Enhanced efficacy and reduced toxicity of multifactorial adjuvants compared with unitary adjuvants as cancer vaccines

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Identification of Toll-like receptors (TLRs) and their ligands, and tumor necrosis factor–tumor necrosis factor receptor (TNF-TNFR) pairs have provided the first logical, hypothesis-based strategies to molecularly concoct adjuvants to elicit potent cell-mediated immunity via activation of innate and adaptive immunity. However, isolated activation of one immune pathway in the absence of others can be toxic, ineffective, and detrimental to long-term, protective immunity. Effective engineered vaccines must include agents that trigger multiple immunologic pathways. Here, we report that combinatorial use of CD40 and TLR agonists as a cancer vaccine, compared with monotherapy, elicits high frequencies of self-reactive CD8+ T cells, potent tumor-specific CD8+ memory, CD8+ T cells that efficiently infiltrate the tumor-burdened target organ; therapeutic efficacy; heightened ratios of CD8+ T cells to Fox3+ cells at the tumor site; and reduced hepatotoxicity. These findings provide intelligent strategies for the formulation of multifactorial vaccines to achieve maximal efficacy in cancer vaccine trials in humans. (Blood. 2008;111:3116-3125)

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Introduction

The molecular identification of Toll-like receptors (TLRs) and their ligands, as well as tumor necrosis factor (TNF)–tumor necrosis factor receptor (TNFR) pairs that control adaptive immunity, has provided the first logical, hypothesis-based strategies to molecularly concoct adjuvants that elicit potent cell-mediated immunity. Paralleling TLRs in mobilizing the innate immune response, CD40 and its ligand represent the primary ligand-receptor pair essential for development of the adaptive immune response. Individually, TLR agonists1 and CD40 agonists2-3 have entered clinical trials as adjuvants for eliciting protective immune responses to cancer. Inherent in these monotherapeutic approaches are limited induction of immunity, lack of clinical efficacy and, in some cases, hepatotoxicity.1,4

TLRs are widely expressed on both hematopoietic and nonhematopoietic cells and elicit proinflammatory responses upon receptor engagement. Indeed, use of TLR agonists as solitary adjuvants triggers dendritic cell (DC) maturation, leukocyte migration, and release of chemokines and cytokines, and enhances immunity.5,6 Studies in which TLR agonists have been scrutinized for their ability to induce cross-presentation and antigen-specific CD8+ responses in vivo7 show some level of activity that is minimal compared with that observed when combined with a CD40 agonist.8,9 TLR agonists as unitary adjuvants in murine tumor models have demonstrated marginal efficacy, as reviewed,10,11 but have proven effective when combined with other vaccine modalities.11-13 Finally, clinical use of a TLR9 agonist in lung cancer trials has been recently suspended due to lack of clinical response.1

Studies from animal models underscore the utility of anti-CD40 (aCD40) as a unitary adjuvant.14,15 We previously demonstrated that the magnitude of immune responses elicited by TLR or CD40 agonists alone is minimal compared with the magnitude of immune responses generated by combined use of CD40 and TLR agonists.8 Less than 1% of the CD8+ T-cell population is antigen specific following immunization with aCD40 alone plus antigen, while extremely high frequencies of antigen-specific CD8+ T cells (> 25% of the total CD8+ T cells) can be generated by the coadministration of TLR and CD40 agonists plus antigen.8 This synergy was observed with all TLR agonists tested (TLR 2,3,4,6,7,9).8 Furthermore, use of CD40 agonists in the absence of any other coactivation signals leads to the early demise of antigen-specific CD8+ T cells16 and has been reported to ablate tumor-specific memory.14 Finally, phase I/2 clinical trials implementing CD40 agonistic monotherapy have resulted in minimal therapeutic efficacy and dose-limiting toxicities.2,4

The present study comprehensively compares the impact of combination therapy with that of monotherapy on the antigen-specific immune responses to melanoma at the cellular and molecular levels. The studies presented demonstrate the profound utility of CD40 and TLR agonists when combined in an adjuvant platform in a murine model of cancer. The data show that vaccination induces extremely high frequencies of primary and memory self-reactive CD8+ T cells that infiltrate metastatic target organs and control tumor growth. Combination therapy also reduces the ratio of regulatory T cells (Treg) to CD8+ T cells at the
tumor site and allows persistent effector CD8⁺ T-cell function. Finally, the overt hepatotoxicity induced by CD40 monotherapy is ablated by combination therapy. Our studies show that combinatorial use of CD40 and TLR agonists provides greater therapeutic efficacy with limited toxicity and provides the principles on which to build new multifactorial adjuvants for use in clinical trials.

