Brief Definitive Report

Induction of an Antigen-specific, CD1-restricted Cytotoxic T Lymphocyte Response In vivo

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Summary

The majority of T cell responses are restricted to peptide antigens bound by polymorphic major histocompatibility complex (MHC) molecules. However, peptide antigens can be presented to T cells by murine non-MHC–encoded CD1d (mCD1) molecules, and human T cell lines specific for nonpeptide antigens presented on CD1 isoforms have been identified. It is shown here that antigen-specific, mCD1-restricted lymphocytes can be generated in vivo by immunizing mice with a combination of plasmids encoding chicken ovalbumin, murine CD1d, and costimulatory molecules. Splenocytes from immunized mice have CD1d-restricted, MHC-unrestricted, ovalbumin-specific cytolytic activity that can be inhibited by anti-CD1 antibodies as well as a competing CD1-binding peptide. These results suggest a physiologic role for murine CD1d to present exogenous protein antigens.

Classically, MHC class I or class II molecules are responsible for presenting peptide antigens to T cells. However, in both humans and mice, the non-MHC–encoded CD1 family of cell surface proteins has been implicated to also have an antigen-presenting function (1, 2). Although MHC class I molecules mediate recognition of nonself or infected tissues by the immune system, the function of CD1 molecules is still unclear. Unlike the MHC proteins, CD1 molecules are nonpolymorphic and have five isoforms: CD1a, -b, -c, -d, and -e (3). The isoforms are conserved in several mammalian species (4) and have been divided into two groups based on the sequences of their external domains (5). CD1a, -b, -c, and -e comprise group 1, while group 2 contains CD1d. Although all five isoforms are found in humans, only the group 2 isoforms are conserved from rodents to humans.

CD1 molecules share some characteristics with both MHC class I and MHC class II ligands. CD1 proteins bear some resemblance to the classical MHC class I proteins both in overall sequence homology, especially in the $\alpha3$ domain, and by their usual association with β_2-microglobulin ($\beta2m$; references 5 and 6). However, unlike MHC class I molecules, CD1 proteins have been reported to be expressed without $\beta2m$ (7) and do not require the transporter proteins associated with antigen processing (TAP) for stable expression (8–10). The mechanism for antigen processing for CD1 is more similar to that of MHC class II than class I (11–13). Like MHC class II, human CD1b is localized to endocytic compartments, including the specialized endosomes where MHC class II proteins are believed to bind endocytosed antigens (14–17). The non-MHC–encoded CD1 family of nonpolymorphic glycoproteins is, therefore, similar to, yet distinct from, other antigen-presenting molecules in its similarity to MHC class I by sequence, structural homology, and association with $\beta2m$, as well as its similarity to MHC class II by its cellular localization and dependence on the endosomal compartment for presentation of exogenous antigens.

Unlike classical MHC, CD1 can present nonpeptide ligands such as mycolic acid (18), lipoarabinomannan (19), and mycobacterial lipid antigens (20) to $\alpha\beta$ T cell receptor–bearing lymphocytes. The presentation of foreign nonpeptide antigens by CD1 has been demonstrated for the human CD1b and CD1c isoforms from which human CD1d and its related murine isoforms are divergent (5). Castaño et al. (2) have reported that murine non-MHC–encoded CD1d (mCD1) can bind long peptides with hydrophobic and bulky amino acids. Immunization of mice with CD1-transfected cells preincubated with peptide generated, CD1-restricted, peptide-specific CTL. These data suggest that mCD1 may have a antigen-presenting function by binding peptides with hydrophobic residues (2).

