$-CD8 interactions are essential for T cell signaling.)

Regarding the possibility of TCR anchoring at a distance from CD8, it has been proposed that N-glycosylated TCR molecules belong to a surface glycoprotein lattice clustered by extracellular galectin-3 (Demetriou et al., 2001; Morgan et al., 2004). This lectin binds with high affinity to N-acetyllactosamine (LacNAc) motifs located near the end of N-linked branched sugar structures bound to asparagine residues of surface proteins. Lattice formation occurs because galectin forms homopentamers (Ahmad et al., 2004). FRET analysis revealed a colocalization between galectin-3 and TCR on $tetramer^{lo}$ CTLs, but not on $tetramer^{hi}$ CTLs, whereas no proximity of galectin-3 and CD8 was observed on either type of cell (Figure 4).

When $tetramer^{lo}$ CTLs collected 4 days after stimulation were treated for 2 hr with LacNAc, a competitive binder to galectin, TCR-CD8 colocalization as measured by FRET was largely restored together with tetramer binding and IFN-γ release (Figure 3). A similar but smaller effect was observed with lactose, a less potent galectin ligand. Treatment of $tetramer^{lo}$ cells with sucrose, which does not bind to galectin-3, had no effect. Treatment with neuraminidase, which cleaves sialic acid from many glycans, had an effect similar to lactose (data not shown). This effect, which was observed previously with murine CTLs (Kao et al., 2005), may be due to the presence of a sialic acid, which may increase the affinity for galectin, next to the LacNAc motif of galectin-binding proteins. Swainsonine is an inhibitor of α-mannosidase II, which is involved in the N-glycosylation pathway that produces the branched sugar structures carrying LacNAc motifs. Swainsonine was added to the CTL medium before antigenic restimulation. On day 4, the cells showed increased tetramer staining, increased TCR-CD8 FRET efficiency, and an increased ability to release IFN-γ upon antigenic stimulation compared to untreated cells (Figure 3). Treating $tetramer^{hi}$ cells with any of the compounds had no effect on tetramer binding.

An increased interaction of TCR with galectin-3 upon CTL stimulation could result from increased galectin-3 secretion after stimulation, a higher abundance of LacNAc motifs on TCR, or both. We observed that antigenic stimulation induced galectin-3 secretion by CTLs (data not shown), but this did not appear sufficient to explain the loss of tetramer binding by more than 80% of CTLs, as we found that only 30% of CTLs became $tetramer^{lo}$ when galectin-3 was added to the medium of $tetramer^{hi}$ CTLs at a concentration about 5-fold higher than that found in the medium of stimulated CTLs (Figure 5). This suggests that stimulation of CTLs also results in a modification of the glycosylation of surface glycoproteins that renders them more susceptible to bind to galectin-3. β1,6-N-acetylglucosaminyltransferase V (MGAT5) appears to be a key enzyme for the biosynthesis of N-glycans with high affinity for galectin-3 (Demetriou et al.,

