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Targeting the Gut Vascular Endothelium Induces Gut Effector CD8 T Cell Responses Via Cross-Presentation by Dendritic Cells

Dorothee Bourges,* Yifan Zhan,* Jamie L. Brady,* Hal Braley, † Irina Caminschi,* Sandro Prato,*‡ José A. Villadangos,* and Andrew M. Lew2∗

Systemic delivery of Ag usually induces poor mucosal immunity. To improve the CD8 T cell response at mucosal sites, we targeted the Ag to MA
dCAM-1, a mucosal addressin cell adhesion molecule expressed mainly by high endothelial venules (HEV) in mesenteric lymph nodes (MLN) and Peyer’s patches of gut-associated lymphoid tissue. When chemical conjugates of anti-
MA
dCAM-1 Ab and model Ag OVA were injected i.v., a greatly enhanced proliferative response of Ag-specific OT-I CD8 T cells was detected in MLN. This was preceded by prolonged accumulation, up to 2 wk, of the anti-MA
dCAM OVA conjugate on HEV of Peyer’s patches and MLN. In contrast, nontargeted OVA conjugate was very inefficient in inducing OT-I CD8 T cell proliferation in MLN and required at least 20-fold more Ag to induce a comparable response. In addition, MA
dCAM targeting elicits an endogenous OVA-specific CD8 T cell response, evident by IFN-γ production and target killing. Induced response offers protection against an OVA-expressing B cell lymphoma. We propose that the augmentation of gut CD8 T cell responses by MA
dCAM targeting is due to both accumulation of Ag in the HEV and conversion of a soluble Ag to a cell-associated one, allowing cross-presentation by DCs. The Journal of Immunology, 2007, 179: 5678–5685.

Mucosal surfaces are the first line of defense against the many infectious agents (including Helicobacter pylori, rotavirus, influenza, HIV) that enter the body via the mucosa. Thus, efforts have been made to develop effective mucosal vaccines and adjuvant systems (1, 2). Mucosal and systemic immunity are largely separate compartments (3, 4) due to differences in homing receptors, such that systemic immunization usually does not generate a mucosal response (5–7). Ag stimulation within the gut associated-lymphoid tissue (GALT), 5 Peyer’s patches (PP), and mesenteric lymph nodes (MLN), induces secretory IgA, CD4, and CD8 T cell responses. However, the accessibility of orally administered Ag to the GALT is limited by a number of barriers including mucus barrier, degradative gastric acid, alimentary enzymes, and commensal flora. Therefore, these barriers limit the success of oral immunization, which is the most common delivery mode of Ag to induce mucosal responses (8). Perhaps this is not surprising given that one of the functions of the GALT is to avoid immune responses against Ags derived from ingested food, airborne matter, and commensal microorganisms (4). Thus, the induction of a mucosal protective immune response is dependent on the delivery and accessibility of the Ag to the GALT.

To circumvent these problems, we have taken a strategy to enhance mucosal immune response by targeting Ag to the GALT using parenterally administered anti-MA
dCAM-1 Ab. MA
dCAM-1 is a mucosal addressin cell adhesion molecule expressed by high endothelial venules (HEV) of PP and MLN as well as by flat endothelial venules of the gut lamina propria (9, 10). Lymphocytes use MA
dCAM-1 to home to the GALT via the expression of α5β1 integrin (11).

Induction of CTL in the gut is particularly pertinent for HIV infection, as the gut and indeed the GALT, are the major reservoir for HIV replication. It is generally thought that CTL are critical for immunological curing of HIV infection (12, 13). We previously showed that the parenteral immunization of mice using this targeting anti-MA
dCAM Ab leads to the development of a secretory IgA response in the gut, a systemic IgG response, as well as T cell cytokine and proliferative response (14). By using this targeting strategy (“posting to the correct address”), we investigated how the CD8 T cell response can be efficiently induced. Furthermore, we identified the APCs responsible for CD8 T cell priming.

Materials and Methods

Chemical conjugation of Abs

The Abs were used rat IgG2a Ab against mouse MA
dCAM-1 (MECA367; BD Biosciences), rat IgG2a Ab against FIRE (6F12), and rat IgG2a isotype control (GL117; which recognizes Escherichia coli β-galactosidase). Abs were isolated from hybridoma culture supernatant and purified on protein G-Sepharose (Amersham Biosciences).

