CD40 Engagement Enhances Antigen-Presenting Langerhans Cell Priming of IFN-γ-Producing CD4+ and CD8+ T Cells Independently of IL-12

Anton V. Gorbachev and Robert L. Fairchild

*J Immunol* 2004; 173:2443-2452; doi: 10.4049/jimmunol.173.4.2443

http://www.jimmunol.org/content/173/4/2443

**Why The JI?**

- **Rapid Reviews!** 30 days* from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

**References**  This article cites 34 articles, 22 of which you can access for free at: http://www.jimmunol.org/content/173/4/2443.full#ref-list-1

**Subscription**  Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**  Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**  Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
CD40 Engagement Enhances Antigen-Presenting Langerhans Cell Priming of IFN-γ-Producing CD4⁺ and CD8⁺ T Cells Independently of IL-12

Anton V. Gorbachev* and Robert L. Fairchild†‡

The delivery of CD40 signaling to APCs during T cell priming enhances many T cell-mediated immune responses. Although CD40 signaling up-regulates APC production of IL-12, the impact of this increased production on T cell priming is unclear. In this study an IL-12-independent T cell-mediated immune response, contact hypersensitivity (CHS), was used to further investigate the effect of CD40 ligation on the phenotypic development of Ag-specific CD4⁺ and CD8⁺ T cells. Normally, sensitization for CHS responses induces hapten-specific CD4⁺ T cells producing type 2 cytokines and CD8⁺ T cells producing IFN-γ. Treatment of mice with agonist anti-CD40 mAb during sensitization with the hapten 2,4-dinitrofluorobenzene resulted in CHS responses of increased magnitude and duration. These augmented responses in anti-CD40 Ab-treated mice correlated with increased numbers of hapten-specific CD4⁺ and CD8⁺ T cells in the absence of IL-12. Engagement of CD40 on hapten-presenting Langerhans cells (hpLC) up-regulated the expression of both class I and class II MHC and promoted hpLC migration into the T cell priming site. These results indicate that hpLC stimulated by CD40 ligation use a mechanism distinct from increased IL-12 production to promote Ag-specific T cell development to IFN-γ-producing cells. The Journal of Immunology, 2004, 173: 2443–2452.

A ntigen-specific T cells require delivery of costimulatory signals as well as recognition of Ag/MHC complexes to become fully activated and develop into effector cells that mediate immune responses (1, 2). T cell engagement of the CD40 costimulatory molecule expressed by APCs via CD154 (CD40L) is required for T cell activation during the development of many responses (3–5). Cross-linking CD40 on APC via agonist anti-CD40 mAb has been shown to augment many immune responses (6–8). One effect of CD40 ligation is increased APC production of IL-12 that has been proposed to contribute to the enhanced Th1-mediated immune responses observed in these studies (9–11).

Contact hypersensitivity (CHS) is a T cell-mediated, cutaneous immune response to sensitization and subsequent challenge with a hapten. During sensitization, hapten-presenting Langerhans cells (hpLC) migrate from the sensitized skin into draining lymph nodes and prime specific CD4⁺ and CD8⁺ T cells (12, 13). After this initial activation, these T cell populations develop into cells producing the cytokines that mediate and regulate the CHS response. Subsequent contact of the skin with the sensitizing hapten (e.g., challenge) directs the hapten-primed T cells into the challenge site to mediate local inflammation, resulting in the characteristic tissue edema or swelling that peaks 24–48 h after challenge and then quickly resolves (14, 15). Studies from this and other laboratories have indicated that CD8⁺ T cells are the primary effector cells in CHS responses to many hapten and that CD4⁺ T cells downstream regulate CHS (16–18). Consistent with their roles in the response, hapten-primed CD8⁺ T cells produce IFN-γ after in vitro stimulation, whereas the majority of hapten-primed CD4⁺ T cells produce type 2 cytokines such as IL-4, IL-5, and IL-10 (17). Recent studies from this laboratory indicated that CD28-B7-2 costimulation is required for priming of T cells for CHS responses (19). In contrast, CD40-CD154 interactions are not essential for priming of the hapten-specific CD4⁺ and CD8⁺ T cell compartments in CHS responses (20).

