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Combined TLR/CD40 Stimulation Mediates Potent Cellular Immunity by Regulating Dendritic Cell Expression of CD70 In Vivo

Phillip J. Sanchez,* Jennifer A. McWilliams,* Catherine Haluszczak,* Hideo Yagita, † and Ross M. Kedl2*

We previously showed that immunization with a combination of TLR and CD40 agonists (combined TLR/CD40 agonist immunization) resulted in an expansion of Ag-specific CD8 T cells exponentially greater than the expansion observed to immunization with either agonist alone. We now show that the mechanism behind this expansion of T cells is the regulated expression of CD70 on dendritic cells. In contrast to previous results in vitro, the expression of CD70 on dendritic cells in vivo requires combined TLR/CD40 stimulation and is not significantly induced by stimulation of either pathway alone. Moreover, the exponential expansion of CD8+ T cells following combined TLR/CD40 agonist immunization is CD70 dependent. Thus, the transition from innate stimuli (TLRs) to adaptive immunity is controlled by the regulated expression of CD70.


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t is generally agreed upon that the successful immunotherapeutic treatment of chronic infectious diseases and cancer requires the generation of a strong cellular immune response. Vaccines based on attenuated infectious agents typically generate some degree of cellular immunity, but their use in the clinic is often complicated by a number of problems ranging from the practical concerns of vaccine production and storage to public health issues, such as adverse reactions or reversion to virulence in some portion of the population. Additionally, not all pathogens can be attenuated for use as a vaccine. Therefore, one of the main goals of vaccine development is to create a noninfectious vaccine that mimics a natural infection’s ability to stimulate a strong cellular immune response. As such, much effort has concentrated on the development of novel and potent vaccine adjuvants. Unfortunately, the majority of vaccine adjuvants developed thus far have not facilitated the generation of clinically significant cell-mediated immunity. At this point, the only approved vaccine adjuvant for general clinical use is alum, which is inadequate for generating cellular responses (1). Additionally, alum preferentially promotes the formation of Th2-type responses, which can be counterproductive to the kinds of inflammatory responses necessary to eradicate established chronic diseases (2–4).

Agonists for TLRs represent a class of vaccine adjuvant that has been the topic of intense study in recent years. Various bacterial- or viral-derived molecular structures (di- and triacylated lipoproteins, ssRNA and dsRNA, LPS, flagellin, unmethylated CpG sequences) are agonists for 1 of 10 known human TLRs and are potent activators of innate immunity (5). Cells important in both innate and adaptive immunity, such as dendritic cells (DCs), express high levels of various TLRs, and stimulation of a DC through its TLR(s) generally results in the activation of the DC to produce cytokines, upregulate costimulatory marker expression, and migrate into T cell areas of lymphoid tissue (4, 6). As such, TLR agonists appear to be optimal for use as vaccine adjuvants for the generation of cellular immunity. However, the use of purified TLR agonists as vaccine adjuvants has been disappointing at best, demonstrating an inability to generate T cell responses on par with the responses observed during an actual infection (7–16). These results have been both surprising and frustrating to those in the field attempting to construct molecular vaccine formulations as alternatives to attenuated infectious vaccines. Collectively, the data are most consistent with a necessary but not sufficient role of TLR stimulation in the progression of events that leads to protective cellular immunity.

Although the development of a cellular immunity-generating vaccine has been fraught with complications, a number of signaling pathways and receptor/ligand pairs have been identified which have the potential, if they could be targeted, to significantly influence the development of CD4 and CD8 T cell responses. One such signaling receptor/ligand pair is CD27/CD70. Although the downstream signaling events are not completely defined, CD27/CD70 interactions are critical for the development of potent cell-mediated immunity in a number of model systems. Mice deficient in CD27 exhibit both impaired primary and memory antiviral T cell responses (17). Conversely, enhanced stimulation of CD27 through either forced cellular expression or soluble administration of CD70, results in enhanced T cell expansion, antiviral immunity,

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and antitumor CD8 T cell responses (18–29). Thus, the development of a vaccine strategy capable of facilitating the CD27-CD70 interaction would be invaluable.