Concentrated Abs (5 mg/ml) were incubated for 2 h at room temperature with a 10-fold excess of EMCS (N-succinimidyl-6-maleimido-caproate, dissolved in dimethylformamide; Sigma-Aldrich). After buffer exchange, the EMCS modified Abs were injected onto a degassed PBS-equilibrated column, and the first peak at 280 nm was collected and retained. OVA (10 mg/ml; Sigma-Aldrich) was reduced with 5 mM tri-n-butyl phosphine (Sigma-Aldrich) for 2 h at 50°C, followed by buffer exchange into degassed PBS. The reduced OVA (in 10-fold molar excess) and EMCS activated Abs were immediately combined. The conjugation reaction was

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allowed to proceed for 3 h at room temperature and then stopped by addition of 2 mM cysteine. Conjugates were separated from free OVA and nonconjugated IgG by size exclusion chromatography and fractions were analyzed by SDS-PAGE. Fractions containing free OVA or high m.w. aggregates were discarded. Protein concentration of the conjugates was assayed by a Bradford type assay (Pierce), and conjugates were then filtered and stored at 2–8°C.

**Mice**

C57BL/6 (B6), TCR-transgenic OT-I mice (MHC class I (H-2Kb)-restricted, OVA-specific, expressing the TCR Vα2 and Vβ5), B6.C-H-2bm1/ByJ (b1), and B6.Ly5.1 (Ly5.1) mice were used at 6–8 wk of age. pCD11c-GFP-DTR mice were originally obtained from D. Littman (New York University Medical Center, New York, NY) (15). Lethally irradiated B6 mice were reconstituted with 5 × 10^6 B6, b1m, or pCD11c-GFP-DTR bone marrow cells and were used 6–8 wk after reconstitution.

**Immunohistochemistry**

B6 mice were immunized i.v. with 20 μg of MECA367-OVA or GL117-OVA conjugates. Three days, 1 wk, and 2 wk later, spleen, inguinal lymph nodes (ILN), and MLN were harvested in OCT (Sakura) and frozen immediately on dry ice. Sections (5 μm) were cut onto polylysine-coated slides (Menzel-Glaser), fixed in cold acetone and kept at −70°C until use. Sections were rehydrated in PBS, blocked with PBS containing 10% FCS, then stained with anti-rat IgG (H + L) Texas Red-adsorbed against mouse Ig (Southern Biotechnology Associates). To detect OVA, sections were stained with a polyclonal rabbit anti-OVA serum (raised in the laboratory) followed by a goat anti-rabbit Ig-FITC adsorbed against human, mouse, and rat Ig (BD Biosciences).

**In vivo OT-1 proliferation**

Lymph nodes (LN) were harvested from OT-1 mice, and single cell suspension was prepared. OT-1 lymphocytes were resuspended at 10^6 cells/ml in PBS containing 1% FCS and labeled with 5 μM CFSE (Molecular Probes) for 10 min at 37°C. After two washes in PBS containing 1% FCS, cells were counted and resuspended in HEM without FCS. Each recipient mouse received 3 × 10^6 CFSE-labeled unpurified OT-1 cells i.v. On the following day, recipient mice were immunized i.v. with the indicated dose of MECA367-OVA or GL117-OVA conjugates. In the case of the pCD11c-GFP-DTR chimera experiment, mice received 100 ng of anti-rabbit IgG (Sigma-Aldrich) in 0.1 M sodium acetate (pH 6), and reactions were allowed to proceed for 3 h at room temperature and then stopped by addition of 2 mM cysteine. Conjugates were separated from free OVA and nonconjugated IgG by size exclusion chromatography and fractions were analyzed by SDS-PAGE. Fractions containing free OVA or high m.w. aggregates were discarded. Protein concentration of the conjugates was assayed by a Bradford type assay (Pierce), and conjugates were then filtered and stored at 2–8°C.

**Dendritic cell (DC) purification**

In the pCD11c-GFP-DTR experiments, we evaluated the depletion of DCs after treatment with DT (CSL). Splenic DC purification was performed as previously described (16) and briefly involved collagenase/Dnase digestion of spleen fragment and selection of light-density cells. This preparation (30–40% DC) was then stained with anti-mouse CD11c-PE Ab (BD Biosciences) and analyzed for expression of GFP and CD11c using a FACSscan (BD Biosciences).