Murine autoreactive, CD1-restricted T cells have been identified in unimmunized mice (21, 22). To test the biological significance of mCD1 presentation of foreign protein antigens, we generated an antigen-specific, CD1-restricted response by plasmid DNA immunization. This immunization protocol raised a CD1-restricted, ovalbumin-specific CTL response, demonstrating that protein antigen is recognized in the context of mCD1 and elicits a

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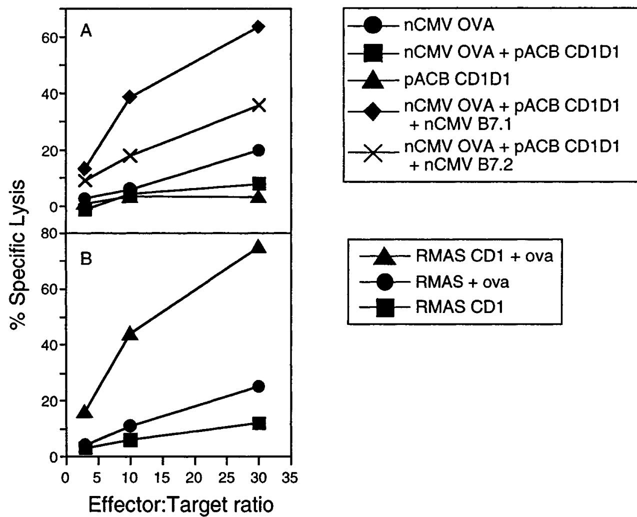

Figure 1. Recognition of ovalbumin by CD1d1-restricted CTL. Three mice per group were immunized with 50 μg of each plasmid at days 0 and 7. 2 wk after the first injection, mice were killed and restimulated in vitro with CD1d1-transfected EL4 cells pulsed with ovalbumin protein for 5 d in the presence of 50 IU rIL-2. The specificity of the resulting pooled CTL was determined by measuring the lysis of CD1d1-transfected RMAS cells incubated with ovalbumin in triplicate. (*A*) Immunizing mice with a combination of plasmids enhances the CD1-restricted CTL response. Mice were immunized with plasmids encoding ovalbumin, mCD1d1, and B7.1 (*diamonds*); ovalbumin, mCD1d1, and B7.2 (*crosses*); ovalbumin (*circles*); ovalbumin and mCD1d1 (*squares*); or mCD1d1 (*triangles*). The values plotted are average protein-specific lysis − background lysis. (*B*) CTL are specific for CD1d1 and ovalbumin. The splenocytes from the three mice injected with plasmids encoding ovalbumin, mCD1d1, and B7.1 were tested for their ability to lyse CD1-transfected RMAS cells pulsed with ovalbumin (*triangles*), CD1-transfected RMAS (*squares*), or RMAS cells pulsed with ovalbumin (*circles*) in triplicate. The values plotted represent the average lysis for three mice. These experiments were repeated twice.

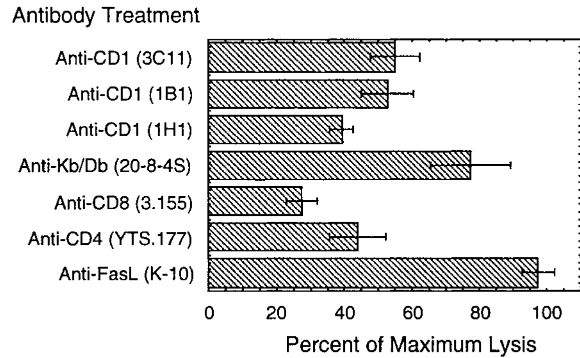

Figure 2. Antibody inhibition of CD1-restricted CTL lysis. Four C57BL/6 mice were immunized with plasmids encoding OVA, mCD1d1, and B7.1 as in Fig. 1. Spleens were harvested after 2 wk and restimulated in vitro for 5 d with CD1d1 transfected RMAS cells pulsed with ovalbumin protein for 5 d with rIL-2. The splenocytes were pooled and assayed for CTL activity in duplicate. The percentage of maximum lysis relative to the value in the presence of targets and antigen alone ± SEM is shown. This experiment was repeated five times.

cellular immune response in vivo. Lysis by these cytotoxic lymphocytes are antigen and CD1 dependent, can be partially abrogated by anti-CD1 antibodies, and are competitively inhibited by an established CD1-binding peptide. Furthermore, these CTLs lyse allogeneic targets in an antigen-specific manner.