Although it is clear that the forced expression of CD70 can significantly augment T cell expansion, the natural regulation and cellular expression of CD70 in vivo is largely unknown. Most studies describing the induction of CD70 expression on various cell types have used in vitro model systems examining in vitro-stimulated cells (20, 27, 29, 30). For example, studies have shown that bone marrow-derived DCs, T cells, and B cells could be induced by various stimuli to express CD70 in vitro (20, 27, 30, 31). However, the little in vivo data available have examined CD70 expression/induction on various cell types in nonlymphoid tissues (30, 32), or in Rag-deficient hosts (33), and essentially no data are available regarding CD70 expression on normal DC subsets in vivo. As a result, the exploitation of the CD27-CD70 interaction for vaccine purposes has remained elusive.

We previously demonstrated that immunization with Ag in combination with both a TLR agonist and an anti-CD40 Ab (combined TLR/CD40 agonist immunization) induces potent CD8 T cell expansion (8). After a single immunization, this response is 10- to 20-fold higher than immunization with either agonist alone and is on par with the magnitude of T cell expansion often observed in response to infectious agents such as lymphocytic choriomeningitis virus or Listeria monocytogenes.

The only mechanistic data available at the time determined that the T cell response to this form of immunization demonstrated an intriguing variable dependence upon type I IFN (8). In our present findings, we demonstrate that the mechanism underlying the CD8 T cell expansion in response to combined TLR/CD40 agonist immunization is determined by the expression of CD70. In contrast to previous in vitro (20, 27, 29, 30) and in vivo (33) data, the up-regulation of CD70 expression on normal DC subsets in vivo is not induced by either TLR or CD40 stimulation alone, but requires the combined stimulation of TLR/CD40. This expression of CD70 plays an essential role in mediating the expansion of CD8 T cells following combined TLR/CD40 agonist immunization. In addition to providing a mechanism behind the CD8 T cell responses elicited from combined TLR/CD40 agonist immunization, the data show that the induction of CD70 on DCs is a central mechanism by which innate signaling pathways integrate with CD40 for the induction of cellular immunity.

Materials and Methods
Mice and injections
Six- to 12-wk-old C57BL/6 mice purchased from The Jackson Laboratory were immunized with 0.1–0.5 mg of whole OVA as previously described (8). In brief, whole protein was injected (i.p. or s.c.) in combination with a TLR agonist (50 μg of N-palmitoyl-S-[2,3-bis(palmitoyloxy)-(2,5)-propyl]- (R)-cysteine-(S)serine-(S)lysine 4 (PamCys), 100 μg poly(I:C), 150 μg of polyinosinic-polycytidylic acid (poly[I:C]), 10 μg of flagellin, 100 μg of polyinosinic-polyribidylic acid (poly[I:C]), 150 μg of CpG2006, 100 μg of CpG1826, and 50 μg of CpG1826), the anti-CD40 Ab FGK45 (50 μg), or both. OVA was purchased from Sigma-Aldrich and contaminating LPS was removed using a Triton X-114 LPS detoxification methodology as previously described (34). Following this detoxification method, the OVA was tested for LPS contamination by Limulus assay and found to have <1 endotoxin unit of LPS activity (−0.1 ng) for the amount injected in vivo. Injection of this amount of LPS has no observable effects on spleen DCs in vivo (data not shown). The TLR agonists used were either purchased (PamCys, InvivoGen; poly(I:C), Amersham Biosciences/GE Healthcare), provided through a material transfer agreement (27609, 3M012, 3M Pharmaceuticals), or isolated in house (flagellin). Flagellin was decontaminated of LPS using the same protocol as described above for OVA detoxification. TNF ligand (TNF-L)-blocking Abs (see below) were injected i.p. between days −1 and +2 of Ag challenge. For vaccinia virus challenge, mice were injected i.p. with 0.5–1 × 107 PFU of an OVA-expressing vaccinia virus (VVova (35)).

Antibodies
CD1c allophycocyanin (clone HL3), CD11b PerCP-Cy5.5 (clone M170), CD6 allophycocyanin-Cy7 (clone 53-6,7), CD70 PE (clone FR70), CD86 FITC (clone GL1), CD80 FITC (clone 16-10A1), CD40 FITC (clone 3/23), and H-2K β FITC (clone F6-88.5) were purchased from BD Pharmingen. CD30L PE (clone RM153), OX40L PE (clone RM134L), and 4-1BBL PE (clone TKS-1) were purchased from eBioscience. PDCA-1 biotin (clone JF05-1C2.4.1) was purchased from Miltenyi Biotec. Streptavidin-Pacific Blue was purchased from Molecular Probes.