**Methods**

**Mice and tumor cell lines**

Male 6- to 8-week-old C57BL/6 mice were obtained from the National Cancer Institute (Bethesda, MD) and were maintained under pathogen-free conditions. All experiments were approved by the Institutional Animal Care and Use Committee of Dartmouth College. B16.F10 melanoma cells were a kind gift from Mary Jo Turk (Dartmouth-Hitchcock Medical Center, Lebanon, NH) and were maintained in complete medium (RPMI 1640 containing 10% fetal calf serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM glutamine, and 50 µM 2-mercaptoethanol).

**Cell lines, antibodies, and reagents**

Mouse monoclonal antibodies (mAbs) to CD8 (53-6.7), CD4 (GK1.5), CD44 (IM7), CD127 (A7R34), CD122 (5H4), IL-2 (JES6–5H4), IFNγ (XMG1.2), FoxP3 (FJK-16s), Granzyme B (16G6), and the isotype control rat IgG2a were purchased from eBioscience (San Diego, CA) as were both Ova(257-264) or TRP2(180-188) peptide. Antibodies were delivered 4 days before the start of experiment and weekly thereafter. Depletion was confirmed by flow cytometry and resulted in greater than 95% reduction of relevant cell types.

**In vivo depletion of cell subsets**

Depletion of lymphocyte subsets was accomplished by intraperitoneal administration of 250 µg anti-CD4 (GK1.5), anti-CD8 (2,43), and anti-NK1.1 (PK136). Antibodies were delivered 4 days before the start of experiment and weekly thereafter. Depletion was confirmed by flow cytometry and resulted in greater than 95% reduction of relevant cell types.

**Flow cytometry**

Single cell suspensions were incubated with antibodies labeled with FITC, PE, PerCP, PC5, or APC. Antibodies, as listed under “Cell lines, antibodies, and reagents,” were from eBioscience, BD Pharmingen, and Invitrogen. Four-color analyses were performed on a modified Becton Dickinson FACScan running CellQuest software (BD Bioscience).

**Intracellular cytokine staining and degranulation assays**

Cells from lung, spleen, or peripheral blood (peripheral blood lymphocytes [PBLs]) were incubated with 1 µg/mL Ova(257-264) or TRP2(180-188) peptide plus 10 U/mL IL-2 and 3 µg/mL brefeldin A in complete medium at 37°C for 5 to 18 hours. Cells were stained with either PerCP or PC5-labeled anti-CD8 and FITC-labeled anti-CD44 antibodies prior to being fixed and rendered permeable following staining with either PE- or APC-labeled anti-IFNγ (XMG1.2), PE-labeled anti-TNFα (MP6-XT22), PE-labeled anti-IL-2 (JES6–5H4), FITC-labeled anti-CD127 (A7R34), or PE-labeled anti-granzyme B (16G6). The percentage of IFNγ⁺ cells was calculated by subtracting the background observed with the irrelevant peptide control. For the degranulation assay, cells were treated as above but with the inclusion of monensin and 2.5 µg/mL FITC-labeled anti-CD107a (1D4B) during the initial 5- to 18-hour incubation period.

**In vivo cytotoxicity assay**

In vivo cytotoxic activity was performed as previously described. Briefly, naive syngeneic splenocytes were differentially labeled with either 0.5 µM or 5 µM carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) for 10 minutes at 37°C, washed, and then pulsed for 1 hour with 20 µg/mL irrelevant Ova(257-264) (SIINFEKL) or antigen-specific TRP2(180-188) peptide, respectively. Labeled and pulsed cells were subsequently mixed at a 1:1 ratio and approximately 10⁵ cells were injected intravenously. One day later, mice were killed and splenocytes were analyzed by flow cytometry. Specific lysis was calculated by first determining the ratio of the number of SIINFEKL-labeled targets to the number of TRP2-labeled targets for each mouse and percentage antigen-specific lysis was subsequently calculated as follows: % specific lysis = (1 – [ratio of CFSE⁺/CFSE⁻ in naive mice ÷ ratio of CFSE⁺/CFSE⁻ in immunized mice]) × 100.