Materials and Methods

Mice. C57BL/6 mice were purchased from The Jackson Laboratories (Bar Harbor, ME) and maintained under standard conditions in the University of California, San Diego Animal Facility accredited by the American Association of Laboratory Care. Mice of either sex were used at 2–4 mo of age.

Preparation of Plasmid DNA. The plasmid pACB-CD1 was constructed by subcloning the BamHI–XhoI fragment from the pBluescript vector encoding murine CD1D1 (reference 6; provided by S. Balk, Beth Israel Hospital, Boston, MA) into the BamHI–SalI site of the pACB vector (23). The nCMVOVA, nCMVB7-1, and nCMVB7-2 plasmids have been previously described (24). DNA was prepared using maxiprep kits (Qiagen Inc., Chatsworth, CA), with the modification of adding 0.1 vol 10% Triton X-114 (Sigma Chemical Co., St. Louis, MO) to the clarified bacterial lysate before applying it to the column in the kit. Before injection, the residual endotoxin level was quantified using a limulus extract clot assay (Associates of Cape Cod, Woods Hole, MA). Plasmid DNA with a level of <5 ng endotoxin/mg DNA was resuspended in sterile isotonic saline solution before injection.

Immunizations. Mice were immunized once a week for 2 wk. Plasmids were injected intradermally in the tail base with 50 μg of each plasmid indicated in 50 μl normal saline using a 25-gauge needle.

Cytotoxic T Lymphocyte Assay. Mice were killed and their spleens were removed and teased apart in RPMI 1640 media (BioWhittaker Inc., Walkersville, MD) supplemented with 2% fetal bovine serum (FBS). In 24-well plates (Costar Corp., Cambridge, MA) 7×10^6 responders were incubated with 1×10^6 mitomycin C–treated stimulator cells in the presence of 50 IU/ml recombinant human IL-2. The culture media was RPMI 1640 supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 50 μM 2β-mercaptoethanol, and 1% penicillin and streptomycin.

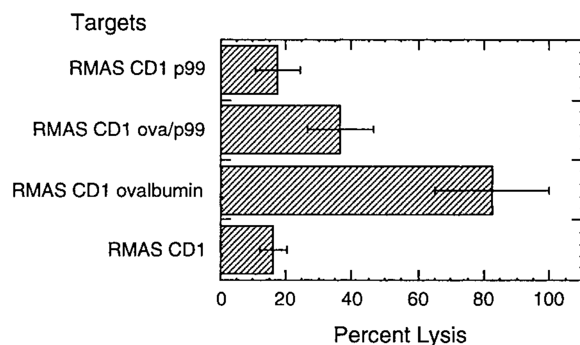

Figure 3. Peptide inhibition of ovalbumin-specific, CD1-restricted CTL lysis. T cell lines from mice injected with plasmids encoding OVA, mCD1d1, and B7.1 were assayed for cytolytic activity. $CD1^+$ RMAS targets incubated with effector cells in triplicate wells were either unpulsed, pulsed for 2 h with 4 μg/ml of p99, pulsed with 200 μg/ml of ovalbumin protein for 1 h, washed, and then pulsed with 12 μg/ml of p99 for 1 h, or pulsed with ovalbumin protein only. The percentage of lysis ± SEM is shown at an E/T ratio of 30:1. This experiment was repeated twice.