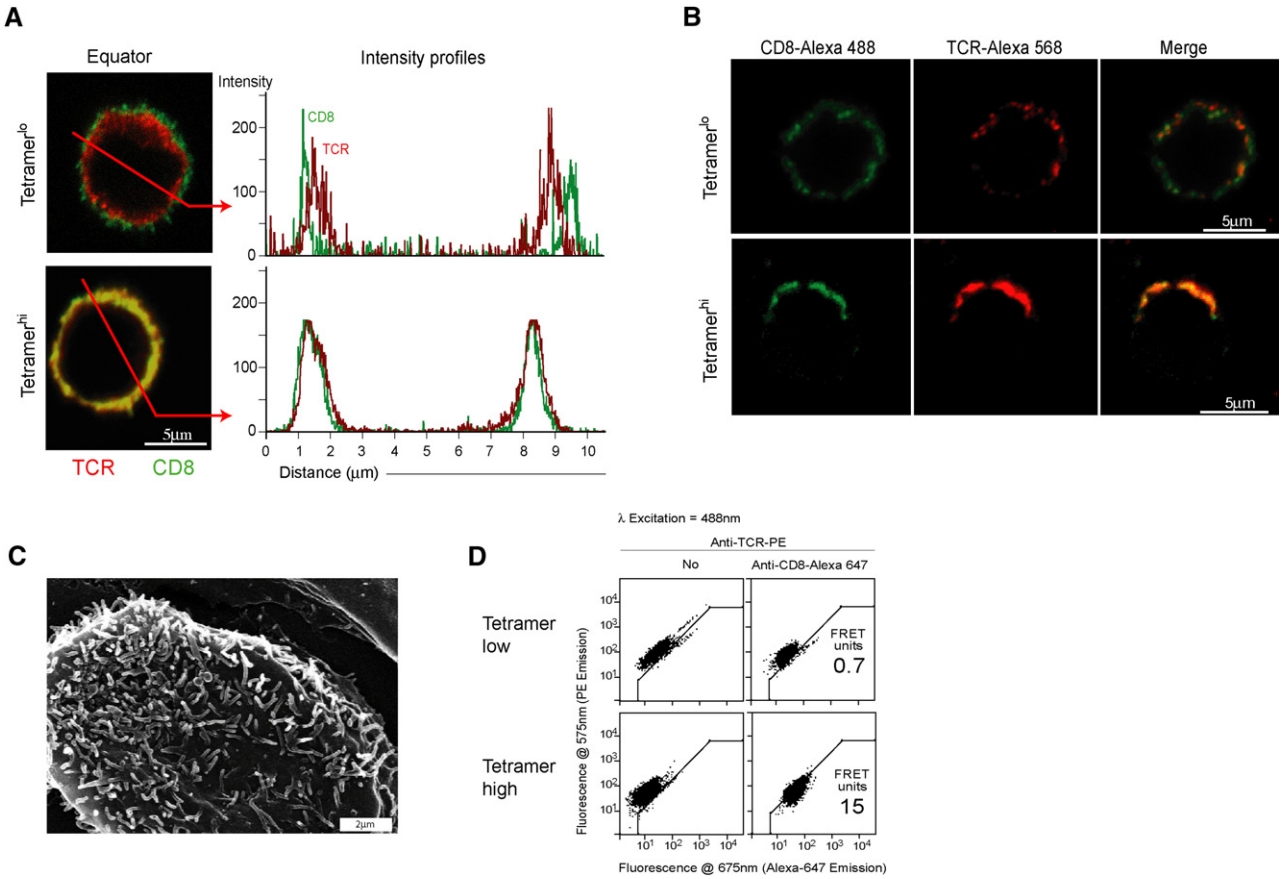

Figure 2. Absence of TCR and CD8 Colocalization on Tetramer^{lo} CTLs

Tetramer^{hi} cells of CTL clone A10 had not been in contact with antigen for 15 days, whereas the tetramer^{lo} cells of CTL clone A10 had been stimulated with peptide-pulsed cells 4 days before analysis.

(A) Cells were attached to coverslips, fixed, and double stained with TCRβ antibody and Alexa 568 secondary antibodies (red) and with CD8α antibody and Alexa 488 secondary antibodies (green). The profiles of red and green fluorescence intensities in equatorial sections were analyzed in both subpopulations for 40 cells (Table S1).

(B) Cells were attached to coverslips but not fixed and directly double stained as in (A). Cells were then fixed and incubated overnight at RT.

(C) Scanning electron microscopy image of CTL clone A10.

(D) Cells were stained with anti-TCRβ-PE (donor), anti-CD8α-Alexa 647 (acceptor), or both. Cells were then fixed and analyzed by flow cytometry for fluorescence resonance energy transfer (FRET). Similar results were obtained when CTLs were fixed before being stained with antibodies. FRET efficiency was calculated according to Doucey et al. (2003) and expressed as FRET units. Results shown are representative of five experiments. Additional controls are shown in Figure S4.

2001; Morgan et al., 2004). MGAT5 has been reported to increase after mouse T cell activation (Demetriou et al., 2001), but we found no increase after human CTL activation (data not shown). Identification of the glycosylation modifications of the TCR that result in increased binding to galectin-3 will require further experiments.

TCR and CD8 Do Not Colocalize on Tumor-Infiltrating Lymphocytes

It has been widely reported that human solid tumors and tumor ascites are infiltrated by T cells (Ioannides et al., 1991; Yannelli et al., 1996; Zorn and Hercend, 1999). Detailed study of melanoma tumors indicates that most of these tumor-infiltrating lymphocytes (TILs) are directed against tumor-specific antigens (Germeau et al., 2005; Lurquin et al., 2005). These TILs appear to be ineffective in situ, as the tumors are clearly progressing. It was not possible to analyze tetramer staining on TILs because the HLA-peptide complexes recognized by the TILs have not been characterized. We therefore resorted to FRET analysis to examine whether TCR and CD8 failed to colocalize on these anergic TILs. We were unable to obtain enough TILs from a melanoma metastasis, but we obtained CD8$^+$ TILs in sufficient numbers from a breast carcinoma lung metastasis and from carcinoma ascites. No marked transfer of energy between TCR and CD8 was measured on CD8$^+$ TILs isolated from these tumors (Figure 6A), indicating a lack of colocalization, whereas a clear transfer of energy was measured on CD8$^+$ blood T cells. In contrast, FRET was clearly positive on CD8$^+$ T cells isolated from a kidney allograft resected because of a chronic rejection process. The transfer of energy was equivalent to that measured on the control tetramer^{hi} CTL clone, indicating that TCR and CD8 colocalize on these T cells obtained during graft rejection (Figure 6A). We conclude that TIL anergy is associated with a lack of TCR colocalization with CD8.