**Endogenous T cell activation**

B6 mice were i.v. immunized with 20 μg of MECA367-OVA or GL117-OVA conjugate. One week later, spleens were harvested, and single cell suspensions prepared. An equivalent number of splenocytes were CFSE stained as previously described (17). After two washes, 5 × 10^6 CFSE labeled cells were resuspended in 1 ml of RPMI 1640 supplemented with 10% FCS and incubated with 0 or 100 μg of OVA at 37°C, 5% CO₂. After 4 days culture, cells were harvested and dead cells were eliminated on a Ficoll gradient (Amersham Biosciences). Cells were then stained with H2-Kb SIINFEKL tetramer-PE, a gift from Dr. A. Brooks (University of Melbourne, Parkville, Victoria, Australia), anti-mouse CD8-allophycocyanin Cy7, and anti-mouse CD4-allophycocyanin (BD Biosciences). For the detection of intracellular IFN-γ, GolgiStop (which contains brefeldin A) was added to the culture, according to the manufacturer’s instructions (BD Biosciences) 6 h before harvest cell cultures. Cells were then surface stained as previously described, permeabilized and fixed using BD Cytofix/Cytoperm and BD Perm/Wash reagents, according to manufacturer’s instructions (BD Biosciences). Cells were then stained for intracellular IFN-γ using biotin rat anti-mouse IFN-γ Ab followed by streptavidin-allophycocyanin (BD Biosciences). Cells were analyzed by flow cytometry using a FACSaria (BD Biosciences).

To measure the secretion of IFN-γ, aliquots of supernatant were harvested at different time points and subjected to an ELISA. Briefly, microtiter plates coated with capture Ab (2 μg/ml anti-mouse IFN-γ; BD Biosciences) were incubated with serially diluted supernatant and standard IFN-γ protein at 4°C overnight. Bound IFN-γ protein was detected after 1 h incubation at room temperature with biotin rat anti-mouse IFN-γ (BD Biosciences), followed by 30 min incubation with strept-ABC complex/HRP (DakoCytomation). The substrate used was tetramethylbenzidine (Sigma-Aldrich) in 0.1 M sodium acetate (pH 6), and reactions were stopped with 0.5 M sulfuric acid.

**In vivo CTL assay**

Splenocytes from naive B6 mice were depleted of red cells and divided into two populations. One population was pulsed for 1 h at 37°C with 0.1 μg/ml OVA323-339 peptide and then labeled with a high concentration (5 μM) of CFSE. The other was incubated for 1 h at 37°C without peptide and labeled with a low concentration (0.5 μM) of CFSE. Equal number of cells from the CFSE<sup>high</sup> and CFSE<sup>low</sup> population were injected i.v. into naive B6 mice or mice primed with 20 μg of OVA-conjugates 1 wk before. Mice were killed 16–20 h later, spleen and MLN cell suspensions were analyzed by flow cytometry. The percentage of OVA-specific lysis was determined by loss of the peptide-pulsed CFSE<sup>pp</sup> population compared with the control CFSE<sup>neg</sup> population.

**B cell lymphoma challenge**

Stable Eμ-myc cell lines (18) bearing GFP (Eμ-myc-GFP) or GFP and OVA (Eμ-myc-OVA) were generated by retroviral transduction (19). Three weeks after immunization with 20 μg of MECA367-OVA or
GL177-OVA conjugate, mice were challenged by i.v. injection of 1 × 10^6 Ep-myc-OVA cells. Spleen and MLN were harvested 7 days later, and cells were stained with anti-mouse CD19-PE (BD Biosciences). CD19^+ GFP^+ tumor cells were enumerated per organ using PE beads.

**Statistical analyses**

To assess the difference between MA(d)CAM targeting and the nontargeting groups, two-tailed unpaired t-test was realized using GraphPad Prism 3.0 software.

### Results

**MA(d)CAM targeting prolongs the accumulation of the Ag within the GALT**

Mice were injected with the nontargeting (GL117) or MA(d)CAM-targeting (MECA367) OVA-conjugated Abs, and 3 days later, localization of the Ab was assessed on tissue sections by staining with Texas Red-conjugated anti-rat Ig. The anti-MAdCAM Ab was found on HEV in MLN (Fig. 1A) and PP (data not shown). In contrast, no obvious deposit of the GL117-OVA conjugate could be detected in the GALT. In addition, using an anti-OVA Ab, we showed that OVA also localizes with anti-MA(d)CAM Ab to HEV in the GALT (Fig. 1A). No localization of MECA367-OVA conjugate could be found in peripheral LNs (data not shown). We were also able to show that the anti-MA(d)CAM Ab was detectable in the GALT HEV for at least 2 wk (Fig. 1B). These data demonstrate that the Ag persists into the GALT after MA(d)CAM targeting.