The goal of the current study was to test the potential effects of CD40 ligation on hpLC function during hapten-specific T cell priming for CHS responses using an agonist anti-CD40 Ab. The results demonstrate that delivery of agonist CD40 signaling during hapten sensitization increases maturation of the hpLC and their migration to skin draining lymph nodes. These events amplify the activation and development of both hapten-specific CD4⁺ and CD8⁺ T cells to equivalent levels in the presence or the absence of IL-12. These findings should be useful for the design of new immunotherapeutic strategies using dendritic cells (DC) and anti-CD40 mAb to augment T cell-mediated responses in the skin.

Materials and Methods

Animals

C57BL/6 and BALB/c mice were purchased through Dr. C. Reeder (National Cancer Institute, Frederick, MD), MHC class II⁻/⁻, CD8⁻/⁻, and B cell-deficient μMT⁻/⁻ mice on the C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). IL-12-deficient p55⁻/⁻ /p40⁻/⁻ mice on the BALB/c background were generated by Dr. J. Magram
Antigen uptake and induction of CHS

For sensitization to 2,4-dinitrofluorobenzene (DNFB), mice were painted on days 0 and 1 with 25 μl of 0.25% DNFB (Sigma-Aldrich) on the shaved abdomen and 5 μl on each footpad. On day 5, sensitized and, as a negative control, unsensitized mice were challenged with 10 μl of 0.2% DNFB on both sides of each ear. Ear thickness was measured in a blinded manner at 24-h intervals after challenge using an engineer’s micrometer (Mitutoyo, Elk Grove Village, IL) and expressed in units of 10 μm. To test the effect of CD40 signaling on CHS responses, groups of mice were injected i.p. with 200 μg of control rat IgG or anti-CD40 mAb FGK 115 on days 0 and 1 of hapten sensitization.

**Results**

**CHS responses and hapten-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells**

To examine the potential effect of CD40 ligation on the induction and elicitation of CHS responses, mice were injected with either isotype control IgG Ab (control group) or anti-CD40 mAb FGK115 at the time of sensitization with DNFB. Ear swelling responses to DNFB challenge in control and Ab-treated groups were compared. The typical ear swelling response to sensitization and challenge with DNFB peaked 24 h after challenge and then quickly decreased in the control IgG-treated group. In contrast, both the magnitude and the duration of the CHS response were markedly increased in mice treated with anti-CD40 mAb (Fig. 1).

Because both hapten-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells participate in CHS responses, the effect of CD40 ligation on the development of these cell populations to cytokine-producing cells was tested by ELISPOT assay. Hapten-specific CD8<sup>+</sup> T cells producing IFN-γ were readily detectable on day 5 postsensitization in the lymph nodes of hapten-sensitized mice treated with control IgG and were low to undetectable in naive mice (Fig. 2A). The number of hapten-specific CD8<sup>+</sup> T cells producing IFN-γ was >3 times higher in anti-CD40 mAb-treated mice than in the control group. The enhancing effect of anti-CD40 mAb treatment was dependent on Ag sensitization, as treatment of naive mice with anti-CD40 mAb did not result in increased IFN-γ-producing T cell numbers (not shown). As typically observed (21), low numbers of hapten-specific CD4<sup>+</sup> T cells producing IFN-γ were observed in the lymph nodes of sensitized mice treated with control IgG compared with the number of IFN-γ-producing CD8<sup>+</sup> T cells (Fig. 2B). Anti-CD40 mAb treatment also increased the number of hapten-specific CD4<sup>+</sup> T cells producing IFN-γ; however, the number of these cells was markedly less than the number of IFN-γ-producing CD8<sup>+</sup> T cells in the anti-CD40-treated group (Fig. 2, A vs B). The development of hapten-specific CD4<sup>+</sup> T cells producing IL-4 was also increased in anti-CD40 mAb-treated mice (Fig. 2C).

Together these results indicate that CD40 ligation has the greatest enhancing effect on the development of hapten-specific, IFN-γ-producing CD8<sup>+</sup> T cells, but also increases the development of flow cytometric analysis to assess the phenotype of hPLC was performed as previously described (19). To prevent nonspecific Ab binding, cells were incubated with rat serum (Rockland, Gilbertsville, PA) diluted 1/1000 in staining buffer (Dulbecco’s PBS with 2% FCS/0.2% NaN₃) for 20 min on ice. Then cells were washed and stained with PE-labeled anti-CD11c mAb and biotinylated anti-I-A<sup>+</sup> or anti-I-E<sup>+</sup> and anti-H-2<sup>D</sup> mAbs plus streptavidin-FITC. Stained cells were washed five times, resuspended in staining buffer, and analyzed by two-color flow cytometry using a FACScan (BD Biosciences, San Jose, CA).

