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TCR-Independent Killing of B Cell Malignancies by Anti–Third-Party CTLs: The Critical Role of MHC-CD8 Engagement

Assaf Lask,*1 Polina Goichberg,*1 Adva Cohen,* Rinat Goren-Arbel,* Oren Milstein,* Shraga Aviner,* Ilan Feine,* Eran Ophir,* Shlomit Reich-Zeliger,* David Hagin,* Tirza Klein,† Arnon Nagler,‡ Alain Berrebi,§ and Yair Reisner*

We previously demonstrated that anti–third-party CTLs (stimulated under IL-2 deprivation against cells with an MHC class I [MHC-I] background different from that of the host and the donor) are depleted of graft-versus-host reactivity and can eradicate B cell chronic lymphocytic leukemia cells in vitro or in an HU/SCID mouse model. We demonstrated in the current study that human allogeneic or autologous anti–third-party CTLs can also efficiently eradicate primary non-Hodgkin B cell lymphomas by inducing slow apoptosis of the pathological cells. Using MHC-I mutant cell line as target cells, which are unrecognizable by the CTL TCR, we demonstrated directly that this killing is TCR independent. Strikingly, this unique TCR-independent killing is induced through lymphoma MHC-I engagement. We further showed that this killing mechanism begins with durable conjugate formation between the CTLs and the tumor cells, through rapid binding of tumor ICAM-1 to the CTL LFA-1 molecule. This conjugation is followed by a slower second step of MHC-I–dependent apoptosis, requiring the binding of the MHC-I α2/3 C region on tumor cells to the CTL CD8 molecule for killing to ensue. By comparing CTL-mediated killing of Daudi lymphoma cells (lacking surface MHC-I expression) to Daudi cells with reconstituted surface MHC-I, we demonstrated directly for the first time to our knowledge, in vitro and in vivo, a novel role for MHC-I in the induction of lymphoma cell apoptosis by CTLs. Additionally, by using different knockout and transgenic strains, we further showed that mouse anti–third-party CTLs also kill lymphoma cells using similar unique TCR-independence mechanism as human CTLs, while sparing normal naive B cells. The Journal of Immunology, 2011, 187: 2006–2014.

Development of strategies to generate ex vivo immune cells selectively endowed with graft-versus-leukemia (GVL), while depleted of graft-versus-host (GVH) reactivity, potentially represents a major challenge in bone marrow (BM) transplantation and in cancer immunotherapy. Our previous studies showed that ex vivo stimulation of mouse CD8 T cells against third-party stimulators (carrying an MHC class I [MHC-I] background different from that of the host and the donor, hence, termed “third party”) under IL-2 deprivation led to the selective growth of third-party–restricted CTL clones concomitantly with a loss of other clones, including anti-host clones that are unable to produce their own IL-2 (1–3). Such anti–third-party CTLs were found to be markedly depleted of GVH reactivity upon transplantation into lethally irradiated mice (2) and exhibited strong veto activity in vitro (4) and in vivo (2). Thus, their potential role in tolerance induction, especially in the context of BM allografting under reduced-intensity conditioning (RIC), was demonstrated in mouse models (2). This ability of donor anti–third-party CTLs to kill cognate alloreactive host CTL precursors, when the donor anti–third-party CTLs are recognized by the naive host CTL precursors, is initiated upon immune-synapse formation through TCR recognition of the donor cells by the host naive T cells. Perhaps somewhat confusing is that the anti–third-party CTLs serving as target are capable of killing the recognizing naive T cells. Early studies using Fas and Fas ligand (FasL) knockout (KO) T cells in long-term MLR cultures indicated a role for Fas–FasL killing (4), whereas a more recent study using microscopy imaging indicated that short-term killing through a perforin-mediated mechanism can also occur (5). In both instances, the interaction of CD8 on the veto anti–third-party CTL with α3 domain of MHC on the recognizing host CTL precursor is critical for induction of the killing process.

Based on the insights from the mouse model, a new procedure for the generation of human anti–third-party CTLs was developed (3). When studied in a human–mouse chimeric model, these newly generated human anti–third-party CTLs did not negatively affect the engraftment of fully allogeneic normal B and T cells (6). Surprisingly, we found that such allogeneic or autologous anti–third-party CTLs exhibited potent killing of B cell chronic lymphocytic leukemia (B-CLL) (6) cells. Thus, these host nonreactive anti–third-party CTLs could potentially be used to facilitate engraftment of allogeneic hematopoietic stem cells, as well as provide GVL reactivity.
In the current study, we demonstrated that anti-third-party CTLs also exhibited marked reactivity against different types of B cell lymphoma while sparing normal naïve B cells; therefore, their use could be extended to treat B cell malignancies other than B-CLL. Furthermore, the availability of different B cell lymphoma lines enabled us to investigate the mechanism by which anti-third-party CTLs can induce apoptosis in lymphoma cells in the absence of TCR recognition.