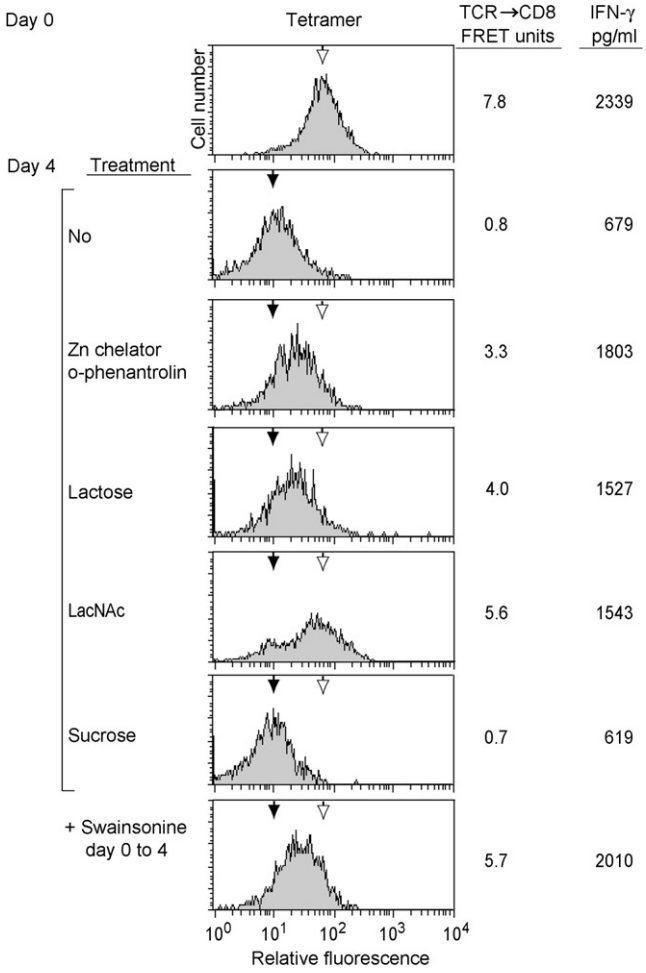

Figure 3. Recovery of CTL Effector Function by Restoring TCR Proximity to CD8

Cells of CTL clone A10 were collected on day 0 and 4 days after stimulation with HLA-A1 MAGE-3$^+$ tumor cells. Swainsonine (0.5 μM) was added on day 0, before stimulation. Cells were stained at RT with an A1/MAGE-3 PE-labeled tetramer. White arrows indicate the MFI for tetramer^{hi} cells collected on day 0; black arrows indicate the MFI for tetramer^{lo} cells collected on day 4. FRET experiments were performed as in Figure 2. Where indicated, cells collected on day 4 were treated for 2 hr with either *o*-phenanthroline (30 μM), lactose (100 mM), N-acetyllactosamine (LacNAc; 1 mM), or sucrose (100 mM). Cells were washed and analyzed for tetramer staining and TCR-CD8 FRET. To assess effector function, 10,000 washed CTLs were cocultured with 10,000 peptide-pulsed EBV-B cells. IFN-γ was estimated in triplicate by ELISA in the supernatant after overnight coculture. Only the averages are indicated. Standard deviations were below 8%, except after sucrose treatment (14%). The same experiment was performed with CTL clone 5, with very similar results.

TCR-CD8 Colocalization and Effector Function Can Be Restored by LacNAc Treatment

CD8$^+$ TILs from a gastric carcinoma ascites were analyzed for TCR-CD8 FRET after treatment with LacNAc. After 5 hr of LacNAc treatment, the TILs had recovered the same amount of TCR-CD8 FRET as that observed on tetramer^{hi} CTLs (Figure 6B). We obtained similar results with TILs isolated from an ovarian carcinoma ascites (Figure 6B, patient 1). In line with the lack of TCR-CD8 colocalization observed on freshly isolated TILs, these TILs failed to

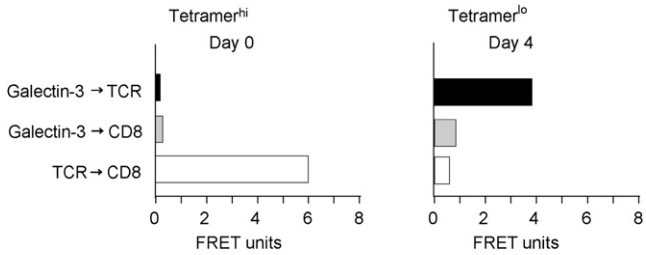

Figure 4. TCR and Galectin-3 Colocalize on Tetramer^{lo} but Not Tetramer^{hi} CTLs

Cells of CTL clone A10 were stained at 4°C with anti-TCRβ-PE (donor) or anti-galectin-3-PE (donor) and with anti-CD8β-Alexa 647 (acceptor) or anti-TCRβ-Alexa 647 (acceptor). Cells were then fixed and analyzed by flow cytometry for FRET. Similar results were obtained with two different CTL clones.

produce IFN-γ during an overnight stimulation either with a mixed population of tumor cells, B cells, and macrophages collected from the same ascites or with allogeneic EBV-B cells (Figure 6C, patient 1), and they showed a very low response to anti-CD3 and anti-CD28 stimulation. Overnight incubation of these TILs with 1 mM LacNAc boosted their IFN-γ response to stimulation with these antigens by more than 10-fold (Figure 6C). The same observation was made for the release of TNF-α, IL-4, IL-5, and IL-10 (data not shown). Similar results were obtained with TILs isolated from other carcinoma ascites (Figure 6C).