**LC migration**

Mice were sensitized by single application of 0.25% DNFB on both sides of the ear. Two hours after sensitization ears were excised from sensitized or naive mice (four animals per group), and peeled ear halves were floated directly on 2 ml of complete RPMI 1640 medium in 24-well plates for 48 h. After this culture, the cells that migrated out of the ear skin into the medium were gently resuspended and counted using trypan blue. Cell suspensions obtained from the ears of mice in each experimental group were pooled and filtered to remove hair and debris, then stained with PE-labeled anti-CD11c mAb to detect Langerhans cells (LC) within the migratory cell population. Cell populations obtained using this method contained ~10–20% CD11c<sup>+</sup> cells with intermediate to high granularity (side scatter) as assessed by flow cytometry.

To assess hapten-presenting DC migration in vivo, mice were painted with 100 μl of 1% FITC on day 0. On day 3 postsensitization, LNC were harvested and stained with PE-labeled anti-CD11c mAb. The CD11c<sup>+</sup> FITC<sup>+</sup> cell population with large size and high granularity (forward scatter/FSC<sup>+</sup>)/side scatter (SSC<sup>+</sup>) was gated and analyzed.

**Cytokine-specific ELISPOT assays**

Hapten-specific ELISPOT assays to enumerate IFN-γ- and IL-4-producing T cells were performed as previously described (20). Briefly, ELISPOT plates (Millipore, Bedford, MA) were coated with 100 μl of 4 μg/ml anti-IFN-γ mAb R26A2 or anti-IL-4 mAb 1B11 and incubated overnight at 4°C. Plates were blocked with 1% BSA in PBS for 90 min at room temperature and washed four times with PBS. LNC from DNFB-sensitized mice were prepared on day 5 after sensitization. CD4<sup>+</sup> or CD8<sup>+</sup> T cell-enriched suspensions were used for additional assays.

**Flow cytometry**

LNC were obtained from hapten-sensitized mice on day 2 postsensitization, and hPLC were enriched by centrifuging LNC suspensions through a 14.5% metrizamide gradient and collecting the interface cells. Two-color flow cytometric analysis to assess the phenotype of hPLC was performed as previously described (19). To prevent nonspecific Ab binding, cells were incubated with rat serum (Rockland, Gilbertsville, PA) diluted 1/1000 in staining buffer (Dulbecco’s PBS with 2% FCS/0.2% NaN₃) for 20 min on ice. Then cells were washed and stained with PE-labeled anti-CD11c mAb and biotinylated anti-I-A<sup>+</sup> or anti-I-E<sup>+</sup> and anti-H-2<sup>D</sup> mAbs plus streptavidin-FITC. Stained cells were washed five times, resuspended in staining buffer, and analyzed by two-color flow cytometry using a FACScan (BD Biosciences, San Jose, CA).

**LC migration**

Mice were sensitized by single application of 0.25% DNFB on both sides of the ear. Two hours after sensitization ears were excised from sensitized or naive mice (four animals per group), and peeled ear halves were floated directly on 2 ml of complete RPMI 1640 medium in 24-well plates for 48 h. After this culture, the cells that migrated out of the ear skin into the medium were gently resuspended and counted using trypan blue. Cell suspensions obtained from the ears of mice in each experimental group were pooled and filtered to remove hair and debris, then stained with PE-labeled anti-CD11c mAb to detect Langerhans cells (LC) within the migratory cell population. Cell populations obtained using this method contained ~10–20% CD11c<sup>+</sup> cells with intermediate to high granularity (side scatter) as assessed by flow cytometry.

To assess hapten-presenting DC migration in vivo, mice were painted with 100 μl of 1% FITC on day 0. On day 3 postsensitization, LNC were harvested and stained with PE-labeled anti-CD11c mAb. The CD11c<sup>+</sup>FITC<sup>+</sup> cell population with large size and high granularity (forward scatter/FSC<sup>+</sup>)/side scatter (SSC<sup>+</sup>) was gated and analyzed.