**Serum transaminase and histologic analysis**

Hepatocellular injury was biochemically assessed by measuring serum liver enzyme activity. Specifically, mice received 100 µg anti-CD40, 100 µg S-27609, or both intravenously or PBS as a control. Serum was harvested 24 to 72 hours later and levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined by standard clinical assays at the National Jewish Medical Center Core Lab (Denver, CO). For histologic analysis, livers from mice treated as above were fixed in buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) prior to being coded and scored on a 0 to 4 scale in a blinded fashion. Numeric scores were assigned as follows: liver; 0 indicates normal liver, no lesions or hepatocellular damage noted; 1, rare portal and perivascular infiltration of lymphocytes; 2, portal and perivascular infiltration of lymphocytes and eosin (H&E) but no necrosis; 3, portal and/or large portal or parenchymal infiltration with occasional isolated islands of coagulative necrosis; and 4, extensive areas of inflammation with bridging coagulative necrosis. H&E images were acquired via an Olympus BX41 microscope (Center Valley, PA) using a 20×/0.05 non-oil objective and 10× or 20× ocular attached to an Olympus DP11 digital camera and were edited with XnView for Windows, version 1.82.2 (Reims, France).
Statistical analysis

Data were expressed as the mean plus or minus SEM and differences between groups were analyzed by one-tailed ANOVA and Tukey analysis unless indicated otherwise. In the case of tumor survival experiments, statistical relevance was determined using log-rank comparison. The extent of hepatitis was scored on an arbitrary scale and the resulting nonparametric data were analyzed using the Mann-Whitney test. Probability (P) values less than .05 were considered statistically significant.

Results

High frequencies of tumor-specific, effector CD8+ T cells are elicited using CD40/TLR7 agonists and tumor-specific peptide

We previously demonstrated that coadministration of CD40 and TLR agonists synergistically enhances expansion of antigen-specific CD8+ T cells to foreign antigen.8 We extend these studies to show that similarly high frequencies of CD8+ T cells can be induced to self-antigens. Recently, a modified peptide variant of the H2Kb-restricted melanoma rejection self-antigen TRP2 (180-188), termed ΔV (SIYDFFVWL), was shown to elicit high-affinity TRP2-specific CD8+ T cells.17 We reasoned that immunization with ΔV plus agonistic CD40 antibody (αCD40) and a TLR7 agonist (TLR7*) would magnify the ensuing CD8+ response and engender increased effector cell function. As seen in Figure 1B, αCD40 increased the relative number of CD8+ T cells in the peripheral blood of immunized mice, regardless of the addition of antigen, TLR7 agonist, or both (P ≤ .001 for ΔV/αCD40, ΔV/αCD40/TLR7*, and αCD40/TLR7* compared with ΔV alone). While αCD40 increased polyclonal CD8+ responses, it failed to generate a substantial population of TRP2-specific CD8+ T cells (Figure 1A,C). Only the combination of tumor antigen, αCD40, and TLR7 agonist resulted in the synergistic expansion of TRP2-specific T cells. To measure cytolytic potential, we assessed the
ability of these cells to degranulate, which can be measured by retention of CD107a (lysosomal-associated membrane protein-1) on the cell surface.\(^{18}\) Cell-surface expression of CD107a is directly correlated with cytolytic activity.\(^{19,20}\) Only approximately 4% and approximately 2% of CD8\(^+\) cells in either the αCD40 or TLR7 agonist alone groups, respectively, expressed CD107a. However, greater than 30% of CD8\(^+\) cells primed with both αCD40 and TLR7 agonists expressed cytolytic activity by this measure (P ≤ .001 compared with ΔV alone). Combination treatment also led to increased lysis of peptide-pulsed targets in an in vivo cytotoxicity assay (data not shown). Together, these data demonstrate that the combination of αCD40 and TLR7 agonists induces high frequencies and high total numbers of self-reactive CD8\(^+\) T cells with cytolytic function.