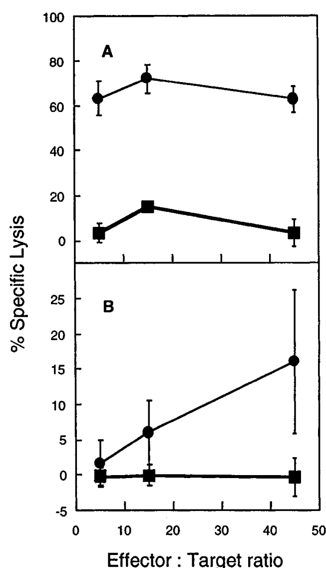

Figure 4. Ovalbumin-specific, CD1-restricted CTL lysis of allogeneic targets. 10 C57BL/6 mice (H-2^b) were immunized with plasmids encoding OVA, mCD1d1, and B7.1 (*circles*), and four mice were immunized with mCD1d1 and B7.1 (*squares*) on days 0 and 10. 2 wk after the first injection, mice were killed and restimulated in vitro with CD1d1-transfected RMAS cells pulsed with ovalbumin protein for 5 d in the presence of 50 IU/ml rIL-2. $CD1^+$ RMAS or $CD1^+$ P815 targets were incubated in the presence or absence of ovalbumin protein (100 μg/ml) for 1 h and washed three times before being plated with effector cells in triplicate wells. The specific lysis was determined by measuring the lysis of $CD1^+$ RMAS cells pulsed with ovalbumin minus unpulsed transfectants (*A*) or $CD1^+$ P815 cells pulsed with ovalbumin minus unpulsed transfectants (*B*). The values plotted represent the average lysis ± SEM. This experiment was repeated twice.

The stimulator cells, EL4-CD1 or RMAS-CD1, were stably transfected with a pCDNA3 vector expressing murine CD1d1 (pCDNA3 CD1D1). These transfectants were incubated with 40–200 μg/ml ovalbumin protein (Sigma Chemical Co.) for 1 h at 37°C and 5% CO_2 and then treated with mitomycin C (Sigma Chemical Co.) 50 μg/ml shaking for 30 min at 37°C. After 5 d, the restimulated cells were harvested and separated from dead cells on a lympholyte M gradient (Accurate Chemical and Scientific Corp., Westbury, NY). In 96-well, round-bottom plates, target cells were incubated in 200-μl volumes with restimulated T cells at titrated E/T ratios for 4 h. The assay medium used was phenol red–free RPMI 1640 supplemented with 2% BSA, 2 mM glutamine, and 1% penicillin and streptomycin. The target cells used were peptide transporter–deficient RMAS cells or RMAS cells stably transfected with pCDNA3 CD1D1. Targets were pulsed with 40–200 μg/ml ovalbumin protein at 37°C and 5% CO_2 for 1 h and then washed three times. 50 μl per well of the supernatant was then transferred to 96-well plates and lysis was assessed by measuring lactate dehydrogenase release using the Cytotox 96 assay kit (Promega Corp., Madison, WI). Controls were included on each plate for spontaneous LDH release from target and effector cells. Percent lysis was calculated according to manufacturer's instructions using the formula: [(test release − spontaneous release)/(maximum release − spontaneous release)] × 100%.

Peptide and Antibodies. The p99 peptide, YEHDFHHIREWGNHWKNFLAVM, that has been reported to bind murine CD1d1 (2) was purchased from PeptidoGenic Research and Company (Livermore, CA) HPLC purified. Antibodies used were anti-K^b and D^b, 20-8-4S (American Type Culture Collection [ATCC], Rockville, MD), anti-CD1, 1B1 (PharMingen, San Diego, CA) or 1H1 (PharMingen) or 3C11 (generously provided by S. Balk), anti-CD8, 3.155 (ATCC), anti-CD4, YTS177, and anti-Fas ligand, K-10 (PharMingen). All antibodies were used at 10 μg/ml with the exception of 3C11 ascites used at a dilution of 1:50, 3.155 ascites used at a dilution of 1:300 and YTS177 ascites used at a dilution of 1:20. The antibodies 20-8-4S and 3.155 were capable of blocking class I–restricted $CD8^+$ T cell responses at these concentrations in pilot experiments.

Generation of T Cell Lines. C57BL/6 mice were immunized with plasmids encoding OVA, mCD1d1, and B7.1 twice. Splenocytes were harvested 2 wk after the first injection and restimulated in vitro for 6 d with CD1-transfected RMAS cells pulsed with ovalbumin protein in IMDM (BioWhittaker) supplemented with 10% Con A supernatant, 10% heat-inactivated FBS, 2 mM glutamine, 50 μM 2β-mercaptoethanol, and 1% penicillin and streptomycin. The cultured splenocytes were restimulated weekly in 24-well plates with 10^5 each of mitomycin C–treated, thioglycollate-activated syngeneic peritoneal macrophages and CD1-transfected RMAS cells pulsed with ovalbumin protein in Con A–supplemented complete IMDM.