**Results**

**CHS responses and hapten-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells**

To examine the potential effect of CD40 ligation on the induction and elicitation of CHS responses, mice were injected with either isotype control IgG Ab (control group) or anti-CD40 mAb FGK115 at the time of sensitization with DNFB. Ear swelling responses to DNFB challenge in control and Ab-treated groups were compared. The typical ear swelling response to sensitization and challenge with DNFB peaked 24 h after challenge and then quickly decreased in the control IgG-treated group. In contrast, both the magnitude and the duration of the CHS response were markedly increased in mice treated with anti-CD40 mAb (Fig. 1).

Because both hapten-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells participate in CHS responses, the effect of CD40 ligation on the development of these cell populations to cytokine-producing cells was tested by ELISPOT assay. Hapten-specific CD8<sup>+</sup> T cells producing IFN-γ were readily detectable on day 5 postsensitization in the lymph nodes of hapten-sensitized mice treated with control IgG and were low to undetectable in naive mice (Fig. 2A). The number of hapten-specific CD8<sup>+</sup> T cells producing IFN-γ was >3 times higher in anti-CD40 mAb-treated mice than that in the control group. The enhancing effect of anti-CD40 mAb treatment was dependent on Ag sensitization, as treatment of naive mice with anti-CD40 mAb did not result in increased IFN-γ-producing T cell numbers (not shown). As typically observed (21), low numbers of hapten-specific CD4<sup>+</sup> T cells producing IFN-γ were observed in the lymph nodes of sensitized mice treated with control IgG compared with the number of IFN-γ-producing CD8<sup>+</sup> T cells (Fig. 2B). Anti-CD40 mAb treatment also increased the number of hapten-specific CD4<sup>+</sup> T cells producing IFN-γ; however, the number of these cells was markedly less than the number of IFN-γ-producing CD8<sup>+</sup> T cells in the anti-CD40-treated group (Fig. 2, A vs B). The development of hapten-specific CD4<sup>+</sup> T cells producing IL-4 was also increased in anti-CD40 mAb-treated mice (Fig. 2C).

Together these results indicate that CD40 ligation has the greatest enhancing effect on the development of hapten-specific, IFN-γ-producing CD8<sup>+</sup> T cells, but also increases the development of
hapten-specific CD4+ T cells producing IFN-γ and IL-4 without an apparent shift toward either a type 1 or type 2 cytokine-producing phenotype.

CD8+ T cells are the major effector component of CHS responses in anti-CD40 mAb-treated mice

To test what cells are the primary mediators of CHS responses in anti-CD40 mAb-treated mice, the effects of anti-CD40 mAb treatment on CHS responses were compared in wild-type mice vs MHC II−/− and CD8−/− mice, which have CD4+ and CD8+ T cell deficiencies, respectively. Consistent with previous findings (18), CHS responses in DNFB-sensitized, MHC II−/− mice treated with control IgG were of higher magnitude and longer duration than those in wild-type mice due to the absence of regulatory CD4+ T cells in the MHC II−/− mice (Fig. 3, A vs B). CHS responses in MHC II−/− mice were further elevated after anti-CD40 mAb treatment. In contrast, CHS responses in sensitized CD8−/− mice were very low and were increased by anti-CD40 mAb treatment to a lesser extent compared with those in wild-type mice (Fig. 3, A vs C). The results indicate that the enhanced development of CD8+ T cells most likely accounts for the augmented CHS responses to hapten sensitization and challenge in mice treated with anti-CD40 mAb.

Endogenous IL-12 is not a crucial factor for CHS responses augmented by anti-CD40 mAb treatment

Because CD40 ligation on APC has been shown to increase the production of IL-12 (9–11), the requirement for this cytokine for anti-CD40 mAb-augmented CHS responses and effector CD8+ T cell development was tested. Preliminary studies indicated that neutralization of endogenous IL-12 by specific Abs at the time of sensitization and anti-CD40 mAb treatment did not decrease the magnitude of ear swelling responses or the development of IFN-γ-producing CD8+ T cells (not shown). To further investigate this, anti-CD40 mAb-mediated effects on CHS responses were compared in wild-type mice vs p35−/−/p40−/− (IL-12−/−) mice. Consistent with our previous studies indicating that IL-12 is not required for CHS responses (21), ear swelling responses in wild-type and IL-12−/− mice were nearly identical. Likewise, anti-CD40
mAb treatment resulted in significantly increased ear swelling responses in both wild-type and IL-12−/− mice (Fig. 4, A vs B).