MHC-I molecules are highly expressed on immune cells, and their major role is to present antigenic peptide fragments to CD8+ T cells (7) through their TCR and its coreceptor CD8 (8, 9). Our novel finding is in agreement with a growing body of evidence highlighting another role for MHC-I molecules in eliciting an intracellular signaling event upon their engagement. This event results in either positive or negative regulation of immune cell function and viability (10–15). Using different blocking Abs and mutated lymphoma cell lines, we showed a novel role for the MHC-I molecule in mediating induction of apoptosis of malignant B cells by anti-third-party CTLs. The initial step in the described killing process is a rapid conjugation formation mediated via an ICAM-1:LFA-1 interaction. Interestingly, this is followed by unique TCR-independent killing, which is dependent on the interaction of CD8 molecules on the CTL and MHC-I molecules on the affected lymphoma cells.

Materials and Methods

Cells

Whole blood was collected from lymphoma patients in the leukemic phase and from healthy volunteers. The lymphoma samples included stage IV follicular lymphoma (one case), lymphoplasmacytic lymphoma (two cases), and splenic lymphoma with villous lymphocytes (one case). The study was approved by the National Ethics Committee, Israel, and informed consent was obtained from patients and healthy donors.

PBMCs were obtained by Ficoll density-gradient centrifugation. HLA typing was performed by molecular methods for patient samples. Briefly, DNA was purified from PBMCs derived from EDTA-treated blood samples using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Hilden, Germany), and HLA-A and -B typing was performed by PCR using RELI SSO HLA-A and HLA-B Typing kits (Invitrogen, Carlsbad, CA). For healthy samples, HLA typing was performed by serology methods.

BL-44 (Burkitt’s lymphoma) and Granta519 (mantle cell lymphoma) lines were cultured in RPMI 1640 supplemented with 10% FCS and antibiotics. Daudi and β2-microglobulin (β2m)-reconstituted Daudi (B2m) Burkitt’s lymphoma cell lines were kindly provided by Dr. R.D. Salters (University of Pittsburgh School of Medicine, Pittsburgh, PA) (16). The H.My2 C1R HLA-A2 wild-type (wt) transfectant B cell lines were also kindly provided by Dr. R.D. Salters (17); the H.My2 C1R HLA-A2 K66A transfectant was kindly provided by Dr. B.M. Baker (University of Notre Dame, Notre Dame, IN) (18). Daudi and H.My2 cell lines were cultured in IMDM with 10% FCS and antibiotics.

Animals

Female 6–12-wk-old BALB/c, CB6, FVB, and C57BL/6 mice were obtained from Harlan Laboratories (Rehovot, Israel). Breeding pairs of Prf−/−, Tnfr2−/−, Tnf−/−, and Trail−/− KO mice of H-2b background and transgenic OT1/rag-2 KO mice of H-2b background and transgenic OT1/rag-2−/− mice of C57BL6 background expressing a TCR specific for OVA 257–264 aa in the context of I-EK on C58 T cells were obtained from The Jackson Laboratory (Bar Harbor, ME). NOD-SCID mice were bred and housed at the Weizmann Institute. All mice were kept in small cages (five animals in each cage) and fed sterile food and acid water.

Generation of nonalloreactive anti-third-party CTLs

As described previously (6), PBMCs were obtained by Ficoll density-gradient centrifugation of buffy coats from healthy donors (allogeneic) or from whole-blood samples of lymphoma patients (autologous). The PBMCs were then stimulated with an allogeneic EBV-transformed B cell line of an HLA background different from that of the host HLA. Cells were cultured at a 40:1 PBMC/stimulator ratio for 10 d. The cultured cells were then mounted on Ficoll to discard dead cells and were restimulated with EBV stimulators at a 4:1 ratio. Four days later, the subpopulation of CD8+ T cells was isolated by one cycle of negative selection with anti-CD56-coated magnetic beads (CD56 MACS; Miltenyi Biotec), followed by positive selection of CD8+ cells with anti-CD8–coated magnetic beads (CD8 MACS; Miltenyi Biotec). CD8+ T cells were then cultured in complete RPMI 1640 containing 300 IU/ml recombinant human IL-2 (EuroCentr, Amsterdam, The Netherlands) and EBV stimulators at a 1:4 ratio. Culture was replenished with IL-2, every 2–3 d, for 7 d post-initial IL-2 addition. Using FACS analysis, CD8 purity of CTLs was shown to be >95% CD8+ on day 14 and on the day of the experiment (day 21).

Mouse anti-third-party CTLs were prepared as previously described (2). Briefly, splenocytes of the donor mice were cultured against irradiated third-party splenocytes for 6 d under cytokine deprivation. Subsequently, cells were subjected to positive selection of CD8+ cells using Magnetic Particles (BD Pharmingen). The isolated cells were restimulated with irradiated third-party splenocytes, and recombinant human IL-2 (40 U/ml) was added every second day.