αCD40/TLR7\(^*\) vaccination elicits potent CD8\(^+\) T-cell memory

We hypothesized that coadministration of CD40 and TLR7 agonists would abrogate the deleterious effects of agonistic CD40–based monotherapies to engender long-term memory. To determine whether concomitant delivery of CD40 and TLR7 agonists in conjunction with tumor antigen elicits the generation of CD8\(^+\) T-cell memory, we vaccinated mice and analyzed effector functions 60+ days later. Vaccination with ΔV and αCD40 primed a minimal, persisting CD8\(^+\) effector population in the lung with limited cytolytic potential (Figure 2A,B,D). TLR7 monotherapy failed to induce a significant pool of persisting antigen-specific CD8\(^+\) T cells. In contrast, vaccination with tumor antigen, αCD40, and TLR7 agonist primed effector cells populating both spleen and lung (Figure 2A,C,D). More importantly, unlike αCD40 or TLR7\(^*\) monotherapy, mice vaccinated with this regimen efficiently lysed peptide-pulsed targets when subjected to an in vivo cytotoxicity assay (Figure 2B,E; P ≤ .001, compared with either ΔV or ΔV/αCD40). In addition, the mean fluorescence intensity of IFNγ staining increased over that seen from αCD40 treatment alone (spleen: 185 ± 30 vs 310 ± 22, P = .0041; lung: 152 ± 6 vs 253 ± 25, P = .0028), demonstrating that CD8\(^+\) T cells primed by αCD40/TLR7\(^*\) are more efficient in producing effector cytokines. Finally, only αCD40/TLR7\(^*\) plus tumor antigen could induce autoimmune vitiligo, a response seen in approximately 36% of vaccinated mice (data not shown). To ensure the identity of the TRP2-specific memory T-cell population, we examined the CD8\(^+\) T cells for expression of CD127 (IL-7R\(\alpha\)), a marker shown to be selectively re-expressed upon differentiation of effector cells into memory cells.\(^{21}\) Indeed, TRP2-specific CD8\(^+\) T cells isolated from spleen and lung expressed CD127 (Figure 2F). Not only did the cells express CD127, but they remained fully functional, being able to produce both TNFα and IL-2. Of the IFNγ\(^+\) cells found in lung and spleen, greater than 70% secreting TNFα while greater than 20% secreting IL-2 (Figure 2G). Furthermore, since a fraction of these cells acquired the ability to secrete IL-2 and express CD127, this indicates that this vaccination regimen generates memory cells of both effector and central memory phenotype.\(^{22}\)

Superior therapeutic efficacy of αCD40/TLR7\(^*\) immunotherapy compared with either monotherapy in control of metastatic melanoma

The ability of different vaccination strategies to alter the progression of metastatic melanoma was compared. Mice were intravenously inoculated with 10\(^5\) metastatic B16.F10 melanoma cells and treatment was initiated 4 days later. Twenty-four days after vaccination, mice were killed and surface lung metastases were enumerated. Treatment with tumor antigen or tumor antigen plus a TLR7 agonist was ineffective in controlling tumor progression (Figure 3A,B). Immunization with tumor antigen plus αCD40 reduced the number of tumor nodules (P ≤ .001 vs ΔV alone). However, addition of a TLR7 agonist to this vaccine resulted in a 3-fold reduction in the number of metastases over αCD40 alone (Figure 3B; P ≤ .01 vs ΔV/αCD40). Furthermore, the protection afforded by αCD40/TLR7\(^*\) relies upon antigen, as the removal of the H2K\(^\beta\) peptide, ΔV, abrogates the effect of treatment (Figure 3A,B). This protection is not unique to TLR7 agonists, as equal efficacy is observed with TLR3 and TLR9 agonists (data not shown). Moreover, changing the route of vaccination did not significantly alter the outcome of treatment (Figure S1, available on the Blood website; see the Supplemental Materials link at the top of the online article). Since αCD40/TLR7\(^*\) vaccination reduced the number of lung metastases, we asked whether combination immunotherapy would afford long-term protection against metastatic disease. All mice vaccinated with tumor antigen, tumor antigen plus TLR7 agonist, or αCD40/TLR7 agonists without tumor antigen succumbed to lung failure (Figure 3A). Mean survival times were 29, 30, and 30 days, respectively. αCD40 monotherapy significantly increased survival times over tumor antigen alone (P ≤ .001) with a median survival time of 35 days and led to 3% of mice surviving greater than 90 days. However, the combination of tumor antigen plus αCD40/TLR7\(^*\) greatly improved survival over αCD40 alone (P ≤ .001). Median survival times increased from 35 to 47 days with 20% of mice alive after 90 days (also see Kaplan-Meier plot in Figure S2). To determine which cellular subset mediates rejection of metastatic melanoma under this vaccination regimen, mice were depleted of CD8\(^+\), CD4\(^+\), and NK1.1\(^+\) cells prior to tumor challenge. Depletion of CD8\(^+\) cells abrogated the protective effect of vaccination (Figure 3C; P = .001 compared with vaccination without depletion). Both CD4\(^+\) and NK1.1\(^+\) cells play a partial role in tumor protection, since their depletion resulted in slightly faster, although not significant, tumor progression (Figure 3C). These data indicate that vaccination with combined immunotherapy, in the presence of antigen, leads to a CD8\(^+\) T-cell–dependent immune response capable of mediating antitumor responses greater than that seen with either αCD40- or TLR-based monotherapy.

