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TLR4 Engagement during TLR3-Induced Proinflammatory Signaling in Dendritic Cells Promotes IL-10–Mediated Suppression of Antitumor Immunity

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Abstract

Toll-like receptor (TLR) agonists are promising adjuvants for immune therapy of cancer, but their potential efficacy as single or combinatorial agents has yet to be fully evaluated. Here, we report that among all TLR agonists tested, dendritic cells (DC) stimulated with the TLR3 agonist polyI:C displayed the strongest activity in stimulating proinflammatory responses and the production of melanoma antigen-specific $CD8^+$ T cells. Simultaneous treatment with TLR7/8 agonists further improved these responses, but the inclusion of bacterial lipopolysaccharide (LPS), a TLR4 agonist, suppressed proinflammatory cytokine production. This inhibition was contingent upon rapid induction of the suppressive cytokine interleukin (IL)-10 by LPS, leading to dysregulated immune responses and it could be reversed by signal transducers and activators of transcription 3 knockdown, p38 blockade or antibodies to IL-10 and its receptor. Our findings show how certain TLR agonist combinations can enhance or limit DC responses associated with antitumor immunity, through their relative ability to induce IL-10 pathways that are immune suppressive. *Cancer Res; 71(16); 5467–76. ©2011 AACR.*

Introduction

Toll-like receptor (TLR) agonists are molecular components associated with microbial pathogens and natural mediators of inflammation. Through ligation of specific receptors on antigen presenting cells (APC), they initiate innate immune responses and facilitate the induction of adaptive immunity. Dendritic cells (DC) are the most potent APCs playing a crucial role at the cross-talk of innate and adaptive immune systems. In the last decade, the unique features of TLR agonists and DCs have been tested in immunotherapy trials for cancer patients (1, 2). Exploiting TLR agonists *in vivo* as adjuvants to tumor antigen delivery has succeeded in inducing potent immune responses and in some cases evidence of tumor regression or delayed time to recurrence. The first adjuvant approved for use in human cancer was live, attenuated *Mycobacterium bovis* (stimulant of TLR2 and TLR4) for the treatment of bladder carcinoma *in situ* and superficial bladder cancers. In the clinic, it performed better than standard chemotherapy (3). Another promising adjuvant is detoxified lipopolysaccharide (LPS), monophosphoryl A (MPL; targeting TLR4) which is a component of many immune therapeutic strategies. It is currently being coadministered with MAGE A3 (a cancer testis antigen) in patients with advanced melanoma and non–small-cell lung cancer (NSCLC; ref. 4). So far, the results in phase I/II trials indicate very low toxicity and suggest clinical benefit with prolonged disease-free survival for patients with resected stages I and II NSCLC (5). Agonists of TLR7 and TLR7/8, imiquimod, and resiquimod, respectively, succeeded in augmenting immunologic responses in melanoma patients treated with either peptide or protein vaccines (6, 7). Combination of TLR7/8 agonists with TRL4 agonist resulted in increased $CD8^+$ T cells retaining CD28 (8). Because the new formulation of the TLR3 agonist, poly-ICLC, also shown to act through the intracellular receptor MDA5, has an improved half-life *in vivo*, it has been reexamined with reported benefits in the induction of the adaptive $CD4^+$ T-cell immune response (9).

In this study, we sought to examine the potential of single and combined TLR agonists for their ability to induce potent antitumor antigen responses, although limiting the potential for activating inhibitory pathways or skewing T-cell responses toward Th2-like profile. We show that certain TLR combinations are synergistic, whereas others are limiting in their ability to induce potent immune responses, and that interleukin IL-10 secretion is a key homeostatic control mechanism that governs this outcome. Upon specific blockade of interleukin IL-10, the potency of tested adjuvants is greatly augmented as shown by an improved induction of both innate

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and adaptive immune responses targeted against the model melanoma antigen MelanA/MART-1.

Materials and Methods

Peptides

For the human studies, Flu $MP_{58–66}$ (GILGFVFTL), MelanA/Mart-$1_{26–35}$ (ELA modified) ELAGIGILTV, and HIV $Gag_{77–85}$ SLYNTVATL peptides were synthesized by Genemed Synthesis Inc.