Purified CD70 (clone FR70), CD30L (clone RM153), OX40L (clone RM134L), and 4-1BBL (clone TKS-1) were produced as previously described (24).

FIGURE 1. Combined TLR/CD40 agonist immunization generates potent primary and memory CD8 T cell responses. A, C57BL/6 mice were immunized i.p. with the indicated combinations of 500 μg of whole OVA (<0.5 endotoxin units), 50 μg of anti-CD40 Ab FGK45, 100 μg of poly(I:C) (TLR3 agonist), or 50 μg of PamCys (TLR1/2 agonist). Seven days after immunization (primary), PBLs were isolated and stained with KIN4F/PE FLJ1 tetramer, tetramer, and CD8 T cells were analyzed by Kb/B8R tetramer staining 7 days after challenge as in A. C, Mice were primed with the combined TLR/CD40 agonist immunization as in A. Seventy-five days later, the mice were challenged with 5 × 107 PFU of VVova. Naïve mice were challenged with VVova as a control. Five days after challenge, the mice were sacrificed and Kb/SIINFEKL tetramer staining was performed on PBLs as described in A. The dot plots shown are representative of two mice per treatment group. The results shown are representative of at least four experiments performed.
TLR agonists
The TLR7 agonist S27609 (27609) (6) and the related molecule 3M012 (9) were synthesized at 3M Pharmaceuticals as previously described (36). They were reconstituted in either water (27609) or DMSO (3M012) at 10 mg/ml and diluted in PBS for injection into mice. Other TLR agonists used were poly(I:C) (Amersham Biosciences/GE Healthcare), Pam3Cys (InvivoGen), monophosphoryl lipid A (MPL) or LPS (Sigma-Aldrich), and flagellin isolated from Salmonella choleraesuis subsp. choleraesuis serovar Minnesota (American Type Culture Collection) as previously described (37, 38).

Cell preparation
Seven days after primary challenge (i.p. or s.c.) or 5 days after secondary challenge, PBLs were isolated via tail vein or dorsal aorta bleed. In some experiments, spleens were removed and homogenized into single-cell suspensions. RBC were lysed using an ammonium chloride buffer followed by washing. Cells were pelleted by centrifugation and subsequently resuspended in ammonium chloride buffer to lyse RBC. Cells were then washed and resuspended in HBSS containing 5 mM EDTA and 0.1% FBS.

Enrichment and phenotype of DCs
B6, B6 MyD88−/−, or B6 Rag1−/− mice were challenged i.p. or s.c. with a given TLR agonist, anti-CD40, or both. At 12–48 h after challenge, spleens were removed, placed into wells of a 6-well plate containing 2 ml of click’s (EHAA) medium containing collagenase D (0.5 mg/ml) and DNase (50 µg/ml), and teased apart with forceps. Plates were incubated at 37°C for 40 min, after which 2 ml of 0.1 MEDTA (in HBSS) was added to each well and incubated an additional 5 min at 37°C. The cell suspension was passed through a strainer into a 50-ml tube and washed with 5 mM EDTA (in HBSS). Cells were pelleted by centrifugation and subsequently resuspended in ammonium chloride buffer to lyse RBC. Cells were then washed and resuspended in HBSS containing 5 mM EDTA and 0.1% FBS. For the DC phenotype, 5 × 10^6 cells were first stained with PDCA-1 biotin and then washed twice in FACS buffer (PBS containing 0.1% FBS and 0.1% sodium azide). The cells were then stained with CD11c allophycocyanin, CD11b PerCP-Cy5.5, CD8 allophycocyanin-Cy7, streptavidin-Pacific Blue, and a FITC- and PE-conjugated Ab specific for the indicated activation marker. Five- or six-color flow cytometry was performed on a CyAn LX flow cytometer (DakoCytomation) and analyzed with Weasel version 2.2 software (The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia).