Enhancement of lung infiltrates with cytolytic potential following αCD40/TLR7\(^*\) immunotherapy

To gain insight into why αCD40/TLR7\(^*\) immunotherapy mediated better antitumor immunity, we performed kinetic analyses of lung infiltrates 10 and 21 days after tumor challenge (Figure 4A). Lymphocytes isolated from tumor-bearing lungs were subjected to intracellular cytokine staining after ex vivo peptide restimulation. Only tumor antigen plus either αCD40 or αCD40/TLR7\(^*\) vaccination primed tumor-specific CD8\(^+\) T cells to migrate into the metastatic target organ (Figure 4B). Flow cytometric analysis of ΔV/αCD40/TLR7\(^*\) vaccinated mice revealed a 5-fold increase in the relative percentage of tumor-specific CD8\(^+\) T cells at day 10 and a 3-fold increase at day 21 over αCD40 monotherapy. On an absolute scale, αCD40 drives migration of polyclonal T cells into lungs of vaccinated mice irrespective of TLR stimulation, but this response wanes with time (Figure 4C,D). In contrast, antigen-specific cells remain elevated, with αCD40/TLR7\(^*\) inducing greater absolute responses at both time points (P ≤ .001 between ΔV/αCD40/TLR7\(^*\) and ΔV/αCD40 at both time points). Furthermore, cells
generated from αCD40/TLR7* vaccination showed cytolytic potential as measured by degranulation and Granzyme B expression (Figure 4E).

Vaccine efficacy must overcome the effect of regulatory T cells, and the ratio of CD8+/FoxP3* cells has been used to assess priming strength.23 At day 10, combination therapy resulted in a 10-fold increase in the absolute numbers of antigen-specific CD8+ T cells to FoxP3* cells, whereas αCD40 monotherapy resulted in a 3-fold increase (Figure 4C). We have shown that optimal reduction in the conversion of FoxP3+ → FoxP3+ T cells requires the maturation of DCs with both αCD40 and TLR agonists (Li Wang, Karina Pino-Lagos, Victor C. de Vries, Mohamed H. Sayegh, and R.J.N., manuscript submitted, November 2007). These data support the hypothesis that one way in which combination immunotherapy
One of the significant dose-limiting safety concerns of the use of immunosuppression.

-mediated increased antitumor immunity is by amplifying CD8+ T-cell numbers and effector function while decreasing the effect of immunosuppression.

αCD40-induced hepatocellular injury is reduced by coadministration of TLR7 agonist

One of the significant dose-limiting safety concerns of the use of αCD40 monotherapies is liver toxicity. Several human2,4 and animal24-27 studies using CD40 agonists report elevated levels of circulating hepatocyte enzymes ALT and AST, indicative of liver damage. To examine the severity of hepatocellular damage with monotherapy versus combination therapy, we measured plasma levels of ALT and AST in mice after vaccination (Figure 5A,B). Both transaminases were significantly elevated in mice treated with αCD40, peaking at 48 hours after treatment. TLR7* had no effect on enzyme levels. In contrast to αCD40 monotherapy, αCD40/TLR7* treatment completely ameliorated the toxicity seen with αCD40 alone. Macroscopic evaluation of livers revealed substantial areas of necrosis, a finding observed only in mice treated with αCD40 (data not shown). Histologic analysis confirmed the severity of hepatocellular damage (Figure 5C-F). Normal liver architecture was seen in mice treated with PBS (Figure 5C). Livers isolated from mice treated with αCD40 exhibited widespread bridging coagulative necrosis (Figure 5D), whereas TLR7* treatment resulted in minor inflammation without any observable coagulative necrosis (Figure 5E). Livers from mice receiving αCD40/TLR7* had some foci of inflammation but little to no coagulative necrosis (Figure 5F). The extent of histologic damage was subsequently scored on a semiquantitative scale (Figure 5G). The data revealed that TLR7* significantly reduces liver toxicity associated with αCD40 monotherapy (P = .026). Although it is not clear why TLR7* attenuates αCD40-induced toxicity, we have shown that this reversion in toxicity is TLR7 dependent, as both CD40/TLR7* (data not shown) and αCD40/TLR7* combination therapy in reversing toxicity remains unclear and requires further investigation, it nonetheless not only provides better therapeutic outcomes but also minimizes adverse side effects.