After DNFB sensitization, the number of hapten-specific CD4+ T cells producing IFN-γ was low to absent in wild-type and IL-12−/− mice. However, hapten-specific CD4+ T cells producing IFN-γ were present in both wild-type and IL-12−/− mice treated with anti-CD40 mAb (Fig. 5A). In contrast to the priming of CD4+ T cells, hapten-specific CD8+ T cells producing IFN-γ were readily detectable, and their numbers were similar in wild-type and IL-12−/− mice sensitized with DNFB (Fig. 5B). Treatment with anti-CD40 mAb during sensitization increased these numbers >3-fold in both wild-type and IL-12-deficient mice. These results indicate that endogenous IL-12 is not a crucial factor for the augmented development of hapten-specific CD4+ and CD8+ T cells to IFN-γ-producing cells induced by anti-CD40 mAb treatment during hapten sensitization.

**CD40 ligation promotes migration of LC from skin**

To further investigate the mechanism of enhanced effector T cell development and CHS responses mediated by CD40 ligation, the potential effects of anti-CD40 mAb treatment on hplC were tested. First, the effect of CD40 ligation on LC migration from hapten-sensitized skin was examined. Groups of control IgG or anti-CD40 mAb-treated mice were either left unsensitized (naïve) or sensitized by applying 0.25% DNFB on the ears. Two hours after hapten application, the ears of the naive or sensitized mice were excised, split, and cultured in RPMI 1640 medium to test LC migration induced by hapten sensitization as described in *Materials and Methods*. After 48 h of culture, the numbers of LC that had migrated out of the ear skin from the different groups were compared. The number of LC that migrated from the epidermis of hapten-sensitized mice was significantly higher than the number of LC that migrated from the epidermis of naïve mice, indicating that hapten sensitization increases LC migration in this ex vivo model (Fig. 6A, gate R7). Consistent with previous observations (22), LC migration from ears of naïve mice treated with anti-CD40 mAb was significantly increased compared with that of naïve mice not treated with anti-CD40mAb (Fig. 6B). Remarkably, LC migration from the ears of hapten-sensitized mice treated with anti-CD40 mAb was increased >2-fold compared with that of LC from the control sensitized group (Fig. 6B). Collectively, these results indicate that CD40 ligation enhances LC migration from hapten-sensitized skin.

To test the effect of CD40 signaling on hplC migration in vivo, the numbers of hapten-bearing DC in the skin-draining lymph nodes of control IgG- or anti-CD40 mAb-treated mice sensitized with FITC were compared. Application of FITC allows detection of hapten-presenting DC, including epidermal LC, as FITC+/CD11c− cells in the skin draining lymph nodes (23). Mice were painted with FITC, and 72 h later LNC suspensions were stained for the DC marker CD11c. Low numbers of FITC−/CD11c− resident DC were present in the lymph nodes of naïve mice. Previous studies have indicated that LC can be distinguished from other cells in lymphoid tissues by their size and granularity (24). To distinguish hplC from other cells, including resident DC labeled with FITC due to hapten flow into lymph nodes, cells with large size and high granularity (FSChigh/SSCmedium) were gated and then analyzed for CD11c and FITC expression. LNC from FITC-sensitized animals contained a majority of FITC+/CD11c− cells within this cell population, indicating the presence of haptenerecting LC that had emigrated from the epidermis to the lymph nodes (Fig. 7A, upper panel, gate R10). CD11c−/FITC+ cells with intermediate size and granularity represent the resident DC expressing hapten (Fig. 7A, lower panel, gate R10). Based on these preliminary studies, the FSCmedium/SSCmedium/CD11c−/FITC+ population was identified as the FITC-presenting LC, and the sizes of this cell population in the lymph nodes of control IgG vs anti-CD40

**FIGURE 4.** CHS responses in control or anti-CD40 mAb-treated mice are independent of IL-12. BALB/c wild-type mice (A) or IL-12-deficient, p55−/−/p40−/− mice (B) were sensitized and treated with rat IgG (⧫) or anti-CD40 mAb (▲). On day 5, sensitized or naïve (○) mice were ear-challenged, and the increase in ear thickness was measured at 24-h intervals. The mean increase in ear thickness after challenge is shown in 10−4 in. ± SEM for groups of four mice. *, p < 0.05.
mAb-treated mice were compared. The number of hpLC in the lymph nodes of anti-CD40 mAb-treated mice was clearly increased compared with that in the control rat IgG-treated group (Fig. 7B, gate R10). These results indicate that CD40 ligation increases hpLC number in the skin draining lymph nodes at the time of hapten-specific T cell priming for CHS responses.