As mentioned above, we measured clear TCR-CD8 FRET on kidney-infiltrating CD8$^+$ T cells isolated from a kidney allograft resected because of a chronic rejection process. As expected, these T cells released IFN-γ upon stimulation with allogeneic EBV-B cells or beads coated with anti-CD3 and anti-CD28. Treatment with LacNAc did not result in increased IFN-γ production (Figure 6D). Thus, treatment with LacNAc appears to be effective only on T lymphocytes where TCR and CD8 do not colocalize.

DISCUSSION

We report here that, after antigenic stimulation, human CD8$^+$ CTL clones enter a transient state characterized by markedly reduced HLA-peptide tetramer binding despite having normal amounts of TCR and CD8 surface expression. The transient loss of tetramer binding is accompanied by an alteration in T cell effector function, in particular a decreased ability to release IFN-γ upon further antigenic stimulation. The time required to recover a tetramer^{hi} phenotype, which can be several weeks, correlates with the strength of the antigenic stimulation. Rapid iteration of the stimulation maintains the CTLs in their tetramer^{lo} phenotype. These results raise the possibility that this phenomenon applies to CTL stimulation in vivo. This may explain why, under persistent antigen exposure such as that occurring within a tumor mass or during chronic viral infection, CTLs are in a blocked, anergic state, which can be reversed in the absence of antigen.

Several groups have reported that, in murine CTLs, reduced tetramer binding is often associated with decreased effector function. After in vitro stimulation of anti-influenza CD8$^+$ T cells from TCR-transgenic animals, a transient loss of tetramer binding was observed from day 1 to day 4 and was associated with reduced TCR signaling and diminished effector activities (Drake

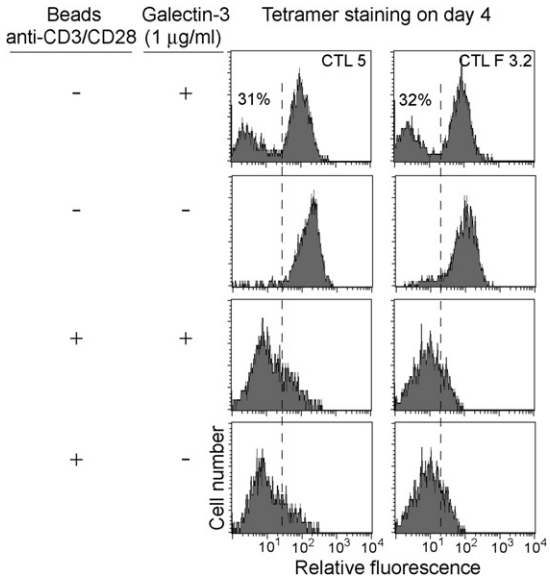

Figure 5. Influence of Galectin-3 and TCR Stimulation on Tetramer Binding

CTL clones 5 and F3.2 were collected 15 days after the last antigenic stimulation and had a homogeneous tetramer^{hi} phenotype. CTLs were stimulated with or without beads coated with both anti-CD3 and anti-CD28 antibodies in the presence of IL-2 but without feeder cells and in medium without human serum (HS). Galectin-3 (1 μg/ml) was added to the medium on day 0 where indicated. Staining with an A1/MAGE-3 PE-labeled tetramer (5 nM) was performed on day 4.

et al., 2005). In a fibrosarcoma model, 20%–30% of the TILs expressed a TCR specific for tumor antigen gp33. TILs had an activated phenotype but lacked effector cell functions. These TILs bound MHC tetramers only after T cell purification and a resting period in vitro (Blohm et al., 2002). Schwartz and colleagues transferred CD4$^+$ T cells from TCR-transgenic mice into hosts constitutively expressing the antigen recognized by the transferred T cells. Within 2 weeks, a massive expansion of the T cells was observed, most of which were hyporesponsive to further in vitro stimulation. This hyporesponsiveness disappeared gradually after the cells were transferred into new hosts that did not express the antigen (Schwartz, 2003). Several groups have reported similar observations with CD8$^+$ and CD4$^+$ T cells (De Mattia et al., 1999; Radoja et al., 2001; Kaech et al., 2002). It has been proposed that this reversible loss of function in the presence of an overwhelming antigen level, which has been named anergy, might protect against excessive side effects of immune responses against viral infection (Oxenius et al., 1998). Murine myeloid-derived suppressor cells, by generating reactive oxygen species and peroxynitrite, induce nitration of tyrosines on TCR and CD8 molecules, making T cells unable to bind soluble peptide-MHC complexes and to respond to the specific peptide (Nagaraj et al., 2007). This loss of function, which may be an important mechanism of tumor escape in mice, is permanent, contrary to the transient anergy described above in murine models and in this report for human T cells.