Results and Discussion

Generation of Ovalbumin-specific, CD1-restricted CTL. Intradermal administration of plasmid DNA induces antigen-specific immune responses (25) and allows for the ectopic expression of membrane-bound molecules such as CD1 and costimulators, as well as the expression of the antigen, chicken ovalbumin. Therefore, we chose this approach to test the biological significance of CD1 presentation of peptide from whole protein antigens. CD1 expression has been detected on gastrointestinal epithelium, thymus, liver, and spleen (6, 26). As murine CD1 is not prevalent in the dermis, a coinjection strategy was used to ectopically express the antigen-presenting molecule (CD1d1) as well as the antigen. Additionally, plasmids encoding costimulatory molecules B7.1 or B7.2 were included to provide the responsive T cell with a nonspecific second signal in the same milieu as the cognate signal (27–29). Chicken ovalbumin was selected as the test antigen as it contains a peptide with the reported mCD1-binding motif (2). In these experiments, antigen loading of target cells was accomplished by incubating them with whole protein, because as recent reports suggest, CD1 antigen loading occurs through a TAP-independent endocytic mechanism (11–13). The TAP independence of CD1 antigen processing made TAP-deficient, CD1-transfected RMAS cells the preferred target cells (9, 10).

Hence, C57BL/6 mice were immunized with different combinations of plasmid DNA encoding mCD1D1 (pACB-CD1), chicken ovalbumin (nCMV-OVA), and costimulator molecules (nCMV-B7.1 or nCMV-B7.2) at weeks zero and one. Mice were killed at week 2, and after in vitro restimulation, their splenocytes were assayed for their ability to lyse H-2^b CD1-transfected RMAS cells. Mice immunized with a combination including B7.1-expressing plasmid repeatedly gave a higher specific lysis than mice immunized with a B7.2-encoding plasmid (Fig. 1 *A*). The in vivo requirement for adding a CD28 ligand is in contrast to in vitro studies with CD1b- or CD1c-restricted human T cell hybridomas that use a pathway of costimulation outside the interaction between CD28 and its counterreceptors (30).

CTL Activity Is Antigen and CD1 Dependent. Splenocytes from mice immunized with the combination of plasmids encoding ovalbumin, mCD1d1, and B7.1 were able to lyse

MHC class I–deficient, $CD1^+$ RMAS transfectants pulsed with ovalbumin more effectively than untransfected RMAS cells pulsed with ovalbumin (Fig. 1 *B*). Although RMAS cells have very low levels of detectable CD1 expression on their surface by cytofluorometric analysis, augmenting the level of surface CD1 by transfection resulted in a threefold increase in cytotoxic lysis in the 4-h period of the assays. To verify that the incubation with ovalbumin did not spuriously result in MHC class I surface loading of peptide that was resulting in CTL recognition, antigen presentation was inhibited with antibodies. An antibody directed against MHC class I (H-2K ^{b}/D ^{b}) partially decreased lysis by 23%, whereas the anti-CD1 antibodies inhibited lysis by ~45–60% (Fig. 2). None of the anti-CD1 antibodies were able to completely inhibit lysis at the concentrations used. In fact, the extent of inhibition varied between experiments, with the 1H1 antibody being the most effective. This variation between the different anti-CD1 antibodies may reflect differences in their affinities for antigen or may represent different epitopes seen by mixed populations of T cells in each experiment.

To confirm that the CTL response was ovalbumin specific and the effector cells were not simply recognizing a conformation of CD1d1 stabilized by binding a peptide, we tested the CTL for lysis activity of $CD1^+$ RMAS cells pulsed with the p99 peptide previously described to bind murine CD1d1 (reference 2; Fig 3). The lysis of targets pulsed with p99 (16%) was not different from unpulsed targets (18%). Furthermore, p99 competitively inhibited lysis of $CD1^+$ RMAS cells pulsed with ovalbumin protein, decreasing lysis from 82 to 37%.