Tetramer staining and flow cytometry
Cells were stained with Kb/OVA tetramer as previously described (35). Briefly, cells were plated in 96-well plates and stained with tetramer for 1.5 h at 37°C. Abs against CD8, CD44, CD27, and B220 were added, and the cells were incubated an additional 20 min at 37°C. The cells were then washed, fixed, and resuspended in FACS buffer for flow cytometric analysis. Four- to five-color FACS data were collected on a BD FACScan flow cytometer, retrofitted with a second laser (Cytek), using CellQuest and Rainbow software, and analyzed using CellQuest software. For analysis,

![FIGURE 2. Combined TLR/CD40 agonist immunization elicits potent protective CD8+ T cell memory. C57BL/6 mice were immunized with 50 µg B8R peptide and the indicated combinations of anti-CD40 (50 µg) and/or TLR agonists (50 µg of CpG1826 or 100 µg of 3M012). A, Peripheral blood was taken by tail vein 7 days later and stained with tetramer to identify B8R-specific CD8+ T cells as described in Materials and Methods. Control mice were challenged with VVova and analyzed for their B8R-specific CD8+ T cell response. Numbers in the dot plots indicate the percent tetramer positive of total CD8+ T cells. B, Sixty days after initial priming, the mice were then challenged with 5 × 10^6 PFU of VVova. Five days after challenge, the T cell responses were again measured by tetramer staining of peripheral blood. Control mice were both primed and boosted with VVova. C, Ovaries from the mice in B were removed, and viral titers were determined by plaque assay. Horizontal line, Lower limit of detection of the plaque assay. Each treatment group had three mice per treatment. Error bars, SD.](http://www.jimmunol.org/DownloadedFromHttp://www.jimmunol.org/ByGuestOnJuly26,2017)
the data were gated on CD8+ B220− events and then analyzed for tetramer staining by the activation marker CD44, the Ag-specific cells being CD44high. CD27/tetramer staining was also analyzed and gave results essentially identical to those of CD44/tetramer.

**Results**

**Combined TLR/CD40 agonist immunization generates primary and secondary CD8+ T cell immunity comparable to viral challenge**

We previously reported that immunization of a host with Ag in the presence of agonists for both CD40 and a TLR (combined TLR/CD40 agonist immunization) generates CD8+ T cell responses exponentially larger than those observed in response to immunization with either agonist alone (8). Given the magnitude of the response to this form of immunization, we sought to determine whether combined TLR/CD40 agonist immunization promoted both primary and secondary protective immunity on par with viral challenge. Mice were immunized with the indicated combinations of Ag (OVA), an agonistic anti-CD40 Ab (FGK45), and a TLR agonist. Seven days later, peripheral blood was isolated from the mice and stained with Kb/SIINFEKL MHC class I tetramers to identify Ag-specific CD8+ T cells. Similar to previously published data of splenic CD8 responses (8), the combined TLR/CD40 agonist immunization elicited a potent expansion of OVA-specific CD8+ T cells, detectable in the peripheral circulation, compared with any other immunization (Fig. 1A). These Ag-specific cells in the peripheral blood were also functional with respect to IFN-γ production and lytic function (data not shown and Ref. 8).

The stimulation of DCs in vitro with combinations of TLR agonists has been reported to have a synergistic effect both on the activation of the DC as well as on the subsequent T cell response stimulated by the DCs (39). However, we found that in vivo, immunization of mice with similar combinations of TLR agonists had little effect on the subsequent T cell response (Fig. 1B). This was also true of other combinations of TLR agonists such as MPL (TLR4) and 27609 (TLR7) and 27609 and CpG1826 (TLR9) (data not shown). At best, the combinations of TLR agonists had an additive effect on the resulting T cell response (data not shown), but synergy to the same degree as combined TLR/CD40 agonist immunization was never observed (Fig. 1B).

In addition to inducing potent primary CD8+ T cell expansion, combined TLR/CD40 agonist immunization resulted in the generation of a competent pool of memory cells capable of even greater...
expansion upon secondary challenge. Seventy-five days after initial combined TLR/CD40 agonist immunization, the mice were challenged with VVova. VVova challenge induced a robust secondary response of the OVA-specific T cells in the peripheral blood (Fig. 1C). Interestingly, a secondary expansion of CD8 T cells was minimally detectable in the spleen or lymph nodes following VVova challenge (data not shown). In previously published work, we also observed minimal memory responses in the lymph nodes and spleen following secondary challenge with either peptide or protein (8). This indicates that the detectable response in these sites is a vast underestimate of the total secondary response, consistent with previous reports from the literature (40, 41).