One of our important findings is the loss of colocalization of TCR and CD8 molecules on CTLs that have low effector function and a low ability to bind tetramers. Our data also suggest a constitutive association of TCR and CD8 on the surface of functional T cells, in agreement with several previous reports (Beyers et al., 1992; Suzuki et al., 1992; Kwan Lim et al., 1998; Doucey et al., 2003) but in contradiction with others (Lee and Kranz, 2003; Yachi et al., 2005). The critical role of the coreceptor CD8 in the binding of TCR to MHC-peptide complexes has been amply documented (Daniels and Jameson, 2000; Arcaro et al., 2001; Pecht and Gakamsky, 2005). The mere separation of TCR from the coreceptor CD8 is therefore sufficient to explain the functional impairment of the tetramer^{lo} CTLs. Our observations were extended in vivo by FRET observation of TCR-CD8 separation on freshly collected TILs, which appeared to be anergic.

We reasoned that some molecules must anchor the CD8 and TCR molecules in different locations of the cell membrane of tetramer^{lo} cells to explain the separation of TCR from CD8. The anchoring of the CD8 molecules in membrane rafts of microvilli is suggested by electron microscopy data (Singer et al., 2001). The anchoring molecule could be p56^{lck}, which is known to bind CD8 in a zinc-dependent manner (Barber et al., 1989; Lin et al., 1998; Arcaro et al., 2000, 2001; Foti et al., 2002). In agreement with this model, we observed that a short treatment of tetramer^{lo} cells with a zinc chelator restored the proximity of TCR and CD8, as well as the ability to bind tetramers. However the importance of p56^{lck}-CD8 interactions for T cell signaling probably precludes the use of a zinc chelator in vivo for restoring effector functions of T cells.

Regarding the anchoring of TCR, murine TCR can be trapped within a lattice formed by extracellular galectin, in particular galectin-3, which binds to surface glycoproteins carrying LacNAc motifs near the end of tetra-antennary N-glycans (Demetriou et al., 2001; Morgan et al., 2004; Grigorian et al., 2007). Several of our observations indicate that galectin-3 is involved in distancing TCR from CD8. First, a close proximity of TCR and galectin-3 was measured by FRET only on tetramer^{lo} CTLs. Second, we observed that incubation of tetramer^{hi} CTLs with galectin-3 converted 30% of them into tetramer^{lo} CTLs. Finally, galectin-3 is known to bind with high affinity to LacNAc motifs, and we observed that LacNAc treatment of tetramer^{lo} T cells rapidly restored TCR-CD8 colocalization, tetramer staining, and effector function. Although these results do not exclude a contribution of other galectins, they support a role of galectin-3.

One possible explanation of the distancing of TCR from CD8 after stimulation could be the secretion of galectin-3 by activated CTLs. This has been observed for activated murine T cells (Joo et al., 2001), and we observed that activation of human CTL clones also induced galectin-3 secretion. However, because incubation of a high concentration of galectin-3 converted only 30% of the tetramer^{hi} CTLs into tetramer^{lo} cells, galectin secretion by CTLs does not appear sufficient to explain TCR-CD8 distancing on almost all CTLs after activation. This must additionally require a modification of the glycosylation pattern of proteins, resulting in an increase in their binding to galectin-3. Activation of murine T cells is known to cause modifications of the N-glycans on surface glycoproteins (Comelli et al., 2006). The biosynthesis of the N-glycans responsible for binding to galectin-3 requires the activity of an enzymatic cascade that includes MGAT5 (Demetriou et al., 2001; Morgan et al., 2004). MGAT5 expression has been reported to increase after mouse T cell activation, but we found no increase after human CTL activation (data not shown) (Demetriou et al., 2001). It has also been reported that