Flow cytometry

The following labeled anti-human Abs were used: CD19-PE (Miltenyi Biotec); CD20-FITC, CD56-PE, and CD8-allophycocyanin (Biosciences Pharmingen, San Diego, CA); and Pan γδ TCR-PE (Beckman Coulter, Krefeld, Germany). Indirect immunolabeling using anti–HLA-ABC (W6/32; Serotec, Oxford, U.K.) was performed using secondary PE-conjugated goat anti-mouse immunoglobulins (Jackson ImmunoResearch Laboratories, West Grove, PA). The following labeled anti-mouse Abs were used: CD8 (allophycocyanin/PE), CD-19 (allophycocyanin/FITC), and B-220 (PE) (BD Bioscience). For the in vitro killing assays, lymphoma cells were prelabeled with 0.15 μg/ml calcein AM (Molecular Probes, Eugene, OR). The MLR was followed by Annexin V labeling (Biosciences Pharmingen).

MLR-killing assay

Anti-third-party CTLs and lymphoma cells were obtained by Ficoll density-gradient centrifugation, after which the lymphoma cells were labeled with 0.15 μg/ml calcein AM, a vital dye that is released upon cell death, according to the manufacturer’s instructions, and brought to a concentration of 1 × 106 cells/ml in the appropriate media. Next, 2.5 × 105 calcein-labeled lymphoma cells were incubated with or without anti-third-party CTLs at the indicated ratio and time intervals in 24-well plates. No exogenous cytokines were added to the MLR. Cells were recovered and analyzed for survival using surface markers or by measuring the number of surviving calcein-stained lymphoma cells by FACS. To obtain absolute values of cells, samples were suspended in constant volume, and flow cytometric counts for each sample were obtained during a constant predetermined period of time and compared with flow cytometric counts obtained with a fixed volume and fixed numbers of input cells (19). Survival rates are presented relative to the survival of lymphoma cells alone.

Preparation of naïve normal B cells

Splenocytes of BALB/c mice were harvested, and single-cell suspensions were prepared. The cell suspensions were fractionated on Ficoll-Paque Plus (Amersham Biosciences, Upsalla, Sweden) to remove RBCs. The isolated mononuclear cells were then subjected to positive selection of B cells using magnetically labeled anti-B220 Abs using the IMAG magnetic separation system (BD Bioscience, San Diego, CA). Purity and efficiency of cleaning were tested by FACS analysis.

Inhibition of B cell lymphoma killing by blocking Abs

CTLS or lymphoma cells were preincubated for 30 min at a minimal volume with the indicated neutralizing Ab at the indicated concentrations. Blocked cells were then incubated with the other nonblocked component of the MLR for 36 h at a 1:5 ratio in favor of the anti-third-party CTLs. Cell survival was analyzed by FACS. The following neutralizing Abs were used: hNKG2D-blocking mAb (clone 149810; R&D Systems, Minneapolis, MN); LFA-1 (CD11a)-blocking Ab (clones TSL18.11.1.2 or 2F12), ICAM-1 (CD54)-blocking Ab (clone MC1615XZ), and HLA-ABC (W6/32)-blocking Ab (AbD (Serotec); ICAM-2 (CD102)-blocking Ab (clone CBR-IC2/2) and CD50 (ICAM-3) (clone CBR-IC3/1)-blocking Ab (Bender MedSystems, Vienna, Austria); and anti-CD8 (LT8)-blocking Ab (GeneTex, San Antonio, TX).

Conjugation assay

Efficiency of cell–cell binding was assayed by conjugate-formation analysis, as previously described (20). Briefly, following incubation with the indicated Ab, calcein AM (0.15 μg/ml)-prelabeled anti-third-party CTLs (1 × 106 cells/ml) were combined with equal numbers of dihydroethidium (DHE) (3 μg/ml; Sigma-Aldrich, Rehovot, Israel)-prelabeled target
B cells, pelleted for 2 min at 500 rpm, and further incubated for 5–10 min at 37°C. Following a 5-s vortex, the percentage of conjugates was determined by FACS.

Detection of apoptosis by Annexin V staining

Samples from in vitro cultures were incubated with a mixture of selected mAbs labeled with different fluorochromes for 20 min at 4°C. After washing off the unbound free Ab, samples were incubated with 5 μl Annexin V-FITC or Annexin V-allophycocyanin (Biosciences Pharmingen) for 10 min at room temperature. Subsequently, unbound Annexin V was washed out, and samples were analyzed by FACS (21).

In vivo analysis of anti–third-party CTL-mediated killing

Target lymphoma cells (20 x 10^6/mice) were labeled with 5 μM CFSE and injected i.p. into NOD-SCID mice in the presence or absence of a 4-fold excess of anti–third-party CTLs. Peritoneal exudates were collected 5 d postinjection, and the numbers of CFSE^+ cells per volume were determined by FACS. Labeling with anti-human CD8-allophycocyanin Ab (Biosciences Pharmingen) was performed to assay the presence of the anti–third-party CTLs in the peritoneal fluids of the coinjected mice.

Statistical analysis

Statistical significance was established using the Student t test.