Human monocyte-derived DCs

Buffy coats were purchased from the New York Blood Center and leukaphereses from BRT Laboratories. These served as sources for peripheral blood mononuclear cells (PBMC). Human monocyte-derived DC (mo-DC) were differentiated from monocyte fractions of PBMCs as described previously (10). Briefly, monocytes that attached to the tissue culture plates following plating of PBMCs were cultured at 37°C on 10 cm^2 plates in 10 mL of culture media containing 300 IU/mL recombinant human (rHu) IL-4 (Immunex) and 116 IU/mL rHu granulocyte macrophage colony stimulating factor (GM-CSF; R&D). On days 2 and 4 of culture, additional IL-4 and GM-CSF were added. Immature DCs were harvested on day 5 for use in experiments.

Tetramers

$Mart_{26-35}$-specific and HIV Gag_{77-85}-specific tetramers were generated by the Vaccine and Cell Therapy facility at New York University School of Medicine.

Human antibodies

Unless otherwise specified, all antibodies (CD3, CD4, CD45RO, CD62L, CD8, CD80, CD86, and PDL-1) for staining human cells were purchased from BD Pharmingen.

Measurement of cytokine and chemokine secretion

Human mo-DCs cultured with various stimuli, LPS at 100 ng/mL (Sigma), polyI:C at 2 to 5 ug/mL from Amersham and InVivogen, or Oncovir's Hiltonol (Figs. 2 and 3 and Supplementary Figs. S3 and S4; all 3 sources gave similar *in vitro* results when compared side by side), Resiquimod (R848) at 1–10 μmol/L (3 mol/L) were incubated for 18 to 24 hours (unless otherwise stated in the figure) at 37°C before their culture supernatants were collected and tested for the presence of the following cytokines: IL-12p70, TNF-α, IL-10, -6, -1β, and -8 by flow cytometry by using a cytometric bead array (BD Biosciences). Alternatively IL-12p70, TNF-α (BD Pharmingen), and IFNα (PBL); were measured by ELISA. Also, a panel of 22 cytokines was measured by using the Luminex platform and a kit from Millipore. Blocking antibodies to IL-10 and -10R and rHu IL-10 (used at 300 ng/mL) were purchased from Biolegend as was control immunoglobulin (IgG)2A/IgG1, all blocking antibodies were used at 5 μg/mL.

In vitro T-cell priming

Human mo-DCs derived from healthy donors were matured in the presence of the agonist of choice and 1 μmol/L $Mart_{26–35}$ peptide overnight at 1×10^6/mL. The day after, cells were washed 2× with RPMI and resuspended at 1×10^5 cells/mL. Naive $CD8^+$ T cells were isolated by depletion of CD45RO-positive cells. Bead isolated $CD8^+$ T cells from autologous donors were resuspended at 1×10^6/mL. DCs were cocultured with $CD8^+$ T cells in 24-well plates at a ratio of 1:10 in 10% pooled human serum in RPMI. After 4 days of culture, 5 ng/mL of IL-7 (R&D) and 5 ng/mL IL-15 (R&D) were added to cultures. Priming cultures were restimulated with autologous irradiated $Mart_{26–35}$ peptide pulsed PBMCs at day 10. IL-7 and -15 were replenished every 2 days. On day 21, the T cells from each well were tested for antigen specificity by staining with Mart-1/MelanA specific tetramers.

Antigen-specific cell capture and expansion

On day 22 of the priming experiments, the cells were stained with Mart-1 tetramer PE for 45 minutes at 4°C, washed 2× with PBS, and then enriched with anti-phycoerythrin beads (Miltenyi) for 15 minutes at 4°C. Cells were placed in 96-well plates containing 1×10^5 allogeneic-irradiated PBMCs per well in CTL media supplemented with 150 U/mL of IL-2 and 1 μg/mL of phytohemaglutinin (PHA-L; Sigma). Cells were then cultured for more than 2 weeks, with media change as needed before they were functionally evaluated.

DC migration assay

Immature human monocyte-derived DCs were treated as described above and then placed in the top chamber of Transwell plates that contained CCR7-specific ligand CCL21 (300 U/mL) in the medium in the bottom chamber. Three hours later, the cells that migrated toward the lower chamber were counted. Fold change over the cells that migrated spontaneously in the absence of CCL21 is reported. Assays were conducted in triplicate and shown is a representative experiment.

Allogeneic T-cell reaction

Naive $CD8^+$ T cells were isolated from PBMCs by using CD45RO and CD8 magnetic beads from Myltenyi Biotech. mo-DCs were prepared as described above. After 24 hours, stimulation with the agonist of choice, mo-DCs were washed 3 times, and then incubated with allergenic naive $CD8^+$ T cells in 96-well round bottom tissue culture plates at different ratios of 1:10 to 1:90, and left in the incubator at 37°C for 6 days in the presence of vehicle (–), rhIL-12 (1 ng/mL) from R&D, anti-IL12 (2 μg/mL), or control IgG (2 μg/mL) from Biolegend before being stimulated with CD3/CD28 dynabeads (Invitrogen), according to the manufacturer's protocol. The following day, supernatants were collected and levels of IFN-γ were assessed.