**FIGURE 6.** CD40 ligation enhances LC migration from the epidermis. Mice were sensitized by DNFB application on the ears (four mice per group). Ears were excised from naive or hapten-sensitized mice 2 h after sensitization, separated into dorsal and ventral halves, and cultured in complete RPMI 1640 for 48 h. Total cells migrating into the medium were harvested and stained with PE-labeled anti-CD11c mAb to detect LC. CD11c-expressing cells with high granularity (SSC) were gated as LC (A). The numbers inside the R7 gate indicate the percentage of LC in the total cell population that migrated from the split ear halves. B, The total numbers of cells that migrated from each ear of naive and sensitized mice treated with or without anti-CD40 mAb were counted, and LC numbers were calculated. The mean number ± SEM of LC per ear is shown for each group of four mice. *, p < 0.05 compared with migration from ears of naive mice not treated with anti-CD40 mAb; **, p < 0.05 compared with migration from ears of control sensitized mice.
CD40 ligation promotes maturation of LC

The phenotypes of hpLC isolated 24 h postsensitization from lymph nodes of hapten-sensitized control and anti-CD40 mAb-treated mice were compared. Because hpLC prime hapten-specific CD4+ and CD8+ T cells in a MHC class II- and class I-restricted manner, respectively, the expression of these molecules on hpLC was assessed by flow cytometry. The hpLC-enriched cell suspensions were obtained from mice treated with control IgG or anti-CD40 mAb during sensitization. CD11c-positive cell populations were gated and analyzed for the percentage of CD11c+/FITC+ (gate R10) or CD11c+/FITC− (gate R11). The numbers indicate the percentage of gated cells in the total CD11c+ cell population. B, Mice were sensitized with FITC and treated with rat IgG(control) or anti-CD40 mAb (anti-CD40 Tx) at the time of sensitization (three mice were used in each experimental group). On day 3 postsensitization, LNC from each individual mouse were stained with PE-labeled anti-CD11c mAb to detect DC. Cells with large size and high granularity (FSC high/SSC high) were gated (gate R7), and hpLC were defined within this population as FITC high/CD11c high cells (gate R10). The numbers in the dot plot panels indicate the number of hpLC per 2 × 10^4 LNC aliquot. The numbers outside the dot plots indicate the mean hpLC number in control vs anti-CD40 mAb-treated groups for three mice per group. p < 0.05.

The expression of MHC class I on hpLC was also up-regulated after anti-CD40 mAb treatment compared with that in the control IgG-treated group (Fig. 8B). These results indicated that CD40 signaling promoted hpLC maturation as well as increased migration from the skin sensitization site.