Results

Allogeneic and autologous anti–third-party CTLs induce apoptotic death of B cell non-Hodgkin’s lymphoma cells

To determine whether anti–third-party CTLs are endowed with antilymphoma cell reactivity, we initially tested their ability to kill the BL-44 and Granta519 cell lines, both of which represent highly aggressive malignancies (22, 23). As can be seen in Fig. 1A, marked killing of 73.5 ± 3% of lymphoma cells was found following incubation with a 5-fold excess of anti–third-party CTLs. The killing kinetics of lymphoma cells by anti–third-party CTLs was much slower than was the killing of cognate third-party stimulators by the same CTLs. Thus, when tested 24 h after the initiation of culture, 41.1 ± 1.3% of noncognate lymphoma cells were killed compared with 92 ± 0.7% of cognate target cells (Fig. 1A).

The killing of lymphoma cells by anti–third-party CTLs was dose dependent. As can be seen in Fig. 1B, 58% of lymphoma cells were viable after incubation at a 1:1 lymphoma cell/CTL ratio, whereas only 23% of lymphoma cells were viable after incubation with a 10-fold excess of anti–third-party CTLs. Likewise, the level of Annexin V^+ lymphoma cells increased from 42 to 77% upon escalation of the lymphoma cell/CTL ratio from 1:1 to 1:10 (Fig. 1B). When the broad-range caspase inhibitor ZVAD-fmk was added to the cell culture, killing was blocked (Fig. 1B). These data are consistent with our previous results on B-CLL eradication (6) and indicated that killing of lymphoma cells by anti–third-party CTLs is mediated by apoptosis.

We next examined the ability of the anti–third-party CTLs to kill primary tumor cells obtained from peripheral blood of stage IV lymphoma patients. We used four patients with HLA-A and -B types that were not cross-reactive with the stimulator cells used for the generation of anti–third-party CTLs. As can be seen in Fig. 1C, following incubation of the primary lymphoma cells with

FIGURE 1. Anti–third-party CTLs are endowed with reactivity against lymphoma cell lines and primary PBLs from lymphoma patients. A, Calcein AM-prelabeled lymphoma cells (BL-44 or Granta519 cell lines) or calcein AM-prelabeled cognate target cells (third-party B cell stimulators) were incubated for the indicated time intervals with or without 5-fold excess of anti–third-party CTLs. Numbers of viable calcein AM^−Annexin V^− cells were determined by FACS, as demonstrated in the representative scatograms (right panels). Data are shown as the percentage of killing compared with target cells incubated in the absence of CTLs (0%). Data shown are mean ± SD of at least three independent experiments, each in triplicate. *p < 0.01, changes in the number of live cells versus no CTL and versus target-restricted CTLs. B, BL-44 or Granta519 lymphoma cell line cells were incubated for 36 h with or without anti–third-party CTLs at the indicated ratios in the absence or presence of ZVAD-fmk and examined by FACS for viability and Annexin V labeling (as described in Materials and Methods). A representative experiment with BL-44 is shown. C, Killing of primary tumor cells obtained from PBLs of lymphoma patients or lymphoma cell lines following 36 h of incubation in the presence or absence of allogeneic anti–third-party CTLs. Data represent mean ± SD of five independent experiments, performed in triplicates (n = 4 patients). *p < 0.01, changes in the number of live cells versus values obtained in the absence of CTLs. D, Killing of primary lymphoma cells following 36 h of incubation in the presence or absence of autologous (Auto) or allogeneic (Allo) anti–third-party CTLs. Data represent mean ± SD of five independent experiments, performed in triplicates (n = 4 patients). *p < 0.01, decrease in the number of live cells versus values obtained in the absence of CTLs.
allogeneic anti–third-party CTLs for 36 h, primary lymphoma cells were killed with a similar efficiency to that found for the lymphoma cell lines described above.

To test whether the killing of lymphoma cells is mediated by residual alloreactivity, we generated anti–third-party CTLs from the patients’ own T cells. As can be seen in Fig. 1D, a similar level of killing of primary lymphoma cells was induced by autologous or allogeneic anti–third-party CTLs. Considering that autologous T cells are markedly depleted of self-reactive clones, the similar killing of lymphoma cells by autologous and allogeneic anti–third-party CTLs strongly indicated that this form of killing cannot be attributed to contamination of the anti–third-party CTLs with residual alloreactive clones.

The killing of lymphoma cells by anti–third-party CTLs is TCR independent and does not use other killing mechanisms commonly used by CTLs

Although the similarity in lymphoma killing exhibited by autologous and allogeneic anti–third-party CTLs ruled out the potential involvement of residual alloreactive clones, it also suggested that the TCR of these CTLs directed against a third party likely does not mediate this killing. The role of the TCR in this killing was more definitively addressed by using mutated B cell lymphoblastoid target cells previously shown to express an MHC-I mutant not recognizable by the TCR. In this experiment, we made use of the previous finding that the K66A mutation in the α1 helix of the HLA-A2 allele, in which the lysine residue is replaced by alanine, disrupts recognition by the TCR (24). These findings were consistent with an earlier study showing that this mutation disrupts recognition of HLA-A2 by a series of alloreactive CTLs (25).