Discussion

Identification of molecular triggers for innate and adaptive immunity will revolutionize adjuvant platforms for vaccines. However, isolated activation of one immune pathway in the absence of others may be toxic, ineffective, and in some cases detrimental to the development of long-term, protective immunity. More effective molecularly engineered vaccines will likely include combinations of agents that trigger multiple immunologic pathways.28-29 Our studies demonstrate that CD40 and TLR agonists in combination, compared with either unitary adjuvant, elicit (1) high frequencies of self-reactive, effector CD8+ T cells, (2) potent, tumor-specific CD8+ memory, (3) CD8+ T cells that efficiently infiltrate metastatic target organs and exert effector functions, (4) superior therapeutic efficacy, (5) heightened ratios of CD8+ T cells to FoxP3+ T cells at the tumor site, and (6) reduced hepatotoxicity.

Heightened frequencies of tumor-specific CD8+ T cells have been primary end points for many human clinical trials13 and are believed to be a necessary component in the emergence of protective antitumor immunity. The frequency of antigen-specific CD8+ T cells that are elicited by the combined administration of αCD40/TLR7* agonist and antigen is an order of magnitude higher than that observed with almost any other adjuvant or cell-based vaccine platform, such as antigen-pulsed DCs.30,32 While the cellular and molecular basis for this striking response is incompletely understood, we have published that the expression of CD70 on DCs is critical for CD8+ T-cell expansion.9 Heightened expression of CD70 on CD8α+ DCs is induced only when both CD40 and TLR agonists are coadministered. The subsequent increased signaling through CD70/CD27 could account for the
Figure 4. Kinetic analysis of lung-infiltrating lymphocytes. (A) Experimental design. (B) Representative dot plots of lymphocytes isolated from metastatic target organs at day 10 or 21 after tumor challenge. Cells were isolated from tumor-bearing lungs as described in “Methods” and subjected to an in vitro restimulation with tumor peptide. Plots are gated on live, CD8^+^/H11001^+^ cells. Numbers in the upper right-hand quadrant reflect the frequency of CD8^+^/H11001^+^ T cells that are positive for both IFN^+^/H9253 and the activation marker CD44. Data are representative of 3 independent experiments with 4 mice per group in each experiment. (C,D) Quantification of lung infiltrates at either 10 (C) or 21 (D) days after tumor challenge. Data are plotted as means (± SEM) and represent pooled data from either 2 (C, n = 8 mice/group) or 3 (D, n = 12 mice/group) independent experiments, with 4 mice/group in each experiment. (E) Effector phenotype of CD8^+^ T cells isolated from lungs of mice vaccinated with tumor antigen plus CD40/TLR7* at either 10 or 21 days following tumor inoculation. The dot plots are first gated on live CD8^+^ cells and then further gated on IFN^+^/CD44^+^ populations. Data are representative of at least 2 independent experiments, with 4 mice/group in each experiment.
Figure 5. Hepatic toxicity associated with αCD40 monotherapy is reversed with TLR7 agonism. (A,B) Kinetic analysis of serum transaminases. Mice were treated with PBS, 100 μg αCD40, 100 μg TLR7*, or both intravenously. Serum was isolated at various time points afterward, and serum levels of alanine transaminase (A) or aspartate transaminase (B) were measured as described. Data are representative of 3 independent experiments, with n = 3 to 8 mice per group, per time point. (C-F) Histologic analysis of livers treated with PBS (C), 100 μg αCD40 (D), 100 μg TLR7* (E), or 100 μg αCD40 and 100 μg TLR7* (F) for 48 hours. (G) Semiquantitative assessment of histopathologic changes in livers from mice treated as above for 48 hours. Data are pooled from 2 independent experiments, with n = 6 mice in each treatment group. \( P = .026 \) by Mann-Whitney nonparametric test.
superior memory responses seen after vaccination. Other data
suggests that CD8α+ DCs acquire the capacity to cross-present
soluble antigen when triggered via CD40 and TLR in vivo, and this
too may contribute to the extremely high frequencies of antigen-
specific CD8+ T cells (A.W., R.S.N., unpublished data, 2006).
Overall, our current hypothesis is that CD40/TLR7* increases the
efficiency of antigen processing and cross-presentation thereby
facilitating enhanced CD8+ T-cell priming and memory. The data
presented herein used peptide antigen, and as such, bypassed the
cross-presentation pathway. However, it is interesting to speculate
that CD40/TLR agonism may facilitate epitope spreading to
alternative tumor antigens after peptide vaccination. Whether this
adjuvant platform enhances epitope spreading is currently being
actively evaluated.