**MA(d)CAM targeting induces specific proliferation of OT-I T cells in the MLN**

To test whether MECA367-OVA conjugate can elicit an OVA-specific CD8 T cell response, we adoptively transferred CFSE-labeled OT-I cells on day 0, followed on day 1 by 5 or 1 μg of MECA367-OVA or GL117-OVA conjugates on day 1. Spleen, ILN, and MLN were harvested 60 h later. Lymphocytes were stained for CD8 and Vα2 and analyzed for CFSE proliferation. A. Plots show proliferation of OT-I CD8 T cells in MLN of mice immunized with 5 μg of conjugate and are representative of three independent experiments. B. Ratio of proliferated to unproliferated OT-I cells in MLN, spleen, and ILN of B6 mice is expressed as mean ± SEM.

![Graph showing proliferation of OT-I CD8 T cells in the MLN](image)

**FIGURE 2.** MA(d)CAM targeting induces a high level of proliferation of OT-I CD8 T cells in MLN. Mice received 3 × 10^6 CFSE-labeled OT-I cells on day 0, followed by 5 or 1 μg of MECA367-OVA or GL117-OVA conjugates on day 1. Spleen, ILN, and MLN were harvested 60 h later. Lymphocytes were stained for CD8 and Vα2 and analyzed for CFSE proliferation. A. Plots show proliferation of OT-I CD8 T cells in MLN of mice immunized with 5 μg of conjugate and are representative of three independent experiments. B. Ratio of proliferated to unproliferated OT-I cells in MLN, spleen, and ILN of B6 mice is expressed as mean ± SEM.

![Graph showing proliferation of OT-I CD8 T cells in the MLN](image)

**FIGURE 3.** High level of OT-I proliferation in the GALT is achieved only after MA(d)CAM targeting. B6 mice received 3 × 10^6 CFSE OT-I cells on day 0, followed on day 1 by 5 μg of MECA367-OVA, GL117-OVA, or 6F12-OVA (targeting DCs) conjugates. Spleen, MLN, brachial, and axillary LN were harvested 60 h later. Lymphocytes were stained for CD8 and Vα2 and analyzed for CFSE proliferation. A. Plots show proliferation of OT-I CD8 cells in MLN. B. Graphs represent the total number of cells, total number of OT-I CD8 cells and the number of proliferating OT-I cells in spleen, MLN and pooled brachial and axillary LN. Cell numbers were determined using counting PE beads.
mucosal-targeting potential of this conjugate. OT-I T cells preferentially in the GALT, demonstrating the induced proliferation of OT-I cells in all lymphoid organs. In peripheral LN, whereas the DC-targeting conjugates in-addition, MAdCAM targeting induced only poor levels of proliferation observed between the two conjugates was due to a different concentration of OVA molecules on each Ab, we performed an in vitro CFSE proliferation assay. OT-I cells proliferated to the same extent with the same amount of both conjugates in vitro (data not shown). In mice immunized with MECA367-OVA conjugate, the proliferative response is higher in the MLN than in the spleen and ILN (Fig. 2B).

To exclude the possibility that the difference of T cell proliferation observed between the two conjugates was due to a different concentration of OVA molecules on each Ab, we performed an in vitro CFSE proliferation assay. OT-I cells proliferated to the same extent with the same amount of both conjugates in vitro (data not shown).

We then assessed the number of total and proliferating OT-I cells per organ after MAdCAM targeting. GL117-OVA conjugate and 6F12-OVA conjugate, an anti-FIRE (F4/80-like receptor, a glycoprotein expressed by CD8\(^+\) DCs and macrophages (20)), which should target to all lymphoid organs. As shown in Fig. 3, the number of OT-I cells recovered in MLN, both total and proliferating ones, was significantly higher after MAdCAM-targeting conjugates than after the nontargeting and DC-targeting conjugates. In addition, MAdCAM targeting induced only poor levels of proliferation in peripheral LN, whereas the DC-targeting conjugates induced proliferation of OT-I cells in all lymphoid organs.

These data show that MAdCAM targeting induces proliferation of OT-I T cells preferentially in the GALT, demonstrating the mucosal-targeting potential of this conjugate.

**FIGURE 4.** Lack of presentation by hemopoietic-derived cells greatly reduced OT-I proliferation after MAdCAM targeting. Lethally irradiated B6 mice were reconstituted with B6 or bm1 bone marrow cells. Six weeks later, mice received 3 \(\times\) 10\(^6\) CFSE OT-I cells followed by 5 \(\mu\)g of MECA367-OVA or GL117-OVA conjugates the next day. Spleen, ILN, and MLN were harvested 60 h later, lymphocytes stained for CD8 and V\(\alpha\)2. A, Plots show the proliferation of OT-I CD8 T cells in MLN after MAdCAM targeting and are representative of three independent experiments. B, Ratio of proliferated to unproliferated OT-I cells in MLN, spleen, and ILN in bm1 chimera mice is expressed as mean \(\pm\) SEM.