Thus, we compared the ability of anti–third-party CTLs to kill the B cell lymphoblastoid cell line H.My C1R, expressing a wt HLA-A2, with the killing of the B cell lymphoblastoid H.My C1R cell line expressing mutated HLA-A2 (K66A) by anti–third-party CTLs. As shown in Fig. 2A, this comparison did not reveal a significant difference in the cytotoxic efficacy. Thus, the killing by anti–third-party CTLs is not disrupted by a mutation in MHC-I, which is known to block TCR recognition of peptide–MHC-I complexes.

Interestingly, other mechanisms generally used by CTLs were also excluded. The potential induction of apoptosis by death molecules, such as TNF, FasL, or TRAIL, was ruled out by using specific blocking Abs that did not affect the killing (data not shown). To test the efficacy of anti-FasL Ab, a positive control study was performed using supernatant containing soluble FasL. Although the soluble FasL induced apoptosis in BL-44 and Jurkat cells, apoptosis was prevented by the addition of the blocking Ab (data not shown).

However, to further define the possible involvement of such death molecules that were previously found to play a role in generating the veto activity by anti–third-party CTLs, we carried out mouse experiments using different KO mice. To that end, we used a mouse model based on the A20 B cell lymphoma/leukemia line (26) spontaneously derived from BALB/c (H-2b) mice. Initially, we tested the ability of mouse anti–third-party CTLs to kill A20 cells. As can be seen in Fig. 2B, allogeneic anti–third-party CTLs exhibited dose-dependent killing of A20 target cells. Similarly to the human setting, this killing is not mediated by alloreactivity, because anti–third-party CTLs derived from F1 mice (CB6 H2bKb), which are not alloreactive against BALB/c alloreactivity, because anti–third-party CTLs derived from F1 similarly to the human setting, this killing is not mediated by anti–third-party CTLs exhibited dose-dependent killing of A20 target cells. For example, in Supplemental Fig. 1A, anti–third-party CTLs from perforin KO mice (Fig. 2D). Furthermore, in addition to the experiments described above indicating that the killing of B cell lymphoma by human anti–third-party CTLs is TCR independent, the mouse model enabled us to directly examine this important question. Thus, CTLs generated from OT-1–RAG−/− transgenic mice, in which the CD8+ T cells express only a TCR specific for the SIINFEKL peptide of OVA in the context of H2-Kb (not expressed by the A20 lymphoma cells), exhibited similar killing to that found when using nonalloreactive F1-derived (BALB × B6) anti–third-party CTLs (Fig. 2E).

Thus, again, as for human anti–third-party CTLs, the killing of lymphoma cells is TCR independent.

In addition, a potential involvement of γδ T cells in the observed killing is highly unlikely, considering that γδ T cells are markedly depleted in the process of anti–third-party CTL generation and could barely be detected at the end of the culture (Supplemental Fig. 1B). Furthermore, we also showed, by using blocking Abs, that this killing does not involve the NKG2D ligand (Supplemental Fig. 1C).

Mouse anti–third-party CTLs spare normal naive B cells

We previously suggested that human anti–third-party CTLs are unable to affect the engraftment of normal B cells, because the human IgG secretion by normal B cells was not significantly affected upon infusion of anti–third-party CTLs into a human/mouse chimera (6). Yet, it could be argued that this indirect evidence based on measuring Abs does not rule out possible elimination of different types of normal B cells. Although it is difficult to maintain human B cells through the time required for HLA typing (needed in our routine assay to avoid cross-reactivity) in the absence of antigenic stimulation (27), this problem can be circumvented by using well-defined mouse strains. Thus, we attempted to directly assess in vitro the reactivity of mouse-derived anti–third-party CTLs against normal naive B cells. Interestingly, in contrast to A20 lymphoma cell eradication, the anti–third-party CTLs did not affect naive B cells (Fig. 2F).

LFA-1– and ICAM-1–mediated interactions are required for lymphoma cell killing by anti–third-party CTLs