Anti-CD40 as a unitary adjuvant has been shown to terminate
both humoral12 and cell-mediated immune16 responses. While
αCD40 monotherapy may provide a minimal enhancement of
short-term immunity, studies have shown that it abbreviates the
generation of CD8+ T-cell memory. Interestingly, even for
humoral immunity, the use of CD40 agonists aborts long-term
memory and the generation of long-lived plasma cells.13 In recent
studies by Murphy and coworkers (Berner et al15), CD40 mono-
therapy resulted in the IFNγ-dependent apoptosis of tumor-specific
CD4+ T cells and the inability to mount protective memory
responses to tumor challenge. A number of αCD40 monoclonal
antibodies have entered the clinic7,4,35-38 only one2 of which has
been reported to be a strong agonist, similar to the antimiture
CD40 used herein and in a wealth of other murine studies, for
example,39,40 In that phase 1 study, 4 patients, each with stage IV
melanoma, were found to have a partial response on restaging at the
end of study. While it may be premature to make any conclusive
statements concerning agonistic αCD40 monotherapy2 as a vaccine
platform, the preclinical studies in mice certainly suggest that it
would be more effective as a vaccine when combined with
activators of innate immunity. Even if not for clinical efficacy, the
toxicity of CD40 monotherapy may be ameliorated with the
addition of other immune activators. One indication where agonis-
tic αCD40 monotherapy may be suitable is in B-cell lymphoma
where, in mice, high-dose monotherapy has been shown to be
extremely effective40,41

Studies in animal models reveal that as unitary adjuvants, TLR
agonists can elicit robust, inflammatory responses and enhance a
wide spectrum of specific immune responses.42 Results of clinical
studies with TLR agonists have been mixed.43 Imiquimod, an
FDA-approved topicalically applied TLR7 agonist, has proven ex-
tremely effective in basal cell carcinoma. Furthermore, 2 improved
adult hepatitis B virus (HBV) vaccines using TLR4 agonists have
been approved. However, in June 2007, Pfizer suspended a clinical
program in non–small cell lung cancer for a TLR9 agonist due to
lack of clinical efficacy in phase 2 and 3 trials when combined with
a variety of chemotherapeutic agents.44 Our data strongly suggest
that, at least in cancer indications, activators of adaptive immunity
will greatly augment the therapeutic potential of TLR agonists.

It is encouraging that single-arm trials with TNFR agonists and
TLR agonists have shown to be largely safe and induce
inflammatory responses. Based on emerging preclinical studies in
mice using admixtures of TLR agonists, TNFR agonists, and other
immune activators, it is anticipated that these admixtures will
greatly improve efficacy in clinical trials, and at the same time
reduce toxicity. Enhanced frequencies of primary effector T cells,
potent long-term immunologic memory, and reduced regulatory
T-cell functions are some of the hallmark end points that likely
need to be achieved for successful therapeutic intervention. The
findings of this and other studies45 provide rational strategies for
the creation of multifactorial vaccines to achieve maximal efficacy
in cancer vaccine trials in humans.

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Authorship

Contribution: C.L.A. designed and performed research, collected
and analyzed data, performed statistical analyses, and wrote the
paper; A.W., S.F., A.A.S., J.D.G., and R.M.K. performed research,
collected data, and analyzed data; M.I.T. and E.I.U. designed
research and analyzed data; M.S.E. analyzed data; and R.J.N.
designed research, analyzed data, and wrote the paper.

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cofounders, stockholders, and consultants of ImmuRx, a company
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