It was noteworthy that the expression of MHC molecules was also increased on CD11c-negative cell populations within the hpLC-enriched fraction from anti-CD40 mAb-treated mice (Fig. 8, A and B). To identify these cells, the CD11c-negative cell population was analyzed for the expression of markers identifying B cells and macrophages. The results indicated that the majority of CD11c-negative cells with up-regulated MHC class II expression were B cells (Fig. 8C, B220+ panel). To test the potential influence of these B cells expressing high levels of class II MHC on the magnitude of CHS responses, groups of wild-type and B cell-deficient μM T−/− mice were sensitized and treated with control IgG.
FIGURE 8. Treatment with anti-CD40 mAb increases class I and class II MHC expression on hpLC and B cells during hapten sensitization. A, The hpLC-enriched cell suspensions from pooled skin draining lymph nodes of sensitized control or anti-CD40 mAb-treated C57BL/6 mice (10 animals/group) were stained with PE-labeled anti-CD11c mAb and biotinylated anti-I-Ab mAb plus streptavidin-FITC. CD11c-positive cell populations were gated and analyzed by histogram for MHC class II expression. The numbers in the histogram panels indicate the mean percentage of gated cells expressing intermediate and high levels of MHC class II. $p < 0.05$ when the percentages in gates M1 and M2 were compared between control and anti-CD40-treated groups. B, The hpLC-enriched cell suspensions from sensitized control and anti-CD40 mAb-treated C57BL/6 mice were stained with PE-labeled anti-CD11c mAb and a mixture of biotinylated anti-H-2Kb and H-2Db mAb plus streptavidin-FITC. CD11c-positive cell populations were gated and analyzed by histogram for MHC class I expression by hpLC isolated from control mice (dashed line) or anti-CD40 mAb-treated mice (solid line). $p < 0.05$ when the mean channel fluorescence was compared in control vs anti-CD40-treated groups. C, The hpLC-enriched cell suspensions from pooled skin draining lymph nodes of sensitized control rat IgG- or anti-CD40 mAb-treated C57BL/6 mice were stained with PE-labeled anti-CD11c mAb, FITC-labeled anti-B220, or anti-CD11b mAb and with biotinylated anti-I-Ab mAb plus streptavidin-PerCP. CD11c-positive and CD11c-negative cell populations were gated and analyzed by histogram for MHC class II expression. The numbers in the histogram panels indicate the percentage of gated cells expressing intermediate and high levels of MHC class II.
Anti-CD40 mAb stimulates cultured DC and macrophages to increase the expression of class II MHC and ICAM-1 and induces or increases IL-12 production (9–11). These studies led us to test the effect of anti-CD40 mAb given during hapten sensitization on the development of hapten-specific T cells and the CHS response. Anti-CD40 mAb increased the magnitude and duration of CHS responses, similar to the effect observed during rIL-12 treatment of sensitized mice. However, the mechanism mediating the increase in CHS responses after anti-CD40 mAb treatment was distinct from that after treatment with rIL-12. First, there was no apparent switch of hapten-specific CD4+ T cell development from Th2- to Th1-cytokine producing cells, as the numbers of both IFN-γ-producing and IL-4-producing CD4+ T cells were increased in the lymph nodes of anti-CD40 mAb-treated mice. This suggests that anti-CD40 mAb stimulated hpLC to promote the development of hapten-specific CD4+ T cells producing IFN-γ as well as augmented the expansion of IL-4-producing CD4+ T cells. Second, the number of IFN-γ-producing CD8+ T cells was increased by anti-CD40 mAb treatment more markedly than the number of IFN-γ-producing CD4+ T cells, indicating that CD40 ligation has the greatest enhancing effect on hapten-specific CD8+ T cells during the development of CHS. Consistent with these results, the magnitude and duration of CHS responses in CD8-/- mice remained low even after anti-CD40 mAb treatment, indicating that CD4+ T cells are unable to substitute for the effector CD8+ T cells in these mice. Furthermore, no differences in the histology of ear swelling responses were observed in control and anti-CD40 mAb-treated mice (A. Gorbatchev, unpublished observations). Together these results suggested different mechanisms mediating the increase in CHS responses and hapten-specific T cell development by rIL-12 vs anti-CD40 mAb treatment.

The role of IL-12 in anti-CD40 mAb augmentation of CHS was directly tested in these studies. Preliminary studies indicated that treatment of sensitized, anti-CD40 mAb-treated mice with a mixture of anti-IL-12 mAb did not decrease CHS responses or hapten-specific IFN-γ-producing CD8+ T cell development. The effects of anti-CD40 mAb on CHS responses were also compared in wild-type and IL-12-deficient p35-/-/p40-/- mice. The development of ear swelling responses and hapten-specific, IFN-γ-producing CD8+ T cells was independent of IL-12 production in both control IgG and anti-CD40 mAb-treated mice. Although IFN-γ-producing CD8+ T cells were absent in control sensitized, IL-12-deficient mice, these CD8+ T cells were readily detectable in anti-CD40 mAb-treated, IL-12-deficient mice. These results indicate that increased IL-12 production during hapten sensitization is not critical for the development of CHS responses augmented by CD40 ligation and suggest that different mechanisms mediate increased CHS responses and hapten-specific T cell development produced by rIL-12 vs anti-CD40 mAb treatment.

As hpLC prime both hapten-specific CD4+ and CD8+ T cells, the potential effect of CD40 ligation on the phenotype of hpLC was examined to further explore potential mechanisms enhancing hapten-specific T cell development. Recent in vitro studies demonstrated up-regulated expression of B7 and MHC class II molecules by CD40 ligation on APC, including DC subsets (9, 11, 26). In the current study, up-regulation of class I as well as class II MHC was observed on hpLC isolated from lymph nodes of anti-CD40 mAb-treated, hapten-sensitized mice. These results suggest that the increased MHC expression on hpLC may account at least in part for
the augmented development of both hapten-specific CD4⁺ and CD8⁺ T cells during sensitization. B cells and macrophages express CD40, and agonist anti-CD40 mAb treatment increases class II MHC expression on lymph node B cells, but more modestly on macrophages. However, anti-CD40 mAb enhanced CHS responses in B cell-deficient mice to the same degree as in wild-type mice. These results indicate that B cells are not critical for the priming and effector function of hapten-specific T cells mediating CHS responses. In contrast to these results, recent studies have suggested a role for B cells in the elicitation of CHS (27). The expression of CD40 has been reported on CD8⁺ T cells (28), raising questions about the effects of anti-CD40 mAb directly on hapten-specific CD8⁺ T cells during sensitization. Intensive Ab staining and analyses failed to detect CD40 expression on CD8⁺ T cells in skin draining lymph nodes after hapten sensitization (A. Gorbachev, unpublished observations).