**FIGURE 5.** Ablation of CD11c\(^+\) DCs by DT injection into pCD11c-GFP-DTR bone marrow chimeras greatly reduced the proliferation of OT-I CD8 T cells in MLN after MAdCAM targeting. Lethally irradiated B6 mice were reconstituted with pCD11c-GFP-DTR bone marrow cells. Six weeks later, mice received 100 ng of DT i.p. on days 0 and 2. Spleen were harvested on day 3 and submitted to DC enrichment. A, Plots show the number of CD11c\(^+\)GFP\(^+\) DCs in the pCD11c-GFP-DTR chimera mice before and after treatment with DT. The graph shows the percentage of GFP\(^+\)CD11c\(^+\) cells after DT treatment. Results are expressed as mean \(\pm\) SEM (\(n=10\) reconstituted mice). B, pCD11c-GFP-DTR chimera mice received 100 ng of DT i.p. on days 0 and 2. 3 \(\times\) 10\(^6\) CFSE OT-I cells on day 0, and 5 \(\mu\)g of MECA367-OVA or GL117-OVA conjugates the next day. Spleen, ILN, and MLN were harvested 60 h later, lymphocytes were stained for CD8 and V\(\alpha\)2. Dot plots show the proliferation of OT-I cells in MLN in the absence of DCs and are representative of two independent experiments. Data show the ratio of proliferated to unproliferated OT-I cells in MLN, spleen, and ILN in CD11c-DTR chimera mice and are expressed as mean \(\pm\) SEM.

**OT-I proliferation after MAdCAM targeting is dependent on MHC class I DC presentation**

Because MECA367-OVA conjugate binds to HEV and because endothelial cells express MHC class I and costimulatory molecules (21), it is possible that endothelial cells present directly the Ag to OT-I T cells. To investigate the mechanism by which OT-I cells are activated after MAdCAM targeting, we reconstituted B6 mice with bm1 bone marrow cells bearing a mutation in the H2-K\(^b\) gene so that they can’t present OVA peptide to OT-I CD8 T cells (22). In this model, bone marrow-derived cells, such as DCs, cannot present the Ag.

OT-I cells did not proliferate in bm1 chimera mice injected with soluble OVA (data not shown) or GL117-OVA conjugate (Fig. 4A). OT-I T cell proliferation was dramatically reduced in MLN of bm1 chimera compared with B6 chimera mice injected with MECA367-OVA conjugate (Fig. 4A), indicating that bone marrow-derived APC, such as DCs, play a central role in the presentation of our OVA conjugate to OT-I T cells. However, a small but consistent proliferative response of CD8 T cells was detected in
FIGURE 6. MAdCAM targeting elicits an endogenous OVA-specific CD8 and CD4 T cell response. B6 mice were immunized with 100 μg of soluble OVA, 20 μg of MECA367-OVA, or GL117-OVA conjugates. One week later, spleens were harvested, cell suspensions stained with CFSE and then cultured with 0 or 100 μg of OVA. After staining for CD8, CD8- and PE-conjugated SIINFEKL tetramer cells were analyzed for CFSE proliferation. A. Plots show proliferation of endogenous CD8 and CD4 T cells after in vitro restimulation. B. Plots are gated on CD8+ cells and show the SIINFEKL-positive CD8 population. Data are representative of three independent experiments (n = 3 animals per group).

the MLN but not in other lymphoid organs (Fig. 4B) with MECA367-OVA as compared with GL117-OVA conjugate. This small residual response may be due to the small number of residual B6 DCs surviving in the irradiation chimera or to a secondary role of HEV presenting Ag directly to T cells.

Depletion of CD11c+ DCs dramatically reduces OT-I proliferation after MAdCAM targeting
To investigate further the nature of APCs in CD8 T cells priming after MAdCAM targeting, we used B6 mice reconstituted with pCD11c-GFP-DTR bone marrow cells. These mice express GFP and the DT receptor under the control of the CD11c promoter allowing the conditional depletion of CD11c+ DCs by DT injection (15).