To establish the degree to which combined TLR/CD40 agonist was able to provide protective immunity, mice were primed against the dominant vaccinia class I peptide epitope B8R (16), in the context of the indicated combinations of TLR agonist, anti-CD40, or vaccina virus (Fig. 2A). Sixty days later, the mice were challenged with vaccina virus, and the secondary expansion of memory B8R-specific CD8 T cells (in peripheral blood) and viral titers (in the ovary) were measured 5 days after challenge. Prior immunization with combined TLR/CD40 agonists again resulted in an elevated secondary expansion of memory B8R-specific T cells (Fig. 2B), as well as the elimination of viral replication (Fig. 2C). In contrast, prior immunization with either TLR or CD40 agonists alone resulted in only minimal reduction in viral titers compared with virus-challenged naive mice. Thus, combined TLR/CD40 agonist immunization elicits potent primary CD8 T cell responses that culminate in the generation of protective memory.

**CD70 expression is regulated in vivo by combined TLR and CD40 signaling**

We next sought to determine the mechanism responsible for the synergy between the TLR and CD40 pathways for inducing such potent CD8 T cell responses. Given their constitutive expression of CD40 and large repertoire of TLRs (4), we reasoned that DCs were the likely cellular target of the coadministered anti-CD40 Ab and the TLR agonist. Upon stimulation through either molecular pathway, DCs are activated to migrate into T cell zones of lymphoid organs, produce cytokines, and increase expression of

![FIGURE 4. TLR/CD40 agonist stimulates a greater increase in surface CD70 than other members of the TNFL superfamily. A. DC subset surface expression of TNFL superfamily members. Mice were injected i.p. with agonistic anti-CD40 Ab (50 μg), poly(I:C) (TLR3 agonist, 100 μg), or both. At 6, 12, 24, and 36 h, mouse splenocytes were stained for six-color flow cytometry. Data shown are the MFI of the indicated surface marker at each time point. The same scale was used for each DC subset within the indicated surface marker to show the relative expression level between DC subsets. Error bars, SD. Graphs are representative of similar data obtained using Pam3Cys (TLR1/2), MPL (TLR4), and 27609 (TLR7) as the TLR agonist. B. Relative increase in surface expression of TNFL superfamily members on DC subsets. Values represent the fold increase, over naive controls, of the surface marker expression at 24 h postinjection (the peak of expression stimulated by TLR/CD40 agonist combination). The fold increase was calculated as the MFI of the surface marker on the DC from stimulated mice divided by the MFI of the same surface marker on DC from unstimulated mice. The CD70 graphs were generated from the means of two independent experiments. Error bars, SE. Values in the 4-1BBL, OX40L, and CD30L graphs represent the mean ± SD from single experiments.](http://www.jimmunol.org/)

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costimulatory markers (4, 6). These changes in DC phenotype during activation are crucial for priming of naive T cells and the development of Ag-specific immunity. We therefore determined whether there was a change in DC phenotype that was unique to the combination of anti-CD40 and TLR agonist compared with either stimulus alone. To examine the phenotype of activated splenic DCs, mice were injected with a TLR agonist, anti-CD40, or both. Splenic CD8<sup>+</sup>, CD11b<sup>+</sup>, and PDCA-1<sup>+</sup> DCs (CD11c<sup>-</sup>) were analyzed directly ex vivo at 6, 12, 24, and 36 h after the injection for the expression of the surface markers CD80, CD86, CD40, and CD70.

The expression of any of these surface markers was relatively unaffected on PDCA-1<sup>+</sup> DCs (Fig. 3B) and, as a result, phenotypic data for this DC subset is not shown in subsequent experiments. In CD8<sup>+</sup> and CD11b<sup>+</sup> DCs, administration of agonistic anti-CD40 Ab or TLR agonist alone induced varying levels of increased CD80, CD86, and CD40 expression, which peaked at 6–12 and 24–36 h, respectively (Fig. 3B). The increase in these activation markers was prolonged in mice challenged with combined TLR/CD40 agonists. However, only combined TLR/CD40 stimulation induced significant up-regulation of CD70 expression on the major DC subsets (Fig. 3B), with little or no increase in CD70 observed in response to challenge with either agonist alone. Surface levels of CD70 were increased within 6–12 h of administration and were sustained at their peak levels between 24 and 36 h (Fig. 3B). Therefore, of the markers analyzed, only the expression of CD70 correlated directly with the magnitude of the CD8<sup>+</sup> T cell response observed following individual or concomitant stimulation of TLR and CD40.