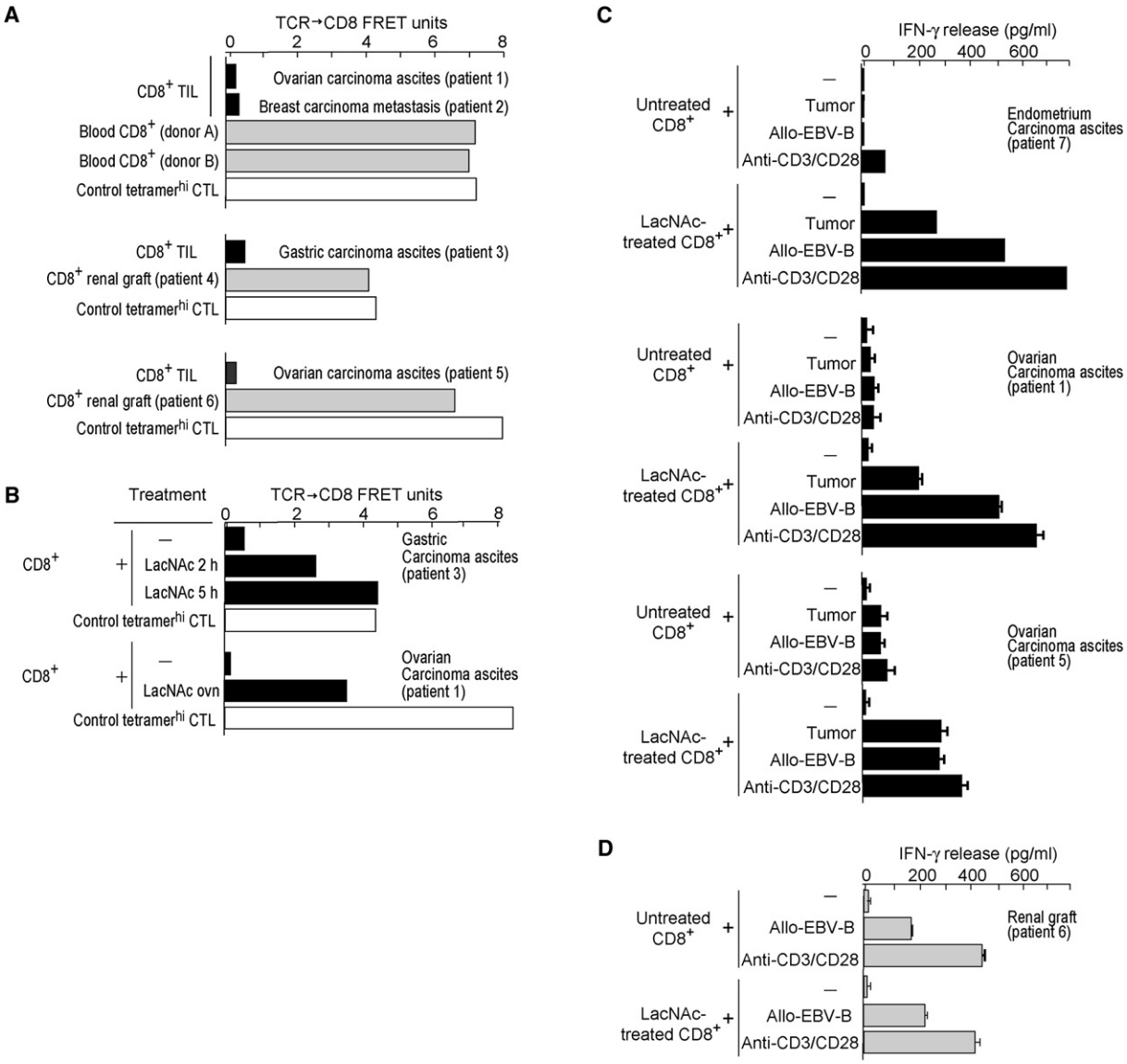

Figure 6. TCR and CD8 Do Not Colocalize on TILs, but LacNAc-Treated TILs Recover TCR-CD8 Proximity and Effector Function

$CD8^+$ T cells were isolated using a depletion strategy from a breast carcinoma metastasis, tumor ascites, blood, or a kidney allograft resected during a chronic rejection process. Control $tetramer^{hi}$ anti-MAGE-3.A1 CTL clone A10 was analyzed 2 weeks after the last antigenic stimulation.

(A) FRET experiments were performed as in Figure 2.

(B) $CD8^+$ TILs were incubated for various time periods at 37°C with 1 mM LacNAc and washed, and FRET experiments were performed as in Figure 2. Control $tetramer^{hi}$ CTL clone A10 was analyzed 2 weeks after the last antigenic stimulation.

(C and D) $CD8^+$ T cells were incubated overnight with or without 1 mM LacNAc. Washed $CD8^+$ T cells (10,000) were cocultured with 10,000 ascites tumor cells, 10,000 of a mix of five types of allogeneic EBV-B cells, or 10,000 beads coated with CD3 and CD28 antibodies. IFN-γ was measured in triplicate in the supernatant of an overnight coculture. The tumor cell fraction corresponds to the nonrosetting fraction of cells collected from the ascites. Error bars represent standard deviation of triplicate measurements. There is no error bar for patient 7 because the results were obtained by CBA analysis.

MGAT5 has an exceptionally high K_M and that an increase in the concentration of its substrate, UDP-GlcNAc, leads to an increase in surface glycoproteins associated with galectin-3 (Sasai et al., 2002; Grigorian et al., 2007; Lau et al., 2007), but we also failed to observe an increase in the intracellular pool of UDP-GlcNAc after human $CD8^+$ T cell activation (data not shown). Further experiments will be required to identify the mechanism of the glycosylation modifications that follow activation of human T cells.