Reconstituted mice received two i.p. injections of 100 ng of DT at days 0 and 2 to ablate DCs. This treatment greatly reduced the number of GFP+ CD11c+ DCs in the spleen as shown in Fig. 5A. The percentage of CD11c+ DCs was 7-fold lower after DT treatment (Fig. 5A).

To ensure that DT has no effect in a B6 mouse, mice were treated with DT and OT-I proliferation was measured after MAdCAM targeting. As shown in Fig. 5B, DT treatment has no effect on the proliferation of OT-I T cells in the MLN of B6 mice immunized with MECA367-OVA or GL117-OVA conjugates. In contrast, depletion of DCs in pCD11c-DTR chimera dramatically reduced OT-I proliferation in the MLN after MAdCAM targeting, as well as after immunization with the irrelevant Ab (Fig. 5B). In addition, OT-I proliferation in peripheral LN after MAdCAM targeting is almost completely abolished (Fig. 5B).

Thus, using this pCD11c-GFP-DTR model, we confirmed that activation of CD8 T cells after immunization with our OVA conjugate is mainly dependent on Ag presentation by DCs.

MAdCAM targeting activates endogenous CD8 and CD4 T cells
The OT-I system allows for easier detection and quantitation, but we also wanted to look at the activation of endogenous T cells after MAdCAM targeting. B6 mice were immunized with 100 μg of soluble OVA, 20 μg of MECA367-OVA conjugate, or 20 μg of GL117-OVA conjugate, and 1 wk later splenocytes were stained with CFSE and restimulated in vitro with various concentrations of OVA for 4 days. As shown in Fig. 6A, stimulation of splenocytes from unimmunized mice induced little nonspecific proliferation of CD8 (Fig. 6A, left) and CD4 T cells (Fig. 6A, right). In contrast, after restimulation with 100 μg of OVA, MAdCAM targeting elicited a higher CD8 T cell proliferative response than the nontargeting conjugate GL117-OVA (Fig. 6A, left). Similarly, the proliferative response of CD4 T cells was higher after MAdCAM targeting than after the nontargeting conjugate (Fig. 6A, right). However, proliferation of endogenous CD4 cells was lower than proliferation of CD8 cells.

Furthermore, a substantial population of OVA-specific proliferating CD8 T cells was detected only in MECA367-OVA immunized mice, as shown by SIINFEKL tetramer staining (Fig. 6A). Initially we tried to detect IFN-γ production. Initially we tried to detect IFN-γ production. However, secretion of IFN-γ after MAdCAM targeting was higher than in MECA367-OVA immunized mice. It should be noted that MAdCAM-targeting immunization induced higher level of proliferation of CD8 T cells than immunization with 100 μg of soluble OVA as well as a higher percentage of tetramer-positive CD8 cells (Fig. 6B). This population of tetramer-positive CD8 cells was not detected in nonrestimulated cell culture (Fig. 6B, bottom).

To assess that these proliferative CD8 T cells were effector cells, we looked at IFN-γ production. Initially we tried to detect IFN-γ+ CD8 T cells directly ex vivo but the results were not robust. We therefore cultured the cells in vitro for 4 days and as shown in Fig. 7A, MAdCAM targeting elicited a substantial population of IFN-γ+ CD8 T cells while soluble OVA and GL117-OVA immunization induced very few IFN-γ+ cells. In addition, we showed that secretion of IFN-γ after MAdCAM targeting was higher than after immunization with the nontargeting conjugate (Fig. 7B). These differences in IFN-γ secretion could be detected both in the spleen and MLN (Fig. 7B).
We have shown that MAdCAM targeting activates an endogenous CD8 and CD4 T cells and results in an increase secretion of IFN-γ production. We then assessed the cytotoxic response elicited by MAdCAM targeting using an in vivo CTL assay. One week after immunization, B6 mice were adoptively transferred with equal number of unpulsed OVA^−Myc-GFP-OVA tumor cells. Spleen and MLN were harvested 7 days later, and cells were stained with anti-CD8, anti-CD19 PE-conjugated Ab. The number of CD19^+GFP^+ cells and allows partial rejection of an OVA-expressing B cell lymphoma. MAdCAM targeting induces mucosal cytotoxic CD8 T cells and allows partial rejection of an OVA-expressing B cell lymphoma. A, B6 mice were adoptively transferred with equal number of unpulsed OVA^−Myc-GFP-OVA tumor cells. Spleen and MLN were harvested 7 days later, and cells were stained with anti-CD19 PE-conjugated Ab. The number of CD19^+GFP^+ tumor cells was determined using PE-conjugated counting beads. The number of tumor cells in MLN is shown and results are expressed as mean ± SEM. Data are representative of two independent experiments.