CD70 expression is uniquely regulated by TLR/CD40 agonist stimulation compared with other TNFLs

CD70 is a member of the TNFR ligand superfamily. Other members of the TNFL superfamily, including CD30L, OX40L, and 4-1BBL, can have costimulatory effects on T cells and can function to sustain T cell responses after initial T cell activation (42). We examined the expression of these molecules on activated splenic DCs from mice that were injected with TLR agonist with or without anti-CD40 as described above.

The expression of CD70, 4-1BBL, OX40L, and CD30L remained unchanged on CD8<sup>+</sup> DCs in mice treated with agonistic anti-CD40 Ab alone (Fig. 4A). The same treatment resulted in a slight increase in the expression of 4-1BBL, OX40L, and CD30L on the CD11b<sup>+</sup> DCs (Fig. 4A). TLR agonist alone stimulated a small transient increase in the expression of 4-1BBL and OX40L and a more sustained increase in CD30L on the CD8<sup>+</sup> DC subset (Fig. 4A). For the CD11b<sup>+</sup> DC subset, TLR agonist alone stimulated a small sustained increase in the expression of 4-1BBL, OX40L, and CD30L, but CD70 expression was unaffected (Fig. 4A). The combined TLR/CD40 agonist challenge stimulated small increases in the expression of 4-1BBL, OX40L, and CD30L on both DC subsets examined (Fig. 4A) but, again, significantly larger increases in CD70 expression compared with any other TNFL (Fig. 4, A and B). Thus, although increased expression of other TNFL family members could be observed, their regulation was more similar to the expression of CD80/86/40, being increased to one degree or another with either CD40 or TLR stimulation alone and prolonged in expression after stimulation of both (Fig. 4A). Additionally, whereas the increase in 4-1BBL, OX40L, and CD30L was no greater than 3-fold on either DC subset in the presence of agonists for both TLR and CD40 (Fig. 4B), the increase in CD70 expression was 6- to 10-fold on CD8<sup>+</sup> DCs and 4- to 10-fold on CD11b<sup>+</sup> DCs, depending on the TLR agonist that was administered. This was true at all time points between 12 and 48 h (data not shown). Thus, even in relation to other TNFL family members, DC expression of CD70 is uniquely regulated, showing a significant increase only after stimulation of both TLR and CD40 pathways.

CD8<sup>+</sup> T cell expansion, following combined TLR/CD40 agonist immunization, is CD70 dependent

Because the expression of CD70 best correlated with the magnitude of the CD8<sup>+</sup> T cell response elicited, we examined the dependency of the CD8<sup>+</sup> T cell response on CD70-mediated signaling following combined TLR/CD40 agonist immunization. Blocking Abs to CD70, 4-1BBL, OX40L, and CD30L (24) were administered to mice 1 day before immunization to OVA with...
agonistic anti-CD40 Ab and TLR agonist. Injection of the blocking Abs was repeated on days 1, 3, and 5 after immunization. On day 7, mice were bled and the percentage of tetramer-staining CD8\(^+\) T cells was determined.

In contrast to the administration of blocking Abs to 4-1BBL, OX40L, and CD30L, administration of the anti-CD70-blocking Ab to the TLR/CD40 agonist-treated mice inhibited the generation of Ag-specific CD8\(^+\) T cells back to the level observed in response to immunization with anti-CD40 alone (Fig. 5A). This was consistent for all the TLR agonists that were tested, including Pam\(_3\)Cys (TLR1/2), poly(I:C) (TLR3), 27609 (TLR7) (Fig. 5B), MPL (TLR4), and flagellin (TLR5) (data not shown). The reduced T cell response in the presence of anti-CD70-blocking Ab was not due to the elimination of DCs by the anti-CD70 Ab, because DC numbers were not significantly different in immunized mice with or without the injection of the anti-CD70 Ab (Fig. 6) or Abs against the other TNFL family members (data not shown). In addition, the DCs in the CD70-blocked mice expressed high levels of CD80, CD86, and CD40, further indicating that the resident DCs were not eliminated by the CD70 Ab and replaced by recent naive immigrants from the circulation.