Previous studies from this laboratory indicated equivalent levels of hapten-specific CD8⁺ T cell priming and CHS responses in wild-type vs CD154⁻/⁻ mice (20). These results were not surprising in light of the CD4 independence of hapten-specific CD8⁺ T cell priming for CHS and the absence of CD154 expression on these CD8⁺ T cells. Similarly, results indicating the ability of exogenously administered IL-12 to enhance hapten-specific CD8⁺ T cell development in CD154⁻/⁻ mice were not surprising in light of recent studies from Mescher’s laboratory indicating that IL-12 provides direct costimulatory signals to CD8⁺ T cells when provided with TCR-mediated signals (29). However, the ability of anti-CD154 mAb to inhibit CD8⁺ T cell priming and CHS was unexpected. This inhibition was found to be dependent on CD4⁺ T cells that up-regulate CD154 expression during hapten priming and is mediated through anti-CD154 mAb enhancement of CD4⁺ T cell regulatory activity (20). The enhanced CD4⁺ T cell regulatory activity is directed at hpLC function and, in turn, attenuates CD8⁺ T cell priming for the CHS response. We posit that during hpLC priming of hapten-specific CD4⁺ T cells, CD40-CD154 interactions are not sufficiently strong to enhance the regulatory activity inhibiting hpLC function and effector CD8⁺ T cell development. However, ligation of CD154 on the CD4⁺ T cells using the agonist mAb enhances regulatory CD4⁺ T cell activity in quickly down-regulating the response through hpLC (20). The current study has focused on LC expressed CD40 as a target molecule. In contrast to the agonist CD154 mAb that enhances regulatory CD4⁺ T cell activity, the agonist CD40 mAb stimulates and enhances the function of hpLC, resulting in increased development of the CHS effector CD8⁺ T cell compartment (Fig. 10). The implication of these results is not necessarily physiological in nature with regard to the role of CD40 engagement during sensitization in unmanipulated wild-type animals, but is nevertheless important in demonstrating a strategy to enhance CD8⁺ T cell-mediated responses to Ags in the skin, where strong CD8⁺ T cell immunity is needed, such as during cutaneous viral infections and tumor development.

In addition to increased T cell stimulation, anti-CD40 mAb treatment during sensitization increased the number of hpLC migrating to the skin draining lymph nodes. Two possible mechanisms could account for this increase. First, CD40 ligation on hpLC could promote their migration to lymph nodes during hapten sensitization. Studies from other laboratories have indicated an increased migratory capacity of DC after Ab-mediated cross-linking of CD40 (22, 30). Consistent with this, increased LC migration from the epidermis induced by hapten sensitization was observed using an ex vivo model. Second, CD40 signaling could prolong...
mediated immune responses to eradicate infections or malignant fi
sensitization promotes hpLC migration and maturation. The in-
during hapten priming is under investigation in this laboratory.
CD40 signal in the increase in hpLC migration and/or survival
periods of speci
protect cells from apoptosis and would sustain the viability and
sources Unit for excellent animal care.
We thank the staff of the Cleveland Clinic Foundation Biological Re-
2452 ANTI-CD40 mAb ENHANCES T CELL PRIMING INDEPENDENT OF IL-12
8. Bour, H. E., Peyron, M. Gaucherand, J. L. Garrigue, C. Desvignes, D. Kaiserlan,
25. Dihlulio, N. A., H. Xu, and R. L. Fairchild. 1996. IL-12 diverts development of CD4+ T cells from regulatory Th2 to effector Th1 cells in contact hypersensitiv-

Acknowledgments
We thank the staff of the Cleveland Clinic Foundation Biological Re-

References
1. LaFerty, K. J., and A. Cunningham. 1975. A new analysis of allogeneic inter-