Initial experiments using Transwell filters suggested that the killing of lymphoma cells is contact dependent (data not shown). As can be seen in Supplemental Video 1, live-imaging experiments revealed that anti–third-party CTLs bind to target lymphoma cells and rapidly form conjugates (on a time scale between seconds and a few minutes). These conjugates remained stable and long lasting during the follow-up period of 1 h. The conjugates were also monitored by FACS in the presence of blocking Abs. Preincubation of the lymphoma cells with anti–ICAM-1 or preincubation of the CTLs with anti-LFA-1 reduced CTL–lymphoma conjugate formation by two thirds (Fig. 3A). In contrast, neutralization of the interactions of TCR (CD3), CD8, MHC-I, CD102 (ICAM-2), or CD50 (ICAM-3), using blocking Abs, did not affect the percentage of cells in lymphoma–CTL conjugates (Fig. 3A). Importantly, LFA-1 neutralization on CTLs, as well as ICAM-1, but not ICAM-3 (CD50) blocking on lymphoma cell lines, prevented the killing of lymphoma cells following coculture with anti–third-party CTLs (Fig. 3B). Thus, in the absence of TCR recognition, the LFA-1:ICAM-1 interaction is
**FIGURE 2.** The killing of tumor B cell lines by anti–third-party CTLs is TCR independent and does not use typical death molecules involved in alloreactivity. A, H.My2 C1R HLA-A2 wt transfectant and H.My2 C1R HLA-A2 K66A mutant transfectant cell lines were incubated for 48 h with or without anti–third-party CTLs at a 1:5 ratio in favor of anti–third-party CTLs. The killing of the cells was analyzed by FACS, as described in Materials and Methods. The percentage of killing was calculated as described in Materials and Methods (mean ± SD of four independent experiments in triplicates). B, The killing of lymphoma cells in the murine setting is dose dependent and not mediated by alloreactivity; calcein AM+Annexin V + expression (mean ± SD of at least three independent experiments, each in triplicate). C, The killing of murine lymphoma cells by anti–third-party CTLs is mediated by apoptosis. Calcein AM-prelabeled murine A20 lymphoma cells were incubated for 16 h with or without a 5-fold excess of allogeneic (C57BL/6) or F1 (CB6-H2b)-derived anti–third-party CTLs for 16 h in the indicated CTL/lymphoma cell ratios. Numbers of viable cells were determined by flow cytometry (mean ± SD of three independent experiments, each in triplicate). D, The killing of murine lymphoma cells by anti–third-party CTLs is mediated by apoptosis. Calcein AM-prelabeled murine A20 lymphoma cells were incubated for 16 h with or without a 5-fold excess of allogeneic (C57BL/6), F1 (CB6), or syngeneic (BALB/c) derived anti–third-party CTLs and were then examined using flow cytometry for excess of allogeneic (C57BL/6), F1 (CB6), or syngeneic (BALB/c) derived anti–third-party CTLs and were then examined using flow cytometry for viability of the cells. E, Anti–third-party CTLs kill lymphoma cells but spare normal naive B cells. Naive B cells harvested from BALB/c mice by magnetic beads or A20 cells were incubated for 16 h with syngeneic anti–third-party CTLs in vivo, we used adoptive-transfer experiments in NOD-SCID mice. As can be seen in Fig. 5, following transplantation into recipient mice, CTLs efficiently killed B2m cells. Thus, although only 10.83% of the B2m cells survived in the presence of a 4-fold excess of anti–third-party CTLs, Daudi cells lacking functional HLA-I were resistant to killing by the same CTL lines, and 86.87% of the Daudi cells survived under the same conditions.

**MHC-I expression is essential for lymphoma cell eradication by anti–third-party CTLs**

Given the importance of the CD8–MHC-I interaction demonstrated in mice (4, 28, 29), we sought to evaluate whether a role for this interaction also exists in the killing of lymphoma cells by human anti–third-party CTLs. Masking of 2a/3 domains of MHC-I on target B cells by neutralizing Ab significantly impaired the anti–third-party CTL-mediated killing. Thus, killing of target lymphoma cells was decreased from 50 ± 4.9% to 18 ± 1.0% when anti HLA-ABC Ab was added to the incubation with anti–third-party CTLs (Fig. 4A, HLA-ABC). A similar decrease in killing was observed upon the addition of anti-CD8 Ab, suggesting that CD8 binding to the MHC-I C region is required for lymphoma cell killing. Inhibition of CD8 binding to the MHC-I C region similarly abrogated the killing of primary PBLs obtained from lymphoma patients (Fig. 4B). Blocking of CD8 molecules using blocking Abs inhibited the killing in the mouse setting as well (Supplemental Fig. 2).

Finally, to further evaluate the importance of MHC-I expression for the eradication of lymphoma target cells by anti–third-party CTLs in vivo, we used adoptive-transfer experiments in NOD-SCID mice. As can be seen in Fig. 5, following transplantation into recipient mice, CTLs efficiently killed B2m cells. Thus, although only 10.83% of the B2m cells survived in the presence of a 4-fold excess of anti–third-party CTLs, Daudi cells lacking functional HLA-I were resistant to killing by the same CTL lines, and 86.87% of the Daudi cells survived under the same conditions.

**TCR-INDEPENDENT KILLING OF LYMPHOMA CELLS BY CTLs**

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Collectively, these data demonstrated that MHC-I expression is required for lymphoma cell eradication by anti–third-party CTLs.

**Discussion**

We recently demonstrated that autologous or allogeneic anti–third-party CTLs can be used to eradicate pathological cells from B-CLL patients (6). In the current study, we demonstrated that anti–third-party CTLs also exhibited significant killing of B cell non-Hodgkin’s lymphoma (B-NHL) cells. By using blocking Abs and mutated cell lines, we elucidated the initial molecular events that trigger this cytotoxic activity. Our data strongly suggested that unlike the classical killing of cognate targets by CTLs, the described killing of B cell malignant cells by anti–third-party CTLs is TCR independent.