CTL Are Antigen Specific and MHC Unrestricted. Given that CD1 is nonpolymorphic, the same immunization protocol that was effective in generating CD1-restricted CTL in H-2^b mice should also generate CTL that recognize CD1 on cells of another MHC haplotype. Splenocytes from C57BL/6 (H-2^b) mice immunized with plasmids encoding mCD1D1 and B7.1 or mCD1D1, B7.1, and ovalbumin were tested for CTL activity to CD1D1-transfected P815 targets pulsed in the presence or absence of ovalbumin protein. The background lysis of CD1D1-transfected P815 in the absence of ovalbumin was ~45% at the highest E/T ratio, suggesting a partial allogeneic response, which was subtracted to give the percent antigen-specific lysis. However, H-2^b mice immunized with the three plasmids were repeatedly able to mount a significant CTL response against both CD1D1-transfected, ovalbumin-pulsed H-2^b RMAS cells and H-2^d P815 cells (Figs. 4, *A* and *B*), when compared to the response in the negative control group.

CTL Effectors Are Predominantly $CD8^+$ and Lysis Is Fas Independent. To further characterize the phenotype of the CD1-restricted CTL, blocking antibodies to CD4 and CD8 were used (Fig. 2). Anti-CD8 repeatedly blocked lysis, whereas anti-CD4 blocked lysis less consistently. The variation of inhibition between experiments by anti-CD4 implies a mixed effector population from the whole spleen preparations. Recently, Stenger et al. (31) have identified two mechanisms of CD1-restricted, cell-mediated cytotoxicity to contribute to the outcome of infection with intracellular pathogens. CD1-restricted T cells that are $CD4^-CD8^-$ lysed targets through the Fas–Fas ligand pathway while having no effect on intracellular bacterial survival, whereas $CD8^+$ effector cells used perforin and granzymes to kill the bacteria as well as their host cell. In our studies, antibodies against Fas ligand had no effect on the lysis of CD1-transfected cells pulsed with ovalbumin (Fig. 2).

In summary, these studies demonstrate that normal mice are able to generate ovalbumin-specific CTL restricted to CD1 in vivo. The highly conserved nature of CD1 proteins suggests that they may have evolved a specialized function to present nonpeptidic ligands to T cells (1). The crystal structure of CD1d1 reveals many differences from classical MHC molecules (32), including a deep and highly hydrophobic groove that would be structurally difficult to bind peptides in the manner of MHC molecules (33). However, there remains the possibility that CD1d may also present hydrophobic peptides that bind in a different configuration as suggested by the CD1d peptide–binding motif and synthetic peptide binding studies (2). The data presented here suggest that in addition to lipids and other nonpeptidic molecules, proteins can be recognized as antigens in the context of CD1 in vivo to prime CTL that are antigen specific.

CD1-reactive cells have been identified in mice, however, the physiological role of CD1 antigen presentation is unknown. One population in particular, the $CD4^+$ $NK1^+$ T cells, has been shown to make IL-4 (21, 34) in response to CD1. Although MHC class II° mice are relatively deficient in $CD4^+$ cells, they have been described to contain greater numbers of $CD4^+$ $NK1^+$ T cells compared to wild-type mice (22). In these mice, with the absence of MHC II–restricted $CD4^+$ T cells, $CD4^+$ $NK1^+$ cells appear to be important in providing help by serving as an early source of IL-2 in the priming of $CD8^+$ CTL in the induction of resistance to infection by the intracellular parasite, *Toxoplasma gondii* (35). The physiologic relevance of CD1 presentation of antigens remains unclear since T cells that recognize these molecules have been described as having similar effector functions as MHC class I– and class II–restricted T cells. Whether CD1-restricted T cells fulfill an ancillary, regulatory, or completely independent role in the immune system warrants further investigation.

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