These data showed that MAdCAM targeting activates endogenous CD8 and CD4 T cells and results in an increase secretion of IFN-γ.

MAdCAM targeting induces OVA-specific cytotoxic response and immunity against an OVA-expressing B cell lymphoma

We have shown that MAdCAM targeting activates an endogenous CD8 T cell response together with IFN-γ production. We then assessed the cytotoxic response elicited by MAdCAM targeting using an in vivo CTL assay. One week after immunization, B6 mice were adoptively transferred with equal number of OVA-peptide pulsed CFSE^high and nonpulsed CFSE^low target cells. OVA-specific lysis was determined by loss of the peptide-pulsed CFSE^high population compared with the control CFSE^low population. As shown in Fig. 8A, MAdCAM targeting induces specific lysis of the OVA-pulsed target cells, both in the MLN and spleen. However, the induction of specific CTL activity after MAdCAM targeting was heterogeneous, ranging from 80% to 10% of lysis (Fig. 8A). On the contrary, the nontargeting conjugate was really inefficient at inducing killing of the target cells (Fig. 8A).

To assess that our MAdCAM targeting strategy could induce immunity against infection/disease, we used a model of GFP-OVA-expressing B cell lymphoma, Eμ-Myc-GFP-OVA cells. After i.v. transfer, these tumor cells spread and engraft in all lymphoid organs, enabling us to look at induction of immunity in the GALT. Three weeks after immunization with the OVA conjugates, mice received 1 × 10^6 Eμ-Myc-GFP-OVA tumor cells. The number of CD19^+GFP^+ tumor cells in MLN was determined 7 days later. As shown in Fig. 8B, the number of OVA-expressing tumor cells was significantly decreased after immunization with MAdCAM targeting compared with unimmunized mice. However, not all the mice immunized with MECAM-7-OVA conjugates did respond to the immunization. This response is not surprising as it reflects the heterogeneity of the CTL responses after MAdCAM targeting shown in Fig. 8A. Nevertheless, the number of tumor cells in the MLN after MAdCAM targeting was 2.7-fold lower than in naive mice. In contrast, the nontargeting conjugation did not induce immunity against the Eμ-Myc-GFP-OVA tumor cells as the number of tumor cells was not significantly decreased compared with unimmunized mice.

These data demonstrate that MAdCAM targeting induces cytotoxic CD8 T cells both in mucosal and “systemic” lymphoid organs. From the OT-I proliferation data, we presume that the CTL activity was initiated in the GALT and some of these GALT-primed T cells migrated to the spleen. In addition, MAdCAM targeting induces immunity in the GALT against an OVA-expressing B cell lymphoma.
Discussion
The mucosal barriers limit the successful induction of a mucosal immune response via the oral route, whereas systemic delivery of Ag does not generally induce an adequate mucosal immune response (4). In this study, we showed that posting Ag to the GALT by targeting the mucosal addressin MAdCAM-1 generates an enhanced CD8 T cell response in the GALT. Prolonged retention of Ag on HEV of the GALT after targeting Ags prompted us to define the nature of APCs responsible for presentation to CD8 T cells.

Soluble Ag can prime CD8 T cells via cross-presentation by CD8 DCs, but is inefficient (23, 24). When soluble Ag is made into a cell-associated form, the efficiency is markedly enhanced (24). We believe therefore, that targeting by anti-MAdCAM Ab performs two functions. It delivers Ag to the GALT, hence stimulating systemic and local gut responses, but also converts a soluble Ag to a cell-associated one. In general, successful induction of a T cell response requires that sufficient amounts of Ag persist long enough in secondary lymphoid organs (25). This could be a major reason why soluble Ag normally does not induce an adequate T cell immune response. Part of the activity of many adjuvants (alum, mineral oil emulsions) is a depot effect i.e., capacity to retain Ag. In our study, we took advantage of the fact that MAdCAM-1 is expressed abundantly and relatively specifically by HEV cells in the GALT. By targeting this mucosal addressin, Ag persistence and a high local concentration can be achieved. This approach greatly enhances the efficiency of induction of an OT-I CD8 T cell response by soluble Ag. In contrast to the targeting approach, a 20-fold higher dose is required to induce a comparable response with the nontargeting conjugate. In the case of oral delivery to obtain gut responses, a single high dose of 60 mg (>60,000-fold more Ag) is required to induce a good CD8 T cell response (26). At the range of Ag doses (1–20 μg) we tested, a greater proliferative response was detected in MLN compared with spleen and peripheral LN. This preferential induction of the OT-I CD8 T cell response in the GALT was not observed with the nontargeting OVA conjugate.