This intriguing attribute of anti–third-party CTLs is supported by several findings. First, the lymphoma cells are killed efficiently both by allogeneic and autologous anti–third-party CTLs. Second, TCR-independent killing is directly demonstrated by using malignant cells (H.My C1R) expressing mutated HLA-A2 at a position critical for TCR recognition (position K66) (24). Although this mutation disrupts recognition of peptide–MHC-I complexes by the TCR, these HLA-A2–mutated cells were depleted at a similar rate to that of H.My C1R cells expressing wt HLA-A2, when cocultured with anti–third-party CTLs. Finally, the most definitive demonstration that the mechanism is TCR independent is afforded by the mouse studies in which anti–third-party CTLs derived from OT1/Rag$^{-/-}$ mice exhibited comparable killing of A20 lymphoma cells to that found for anti–third-party CTLs of (host × donor)F1 origin. Thus, CTLs expressing a transgenic TCR directed against an irrelevant Ag still demonstrated significant killing of lymphoma cells.

In previous studies investigating the veto mechanism and the killing of CTL precursors by anti–third-party CTLs, both FasL–Fas interaction (4) and perforin (5) secretion were shown to play a role. Interestingly, in contrast to these veto mechanisms, the killing modality of malignant B cells is mediated through a different mechanism. Granule-independent killing is indicated in the human setting by testing for BLT esterase and further supported in the mouse model by using anti–third-party CTLs from perforin KO mice. FasL-independent killing is shown in human CTLs by using blocking Abs and in the mouse model by demonstrating that CTLs from FasL-mutated gld mice do not exhibit significantly reduced killing of A20 lymphoma cells.
The number of live cells was determined as in Fig. 1. A, BL-44 or Granta519 line cells were incubated for 36 h with or without a 5-fold excess of anti–third-party CTLs in the presence or absence of 10–20 μg/ml anti MHC-I (HLA-ABC) or anti-CD8 Ab. Data are shown as mean ± SD of 10 independent experiments in triplicates. *p < 0.05, **p < 0.01, changes in the killing percentage compared with samples cultured in the absence of Ab. B, Primary lymphoma cells were incubated as in A, with or without anti–third-party CTLs from the same healthy donors in the presence or absence of anti–MHC-I Ab in two regimens; in the 20 μg × 2, the second dose was added 18 h after the initiation of culture. Data are from two individual experiments using cells from different patients. *p < 0.01, changes in the average killing percentage of cells in the presence of anti–MHC-I versus values obtained in the absence of Ab. C, Daudi, BL-44, or B2m cells were incubated for 36 h in the absence or presence of anti–third-party CTLs at the indicated ratios. The number of live cells was determined as in Fig. 1 (mean ± SD of four to six independent experiments, each in triplicates). *p < 0.01, differences, at each time point, in the killing percentage of Daudi cells versus B2m cells. D, Conjugate-formation capacity of Daudi and B2m cells with anti–third-party CTLs was assayed as in Fig. 3A. Data are shown as mean ± SD (n = 5 CTL lines).

Interestingly, the involvement of other killing mechanisms generally used by CTLs in inducing apoptosis, such as TNF, TRAIL, or NKG2D, were ruled out indirectly in the human setting by using blocking Abs and more directly in the mouse studies by using CTLs derived from TNF or Trail KO mice or by neutralizing Ab for NKG2D. Furthermore, we showed that the level of γδ T cell depletion in our CTL preparation ruled out the potential involvement of these cells.

Indeed, our results suggested that anti–third-party CTLs induce apoptosis in B cell lymphoma through a novel mechanism. Initially, using Transwell filters, we found that the killing of lymphoma cells by anti–third-party CTLs is contact mediated. Moreover, evaluation of conjugate formation by FACS, as well as by live video microscopy, revealed that these interactions resulted in rapid conjugate formation. Ab blockade showed that conjugates are largely mediated by nonspecific adhesion molecules of the integrin family, namely, LFA-1 molecules on the anti–third-party CTLs and ICAM-1 molecules on the lymphoma cells. Our results are consistent with earlier studies indicating that adhesion ring junctions, composed of high surface densities of ICAM-1 and LFA-1 or peripheral supramolecular activation complexes, can be formed between CD8 T cells and target cells in the absence of TCR engagement (30, 31). ICAM-1 is the most potent ligand of LFA-1 in cell-adhesion assays (32), and activation of lymphocytes leads to an increase in ICAM-1 expression (33). However, despite the fact that ICAM-1–LFA-1 interactions are essential for long-lasting (~1 h) conjugate formation between CTLs and lymphoma cells, these interactions alone are not sufficient to induce caspase-mediated apoptosis of lymphoma cells. Thus, further engagement of MHC-I on the lymphoma cells by the CD8 molecules on the CTLs is required to achieve killing following the initial binding. This was first demonstrated using blocking Abs directed against the α3/α2 domain of MHC-I or against CD8, both of which inhibited the killing, but not the formation, of cell conjugates.

However, more definitive evidence for the central role of MHC-I was provided by comparing Daudi cells lacking MHC-I surface expression and a Daudi transfectant re-expressing MHC-I. In the absence of TCR-mediated signaling, the engagement of the MHC-I C region by the CTL CD8 molecules led to the initiation of apoptosis. However, considering that ICAM-1 is expressed on many cell types, it is unlikely that it could afford, together with HLA expression, sufficient conditions for apoptosis induction. Thus, although we showed that, in the absence of these interactions apoptosis will not ensue, other internal-signaling pathways, especially those regulating apoptosis inhibitors, probably underlie the
predisposition of a B cell tumor to induction of apoptosis upon engagement of its MHC with the CD8 on the CTL.