Human cancers progress even though patients mount a substantial anti-tumoral T cell response resulting in the accumulation of anti-tumoral T cells in the tumor (Boon et al., 2006). There is evidence for local T cell anergy in the tumor location, and several hypotheses have been proposed to explain the local immunosuppression (Boon et al., 2006). Therapeutic vaccination of cancer patients with tumor antigens is followed by tumor regressions in only a small minority of patients. We believe that in these patients, a small number of anti-vaccine T cells succeed in

reversing the local immunosuppression and trigger broad activation of a much larger number of other anti-tumoral T cells, possibly the blocked T cells present in the tumor (Germeau et al., 2005; Lurquin et al., 2005). These anti-tumoral T cells then proceed to the destruction of tumor cells. The results reported here confirm once again that $CD8^+$ T cells in ascites and in a solid tumor are anergic and demonstrate that this anergy is correlated with an absence of TCR-CD8 colocalization. This may be due to the persistence of the antigen, resulting in permanent stimulation of TILs, and to the secretion of galectin-3 by tumor cells (Dumic et al., 2006). Compared to sera from healthy donors, galectin-3 concentration is increased in sera of tumor-bearing patients, especially metastatic patients (Iurisci et al., 2000; Vereecken et al., 2006).

A striking observation was our ability to restore effector functions of TILs after overnight treatment with LacNAc. It is possible that, in cancer patients, delivering LacNAc into tumors or ascites may temporarily restore effector functions of TILs and create conditions favorable for an effective anti-tumor T cell response. Therapeutic vaccination combined with LacNAc treatment may therefore induce tumor regressions in a larger fraction of patients than vaccination alone. In addition to LacNAc, other compounds might be tested, such as other galectin-3 ligands, agents preventing pentamerization of galectin-3, and agents inhibiting the N-glycosylation pathway such as swainsonine (Goss et al., 1994, 1997).

EXPERIMENTAL PROCEDURES

Cell Lines, Media, and Reagents

The EBV-B cell lines and HLA-$A1^+$ MAGE-3^+ tumor cell line were cultured in Iscove's modified Dulbecco's medium (IMDM) (Life Technologies) supplemented with 10% fetal calf serum (FCS), 0.24 mM L-asparagine, 0.55 mM L-arginine, 1.5 mM L-glutamine (AAG), 100 U/ml penicillin, and 100 μg/ml streptomycin. Tumor cell line MZ2-MEL (HLA-$A1^+$ MAGE-3^+) was cultured in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) supplemented with 10% FCS, AAG, and HEPES-glucose. Human recombinant IL-2 was purchased from Chiron Healthcare SAS, IL-7 from R&D Systems, PHA (HA16) from Murex, and antibody-coated beads from Dynal. All peptides were >90% pure as indicated by analytical high-pressure liquid chromatography. LacNAc, *o*-phenanthroline, swainsonine, lactose, and sucrose were purchased from Sigma-Aldrich, and galectin-3 was purchased from R&D Systems.

CTL Clones and Stimulation Conditions

Anti-MAGE-3.A1 CTL clones ERL06-3/3E5 (clone 5), ERL04-7A/32 (clone 32), and LB2586-7/1F3.2 (clone F3.2) were isolated from three patients vaccinated with autologous mature dendritic cells pulsed with the MAGE-3 peptide EVDPIGHLY (Thurner et al., 1999; Godelaine et al., 2003). Clone LAU147-810/A10 (clone A10) was isolated from a patient vaccinated with a canarypox virus expressing MAGE-3.A1. Clone LB1965-23 (clone 23) was described in Lonchay et al. (2004). CTLs were restimulated in 2 ml IMDM supplemented with AAG, 10% human serum (HS), and IL-2 (50 U/ml). CTLs (3×10^5) were cultured in 24-well plates with 1×10^6 irradiated allogeneic EBV-B feeder cells (LG2-EBV) and stimulated either with PHA (0.5 μg/ml) or with EBV-B cells (3×10^5) or irradiated tumor cells (1×10^5) previously incubated for 1 hr at 37°C with 1 μg/ml of peptide and washed.

Blood Cells and TILs

Tumor samples, tumor ascites, and resected kidney allografts were collected after approval of the research project by the ethical committee of the Faculté de médecine of the Université catholique de Louvain. Mononuclear cells were isolated from ascites by Lymphoprep (Axis-Shield PoCAS). After mechanical dissociation, tumor samples and kidneys were incubated overnight in IMDM medium supplemented with 1% HS, AAG, 100 U/ml nystatin (Sigma-Aldrich), streptomycin and penicillin, 30 μg/ml gentamycin (Sigma-Aldrich), and 5 μg/ml collagenase P (Roche). Mononuclear cells were then isolated by Lymphoprep. T cells were isolated from mononuclear cells by rosetting with sheep erythrocytes (bioMérieux) previously treated with 2-aminoethylisothiouronium bromide (Sigma-Aldrich). $CD8^+$ T cells were isolated by a depletion strategy in an AutoMACS system using a $CD8^+$ isolation kit II (Miltenyi Biotec). $CD8^+$ and $CD8^-$ cells were cultured in IMDM 1% HS, AAG.

Antigen Recognition Assays for CTL Clones and TILs

CTL clones (2,000 or 5,000) were stimulated for 20 hr in 150 μl of IMDM 10% HS, AAG. Stimulations were performed with 10,000 stimulator cells, beads coated with CD3/CD28 antibodies (1:1 ratio), or PHA at 0.5 μg/ml. Cytokine contents were measured by ELISA using Biosource Cytoset reagents or by flow cytometry with the Cytometric Bead Array kit Th1/Th2 (BD PharMingen).