Flow cytometry and cell sorting

Flow cytometry was conducted by using either the FACS Calibur (BD Biosciences) or the LSR II (BD Biosciences) flow cytometers. Analysis was done by using FlowJo software (Tree Star).

Statistical analysis

All statistical analysis was conducted using Student's *t* test. We have carried out 2-way ANOVA test on the results of kinetics of IL-10 at 5 and 6 hours.

Results

TLR4 engagement inhibits TLR3-induced proinflammatory cytokine production in DCs

Initially, we compared the effects of the TLR4 and TLR3 agonists, LPS and polyI:C, respectively, and their combination to induce cytokine secretion in immature mo-DCs. We chose to begin with LPS as it is known to mature DCs that induce potent antitumor immunity in mice, and its derivative MPL has shown promise in human immunization protocols (11). PolyI:C has been less extensively studied than LPS but *in vitro* studies generated impressive results (9). For most studies, we used poly-ICLC, which is polyI:C stabilized with carboxymethyl cellulose and poly-L-lysine to confer resistance to ribonuclease. In our study, polyI:C induced high levels of the proinflammatory cytokines IL-12p70, TNF-α, IL-1β, and IP-10 (Fig. 1A–D, respectively), in addition to type I IFNs and IL-6 (Supplementary Fig. S1A and B). LPS was a poor inducer of bioactive IL-12, but a potent stimulator of IL-10. Interestingly, when TLR4 and TLR3 were engaged simultaneously, the proinflammatory cytokine response, in particular IL-12, was dramatically reduced ($P = 0.0023$ for IL12p70 and $P = 0.0051$ for TNF-α) in comparison with TLR3 ligation alone, and resembled the pattern induced by LPS (Fig. 1A and B, respectively). In contrast, IL-10 levels were significantly increased when TLR4 and TLR3 were coligated compared with polyI:C or LPS alone (Fig. 1E).

Suppression of polyI:C-induced DC cytokine responses by LPS is mediated by IL-10

To determine whether IL-10 was responsible for the suppression of IL-12, TNF-α, and other cytokines tested, we added increasing doses of LPS to immature mo-DC while keeping the dose of polyI:C constant at a suboptimal level. The addition of as little as 1 pg/mL of LPS noticeably reduced IL-12p70 levels (Fig. 1F), and maximal inhibition was observed at the dose of 100 ng/mL of LPS, which we use routinely. As IL-12p70 levels fell with augmented doses of LPS, there was a corresponding increase of IL-10 secreted (Fig. 1F). This finding may explain discrepancies in the literature as they pertain to relative IL-12p70 induction by LPS and polyI:C. For example, LPS is often considered to be an effective inducer of IL-12 in DCs but levels of only a few hundred picograms per milliliter are reported, similar to our own results. As minute amounts of LPS (far below Limulus assay detection limits) are able to suppress polyI:C-induced IL-12, it is possible that contamination with LPS may obscure the real effects of this and other agonists. To see if the inhibitory potential of LPS could be reversed, we added blocking antibodies to IL-10 and the IL-10 receptor (IL-10R). The inhibition of IL-12p70 in the LPS/polyI:C condition was not only overturned, but IL-12p70 levels increased substantially. Furthermore, LPS only treated DCs now also produced high levels of IL-12p70, indicating that IL-10 is the source of self-induced inhibition (Fig. 1G). Isotype matched antibodies and rHu IL-10 were used as controls (Fig. 1G). The latter inhibited IL-12 (Fig. 1G), TNFα, IP-10, and IL-1β (data not shown) consistent with a direct role for IL-10 in blocking inflammation. Therefore, the inhibitory profile observed during coligation of TLR4 and TLR3 receptors, is mediated by TLR4-induced IL-10.