Although CD70 expression has been demonstrated on numerous cell types (30, 43), we did not observe any significant expression of CD70 on T cells, B cells, or monocyte/macrophages within the first 48 h after challenge (data not shown). Furthermore, we observed that unless the anti-CD70 Ab was administered within the first 12–24 h of TLR/CD40 agonist immunization, effective blocking of CD8\(^+\) T cell expansion was not observed (Fig. 7). Collectively, these data indicate that the expansion of Ag-specific CD8\(^+\) T cells requires interaction with a CD70-bearing DC within 24 h of antigenic challenge.

Interestingly, the minimal CD8\(^+\) response to CD40 alone (Fig. 5B), or even to TLR stimulation alone (data not shown), was also reduced in the presence of CD70-blocking Ab. This indicates that even the fractional induction of CD70 on DCs by these stimuli is apparently important for the generation of a CD8\(^+\) T cell response. However, the effects of CD70 blockade are most readily observed following combined TLR/CD40 agonist immunization where CD70 appears to mediate the synergy that exists between the CD40 and TLR pathways for the induction of CD8\(^+\) T cell expansion.

**Discussion**

CD70-CD27 interactions are known to influence the generation of primary and memory T cell responses (18–20, 22–26, 29, 32, 44, 45). However, the regulated expression of CD70 and other TNFL family members on DCs in vivo has not been extensively examined. Furthermore, the overall importance of these ligands in the generation of immunity has generally been viewed as another example of redundancy in immune signaling. In the present work, we have uncovered mechanisms by which CD70 expression on DCs is uniquely regulated in vivo, even with respect to other TNFL family members, by concomitant signaling of the TLR/CD40 pathways. This expression of CD70 is critical for eliciting potent CD8\(^+\) T cell responses, because blocking CD70 during this immunization all but eliminates Ag-specific CD8\(^+\) T cell expansion.

The results we show here begin to shed some light on the discrepancy between the ability of TLR agonists to activate DCs and their comparable inability to generate cellular immunity in a vaccine setting. Stimulation of DCs with TLR agonists alone instigates increases in the expression of classical markers of DC activation such as CD80, CD86, and CD40, as well as many inflammatory cytokines in many DC subsets (46). At least some of these increases are necessary, because we previously demonstrated that CD8\(^+\) T cell responses to combined TLR/CD40 agonist immunization are B7 dependent (8). This is not surprising, because almost all T cell responses are dependent on initial CD28 stimulation (47). However, the induction of CD80/86 by the TLR agonist alone does not result in a substantial CD8\(^+\) T cell response (Ref. 8 and Fig. 1). Although the prolonged expression of CD80/
86, in response to combined TLR/CD40 stimulation, may play a supporting role in the ensuing CD8+ T cell response, it is clear that the expression of CD80/86 is necessary, but not sufficient, for mediating the potent expansion of Ag-specific CD8+ T cells. Similarly, CD40 agonists alone also activate many DC functions, and yet have also been unsuccessful, both preclinically (48–50) and clinically (51), at generating long-term cellular immunity. Collectively, these results can now be explained by our data showing 1) maximal CD70 expression requires the combined stimulus of both the CD40 and TLR pathways and 2) the potent CD8+ T cell response following combined TLR/CD40 agonist vaccination is CD70 dependent. Given these data, the rational design of novel and potent vaccine adjuvants should use the induction of CD70 expression on DCs in vivo as a clinically relevant readout.