Tetramers and Flow Cytometry

HLA-A*0101 and HLA-A*0301 complexes were folded with the MAGE-3 peptide EVDPIGHLY and the MAGE-1 peptide EADPTGHSY, respectively, as described previously (Coulie et al., 2001; Demotte et al., 2002). HLA-A*0201 complexes were folded with the BMLF1 peptide GLCTLVAML. Phycoerythrin (PE)-labeled tetramers were produced by mixing the biotinylated complexes with Streptavidin-PE (BD PharMingen). Concentrations of tetramers used for labeling refer to the concentration of the monomers. Cells were incubated for 10 min at room temperature (RT) with 5 nM tetramer and washed. Where indicated, cells were then incubated for 15 min at 4°C with 1/30 anti-CD3.APC (BD PharMingen), washed again, and incubated for 30 min at 4°C with 1/30 anti-CD8.FITC from IQ Product (Prosan). Cells were fixed with 1% formaldehyde (w/v in PBS) before being analyzed on a FACSCalibur system (BD PharMingen). In Figure 1A, cells were labeled for 30 min at 4°C with anti-TCRβ.FITC (BMA031; Immunotech), washed, and fixed with 1% formaldehyde.

FRET Analyses

Cells (2×10^5) were washed in Hank's medium 1% HS and resuspended in 50 μl of medium containing antibodies. For TCR-CD8 FRET, cells were incubated with 1/20 PE-labeled anti-TCRβ as donor (BMA031; Immunotech) and 1/300 Alexa 647-labeled anti-CD8 as acceptor (UCHT-4; Molecular Probes). For galectin-3-TCR FRET, cells were labeled with 1/10 PE-labeled anti-galectin-3 as donor (BD PharMingen) and 1/20 APC-labeled anti-TCRβ as acceptor (BMA031; eBioscience). For galectin-3-CD8 FRET, cells were labeled with 1/10 PE-labeled anti-galectin-3 as donor and 1/300 Alexa 647-labeled anti-CD8 as acceptor. After 30 min of incubation at 4°C, cells were washed and fixed with 2% formaldehyde. Analysis was performed on a FACSCalibur system (BD PharMingen) without compensation. The transfer of fluorescence was calculated as FRET units according to Doucey et al. (2003):

$$\text{FRET unit} = (E3_{\text{both}} - E3_{\text{none}}) - ([E3_{\text{AlI647}} - E3_{\text{none}}] \times [E2_{\text{both}}/E2_{\text{AlI647}}]) - ([E3_{\text{PE}} - E3_{\text{none}}] \times [E1_{\text{both}}/E1_{\text{PE}}]),$$

where $E1$ is the fluorescence detected at 580 nm upon excitation at 488 nm, $E2$ is the fluorescence detected at 670 nm upon excitation at 630 nm, and $E3$ is the fluorescence detected at 670 nm upon excitation at 488 nm. The different fluorescence values (E) were measured on unlabeled cells (E_{none}) or cells labeled with PE (E_{PE}), Alexa 647 (E_{AlI647}), or Alexa 647 and PE (E_{both}).

Recruitment of TCR at the Synapse and Localization of TCR and CD8

EBV-B cells (1.5×10^5) were incubated for 1 hr at 37°C with 1 μg/ml of peptide and washed. Cells were distributed on poly-L-lysine-coated glass coverslips for 30 min, and 3×10^5 CTLs were added for 1 hr. Cells were then labeled with anti-TCRβ (BMA031; Immunotech), washed, labeled with an Alexa 488-labeled goat anti-mouse IgG antibody (H^+L; Molecular Probes), and analyzed with a confocal scanning microscope as described previously (Miro et al., 2006). Staining of TCR and CD8 at the cell surface (Figure 2A) was performed as follows. 1×10^5 CTLs were distributed on poly-L-lysine-coated coverslips for 30 min. Cells were fixed with a 4% formaldehyde/0.2% glutaraldehyde mixture (w/v in PBS) for 20 min at RT, washed with PBS, and incubated for 10 min with 10 mM glycine in PBS. Cells were then incubated for 1 hr with anti-TCRβ (BMA031; Immunotech) and anti-CD8 (UCHT-4; R&D Systems) diluted in 0.2%

BSA (Sigma-Aldrich). Cells were washed by three incubations of 5 min in PBS/BSA and then incubated for 20 min with secondary antibodies diluted in the same buffer: Alexa 568-labeled anti-IgG2b for TCRβ, and Alexa 488-labeled anti-IgG2a for CD8α. After a final wash, coverslips were mounted on glass slides using ProLong Gold (Invitrogen). For cocapping studies, cells were not fixed before incubations with the antibodies. Fluorescence images were acquired with an LSM 510 laser scanning microscope and analyzed by AIM software (Zeiss).

SUPPLEMENTAL DATA

One table and six figures are available at http://www.immunity.com/cgi/content/full/28/3/414/DC1/.

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