In addition to OT-I T cell activation, MAdCAM targeting activates endogenous CD8 and CD4 T cells. These CD8 and CD4 T cell responses could be detected even 1 mo after immunization. Furthermore, these activated CD8 T cells are functional as demonstrated by IFN-γ production and by their cytotoxic activity.

CD4 and CD8 T cells may have differential requirements for Ag persistence. In mice expressing a MHC class II-restricted epitope in DCs under the control of a tetracycline-inducible promoter, the proliferation of CD4 T cells was dependent on the presence of Ag throughout their expansion phase, even in the presence of an inflammatory stimulus (27). This was not the case for CD8 T cells. Exposing naive CD8 T cells to the APC for 2 h was sufficient to drive multiple rounds of division over the next several days in an Ag-free culture (28). Similar conclusions have also been derived from studies with bacterial and viral Ags (29, 30). However, full activation of CD8 T cells required concerted signaling by Ag, co-stimulation, and certain cytokines for greater than 40 h. The gene expression program required for cell division and for survival and effector function differs in time of Ag exposure (31). Our targeting strategy prolongs the signaling by Ag, which would allow for full activation of CD8 T cells and the development of effector function.

It has been firmly established that DCs are specialized cells that can prime naive T cells (32, 33). However, vascular endothelial cells have been shown to express MHC class I and class II molecules and costimulatory molecules (21, 34, 35). There is accumulating evidence supporting that endothelial cells can function as APCs. Mouse vascularized cardiac allografts can be acutely rejected via direct recognition of foreign class I molecules by CD8 T cells, even when the graft hemopoietic cells are syngeneic with the host’s (36). In vitro experiments demonstrated that insulin-specific CD8 cells directly recognized pancreatic endothelial cells in islet organ cultures (37) and mouse endothelial cells cross-present Ag to CD8 T cell hybridomas via a TAP-1- and proteasome-dependent pathway (38). However, it is still an unresolved issue whether endothelial cells can activate naive T cells in vivo. Because our data clearly showed that anti-MAdCAM OVA conjugate is mainly deposited at HEV of MLN and PP, it poses a question whether they act as APCs in priming naive CD8 T cells. We took two approaches to address this question. Firstly, bm1 bone marrow was transferred to lethally irradiated B6 mice, so that the reconstituted donor hemopoietic cells could not present OVA to transferred CD8 T cells, due to the mutations of H-2Kb. Secondly, CD11c<sup>−</sup> DCs were ablated using DT in CD11c<sup>+</sup> promoter control-DT receptor transgenic mice (15). In both systems, proliferation of OT-I CD8 T cells was greatly reduced, suggesting that DCs are the principal APCs involved in the priming of CD8 T cells in GALT. Interestingly, a residual proliferative response of OT-I CD8 T cells was observed when DCs were either absent (CD11c<sup>−</sup>DTR mice) or could not present Ag (bm1 chimeras). At this moment, it is not clear whether the residual CD8 T cell response is due to direct presentation of Ag by endothelial cells, or cross-presentation by residual DCs that are resistant to radiation in bm1 chimeras, and to toxin in CD11c<sup>−</sup>DTR transgenic mice. Even for the latter case, the role of Ag presentation by endothelial cells must be minor. The great reduction in CD8 T cell proliferation in DC- ablabeled mice established that DCs are the principal APCs involved in priming of CD8 T cells, even though Ag primarily accumulated at HEV site. We argue that a more important role for endothelial cells in secondary lymphoid organs is to capture Ag and be a source of Ag for cross-presentation by DCs (39, 40), possibly as they extravasate through the HEV.

In summary, we have shown that targeting MAdCAM via par- enteral routes for the delivery of Ag to the GALT is an efficient way to bypass mucosal barriers and enhance mucosal immune response. We were able to induce a high level of OT-I T cell proliferation in the GALT using a low amount of targeted Ag, and we showed that this proliferation was dependent upon Ag presentation by DCs. Targeting also enabled us to specifically activate endogenous CD8 T cells and to generate CTL responses in the gut. We extrapolate that our strategy may be useful for induction of CTL in the gut, which serves as a major reservoir for pathogens such as HIV. Furthermore, our targeting-strategy induces mucosal and systemic immunity against a tumor model. We believe that our findings may have implications in the generation of CD8 T cells against gut infections including HIV, which uses the gut as a reservoir (13).

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References