Inhibition of polyI:C-induced responses by LPS is mediated by Stat3 and partially by p38

IL-10 signals through IL-10R, Janus-activated kinase 1, and ultimately signal transducers and activators of transcription 3 (STAT3) to suppress proinflammatory signaling (12). Therefore, we silenced STAT3 using specific siRNA, achieving about 50% reduction in total STAT3 levels (Supplementary Fig. S1C and D). That level of downregulation was sufficient to completely rescue the LPS-induced inhibition of IL-12 (Fig. 2A). Knockdown of STAT3 also partially reversed rhIL-10 inhibition of polyI:C-induced IL-12 (Fig. 2A). As phosphoinositide 3-kinase (PI3K) has been hypothesized to block IL-12 by blocking p38, c-*jun* NH, kinase, extracellular-signal regulated kinase, and NF-κB pathways in the early phase of TLR signaling (13), we blocked PI3K activity with wortmannin at 100 nmol/L, a concentration that does not inhibit endocytosis (14). Inhibition of PI3K allowed induction of IL-12p70 by LPS alone, as well as reversal of the inhibitory profile of LPS on polyI:C (Fig. 2B). However, it also increased levels of IL-10 (Fig. 2C) suggesting that PI3K plays an important role in overall suppression of TLR signaling, and indicating that its effects are IL-10 independent. Inhibition of p38 suppressed induction of IL-10 and partially reversed the inhibition of IL-12p70 when mo-DC were coligated with TLR4 and TLR3 agonists (Fig. 2B and C, respectively), consistent with the fact that p38 activation participates in LPS-induced IL-10 transcription (15) and that the levels of IL-10 were reduced. In line with these observations, we noted phosphorylation of p38 as early as 10 minutes within mo-DC when both LPS or LPS/polyI:C were used (but not polyI:C), reaching peak levels between 30 and 60 minutes (Fig. 2D). Altogether, these results indicate that LPS-induced suppression is mediated at least in part by p38 that is required for IL-10 induction, and which inhibits IL-12 secretion in a STAT3-dependent manner.

Kinetics of IL-10 secretion account for the differential costimulatory and proinflammatory capacities of TLR agonists

To determine whether the results we observed with polyI:C and LPS applied to other TLR agonists, we tested R848, a TLR7/8 agonist. R848 alone induced very low levels of IL-12p70 as compared with polyI:C (Supplementary Table S1). In every condition inducing appreciable amounts of IL-10, blocking this cytokine during the maturation of mo-DCs subsequently resulted in highly increased CD80 and CD86 costimulatory molecule upregulation (Fig. 2E). Negative costimulation, as measured by the expression levels of PDL-1 was further increased with the addition of rhIL-10, while blocking IL-10 reversed this upregulation in the conditions where IL-10 was secreted (Fig. 2F). Similarly, when IL-10 and IL-10R were