It should be noted that our results showing the requirement for both TLR and CD40 stimulation for optimal DC CD70 expression in vivo are in apparent contradiction to recently published data by Taraban et al. (33). This recent publication documented increased CD70 expression on splenic DCs in vivo after injection with a high dose (500 μg) of anti-CD40 (33). There are two possible explanations for the discrepancies between our data and the data of Taraban et al. (33). First, Taraban et al. (33) used 10-fold more anti-CD40 than we did. We have repeated our experiments using this amount of anti-CD40 and have found a small increase in CD70 expression on the DCs in vivo as well (data not shown). However, this increase in DC70 was abrogated in MyD88−/− mice (data not shown), suggesting that this amount of Ab may contain enough contaminating LPS to synergize with the CD40 in wild-type (WT) mice. Second, Taraban et al. (33) actually used Rag−/− mice, not WT, when examining DC activation and CD70 expression in vivo. We have also repeated these experiments using a high dose of anti-CD40 in Rag−/− mice and have found a surprising difference in the regulation of CD70 expression between Rag−/− and WT mice. Following high-dose anti-CD40 challenge of Rag−/− mice, we find exactly the same results as Taraban et al. (33), namely, that a significant population of CD11c+ cells express high levels of CD70 (data not shown). In contrast, the DCs in WT mice show only a minor, though detectable, increase in CD70 to high doses of anti-CD40, which again is eliminated in MyD88−/− hosts. As a positive control, the addition of poly(I:C) (which does not signal through MyD88 (52)) to the antiCD40 resulted in the greatest up-regulation of CD70 in all strains. Given these data, there appears to be no real contradiction between our data shown here and the previous work by Taraban et al. (33). We would explain the apparent discrepancies as the result of either contaminating LPS, due to the high dose of anti-CD40 used, a fundamental difference between DCs in WT and Rag−/− mice, or a combination of both. Given the phenotype of CD70 expression in the Rag−/− hosts, we find the second explanation the most likely, although it is unclear at present what the significance of these findings are. Given the lack of regulatory T cells in the Rag−/− hosts, an intriguing possibility is that these results have uncovered a role for regulatory T cells in the regulation of DC CD70 expression.

That being said, we do see minimal increases in DC expression of CD70 in vivo following challenge with TLR or CD40 agonists alone, and even these limited amounts of CD70 expression appear to be physiologically relevant, because CD70 blockade reduced even the minimal CD8+ expansion observed in response to immunization with either agonist alone (see Fig. 4B). However, optimal CD70 expression on DCs in WT B6 mice in vivo cannot be achieved with either agonist alone, suggesting that the differences between previous in vitro results and the data presented here are due to differences between bone marrow-derived DCs and normal DC subsets, between in vitro and in vivo stimulation of DCs, or both.

We previously also showed an important role for type 1 IFN in determining the CD8+ T cell response from some TLR/CD40 agonist combinations (8). This IFN dependency was observed only when the TLR stimulation elicited significant IFN production in the host. Interestingly, we have recently determined that this IFN dependency is based on the same immunologic principle that mediates the CD8+ T cell expansion to TLR/CD40 immunization, namely, the induction of CD70 expression on DCs, and is the subject of a manuscript currently in preparation. Thus, we now have evidence that multiple innate signaling pathways integrate with CD40 for the induction of potent cellular immunity through CD70.

Our data further support the role of CD70 as the primary factor mediating the licensing of DCs for productive interaction with CD8+ T cells (27). Previous experimental models showed that CD4-dependent CD8+ T cell responses could be recovered in CD4-depleted or deficient mice by the administration of an agonistic CD40 Ab (53–55). These previous data demonstrated that, following CD40 stimulation, DCs were enabled, or licensed, to productively stimulate CD8+ T cells. Our results are consistent with those of others (27), which demonstrate that CD70 can provide this licensing role and that CD70 may arguably be the definitive licensing signal for CD4-independent, CD8+ T cell responses.

Finally, our data show that the engagement of TLR and one TNFR/L pair (CD40/L) leads to the eventual expression and engagement of another TNFR/L pair (CD27/CD70). It may be that TNFR/L superfamily members generally play a role in the positive feedback regulation of primary, and possibly secondary, T cell expansion. Although we observed a critical role for CD27/CD70 interactions in primary CD8+ T cell responses, secondary T cell expansion appears to be relatively independent of CD70 (P. J. Sanchez and R. M. Kedl, unpublished observations). As shown above, the TNFL OX40L and 4-IBBL are induced to some degree by TLR stimulation alone. Memory cells are known to be more sensitive to secondary Ag challenge (56, 57), and this may be due in part because they are more responsive to TNFL family members whose expression is achieved under conditions of minimal inflammation. Understanding the relationship between the regulated expression of the different TNFL members and primary/secondary T cell expansion will result in the development of clinical prime-boost regimens that optimally promote long-term cellular immunity.

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Disclosures

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