MHC-I clustering was previously implicated in apoptosis induction in several cell types (10, 34). Several early studies suggested that engagement of the α3 domain of the MHC-I by Abs induces apoptosis in resting or in activated B cells (35, 36). In addition, Pedersen et al. (12) observed that cross-linking of MHC-I molecules on B cell lymphoma cells led to mobilization of intracellular Ca²⁺ ions. Hence, it was suggested that apoptosis induction via MHC-I could be mediated by protein tyrosine kinases.

Similarly, Yang et al. (37, 38) discovered that recruiting the MHC-I molecules to the lipid rafts by specific β2M mAbs induced apoptosis of hematological malignant cells while sparing normal cells. This apoptosis was shown to be mediated via activation of JNK signaling. Thus, further studies are warranted to evaluate the possibility that the CTL CD8 molecules induce clustering of MHC-I molecules on the malignant cell, resulting in their recruitment into lipid rafts. Likewise, the signal-transduction pathway that follows the MHC-I engagement by the CTLs requires further elucidation. Such signaling might not be mediated directly through the cytoplasmic tail of MHC-I, because it was shown that this tail lacks phosphorylation sites (14, 15, 39). Furthermore, it was demonstrated that Ab-induced cross-linking of MHC-I on Jurkat cells expressing a truncated MHC-I, lacking the cytoplasmic tail, evoked the same increase in the intracellular Ca²⁺ ions concentration as did that in Jurkat cells expressing native MHC-I molecules (14).

Collectively, our data suggested the following sequence of events for apoptosis induction in B cell lymphoma by anti-third-party CTLs (Fig. 6). First, nonspecific, yet long-lasting, adhesive interactions are formed between anti-third-party CTLs and target cells. These interactions are mediated by LFA-1 molecules on the CTLs and ICAM-1 molecules on the lymphoma cells. Next, CD8 molecules on the CTLs engage the α3 domain of the MHC-I on the lymphoma cells. Upon MHC-I–CD8 engagement, a signal transduction is initiated, which is likely the result of recruitment of signal molecules adjacent to the intracellular tail of the MHC-I molecule and not the tail itself (14, 15, 34). These, as-yet-undefined signals lead to cell death by apoptosis.

In addition to the novel role of MHC–CD8 interaction in the mechanism of action underlying the killing by anti-third-party CTLs, our results extend the potential therapeutic application of CTLs, previously shown to eradicate B-CLL (6), to the treatment of other forms of B-NHL. In particular, the use of these cells is attractive in the context of hematopoietic stem cell transplantation. The use of hematopoietic stem cell transplantation for tolerance induction as a prelude to cell therapy with donor T cells is being investigated by several groups, and major efforts are currently directed at reducing transplant-related mortality by eliminating the risk for GVH disease and by reducing the toxicity of the conditioning protocol. T cell-depleted BM transplants were shown to adequately eliminate GVH disease. However, even in leukemia patients undergoing supralethal conditioning, mismatched T cell-depleted BM transplants are vigorously rejected. This barrier can be overcome through the modulatory activity of CD34+ cells, which are endowed with veto activity, by the use of megadose stem cell transplants (40, 41). Nevertheless, the number of human CD34 cells that can be harvested is not likely to be sufficient to overcome rejection under RIC. To address this challenge, the use of anti-third-party veto CTLs was initially proposed to provide additional veto activity and to enhance engraftment of purified CD34+ stem cells in patients treated with RIC (2). We previously suggested, based on indirect evidence, that these anti-third-party CTLs might not negatively affect normal allogeneic B and T cells (5). Sparing of normal B cells is now directly demonstrated by showing that anti-third-party CTLs do not adversely affect naive B cells in vitro, whereas they kill malignant B-NHL cells both in vitro and in vivo. This selective killing is of great clinical significance, because it will spare the naive B cell population and will not delay humoral immune reconstitution against new Ags (42, 43). Thus, anti-third-party CTLs could be highly attractive for use in B-NHL patients, because, in addition to their prominent tolerizing activity demonstrated in mouse models, they might further improve the clinical outcome by virtue of their GVL reactivity.

Alternatively, our finding that autologous anti-third-party CTLs are as effective as their allogeneic counterparts offers another option for cell therapy in the context of autologous transplantation. Although autologous transplants have lower transplant-related mortality (44), allogeneic transplants exhibit increased overall survival due to the enhanced GVL reactivity mediated by adoptively transferred, as well as newly formed, T cells (45–47). Further studies to evaluate whether the autologous anti-third-party CTLs could effectively eradicate residual disease and, thereby, enhance overall survival compared with allogeneic anti-third-party CTLs in the context of “megadose” purified CD34 cell transplants is warranted.

Disclosures

The authors have no financial conflicts of interest.

References