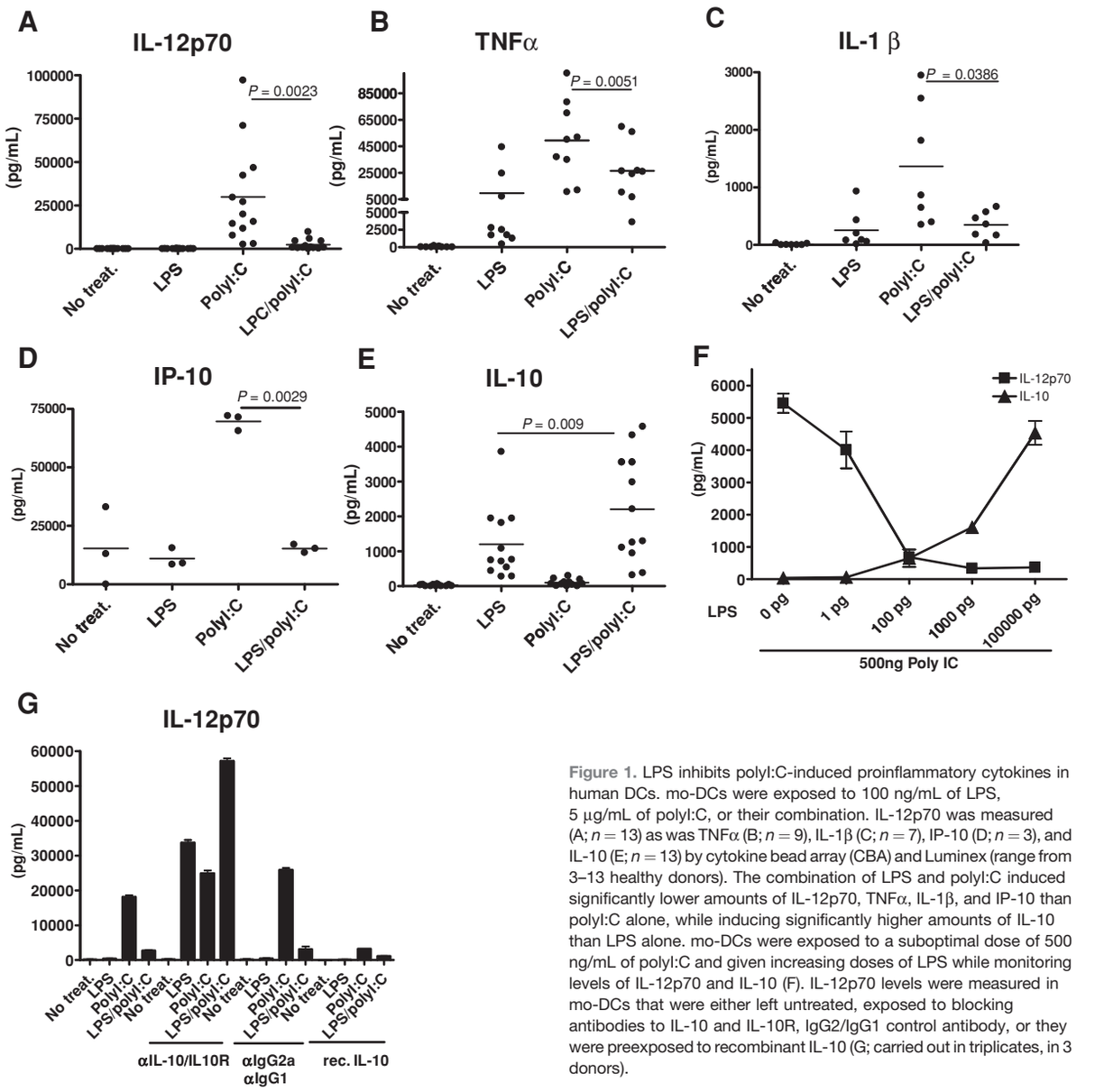

Figure 1. LPS inhibits polyI:C-induced proinflammatory cytokines in human DCs. mo-DCs were exposed to 100 ng/mL of LPS, 5 μg/mL of polyI:C, or their combination. IL-12p70 was measured (A; $n = 13$) as was TNFα (B; $n = 9$), IL-1β (C; $n = 7$), IP-10 (D; $n = 3$), and IL-10 (E; $n = 13$) by cytokine bead array (CBA) and Luminex (range from 3–13 healthy donors). The combination of LPS and polyI:C induced significantly lower amounts of IL-12p70, TNFα, IL-1β, and IP-10 than polyI:C alone, while inducing significantly higher amounts of IL-10 than LPS alone. mo-DCs were exposed to a suboptimal dose of 500 ng/mL of polyI:C and given increasing doses of LPS while monitoring levels of IL-12p70 and IL-10 (F). IL-12p70 levels were measured in mo-DCs that were either left untreated, exposed to blocking antibodies to IL-10 and IL-10R, IgG2/IgG1 control antibody, or they were preexposed to recombinant IL-10 (G; carried out in triplicates, in 3 donors).

blocked with specific antibodies, IL-12p70 and TNF-α levels were dramatically increased ($P < 0.0001$, Fig. 3A, Fig. S1E). The combination of LPS and R848 gave a synergistic profile, as previously described (16), inducing higher amounts of IL-12p70 compared with either agonist alone, despite high levels of IL-10 (Fig. 3B) but still fairly small levels of IL-12p70 in comparison with polyI:C alone (Fig. 3A). However, blocking IL-10 upon stimulation in this dual agonist combination resulted in dramatically increased amounts of proinflammatory cytokines, as exemplified by IL-12p70, suggesting that IL-10 production blocked the synergistic effects of the 2 agonists on IL-12 production (Fig. 3A and Supplementary Table S1). Although the combination of TLR3 and TLR7/8 ligation induced high amounts of IL-12p70, these were similar to levels induced by polyI:C alone ($P = 0.47$, Supplementary Fig. S1F). In contrast, the levels of TNF-α were significantly higher if polyI:C and R848 were combined ($P = 0.02$, Supplementary Fig. S1G) compared with TLR3 or TLR7/8 ligation alone. Interestingly, although the ability of R848 to produce IL-12 was dampened by the simultaneous production of IL-10 (Fig. 3A), it did not seem to inhibit the endogenous IL-12 production by polyI:C when the 2 agonists were combined. However, blockade of IL-10 did further augment the IL-12 levels induced by these 2 agonists ($P = 0.0048$).

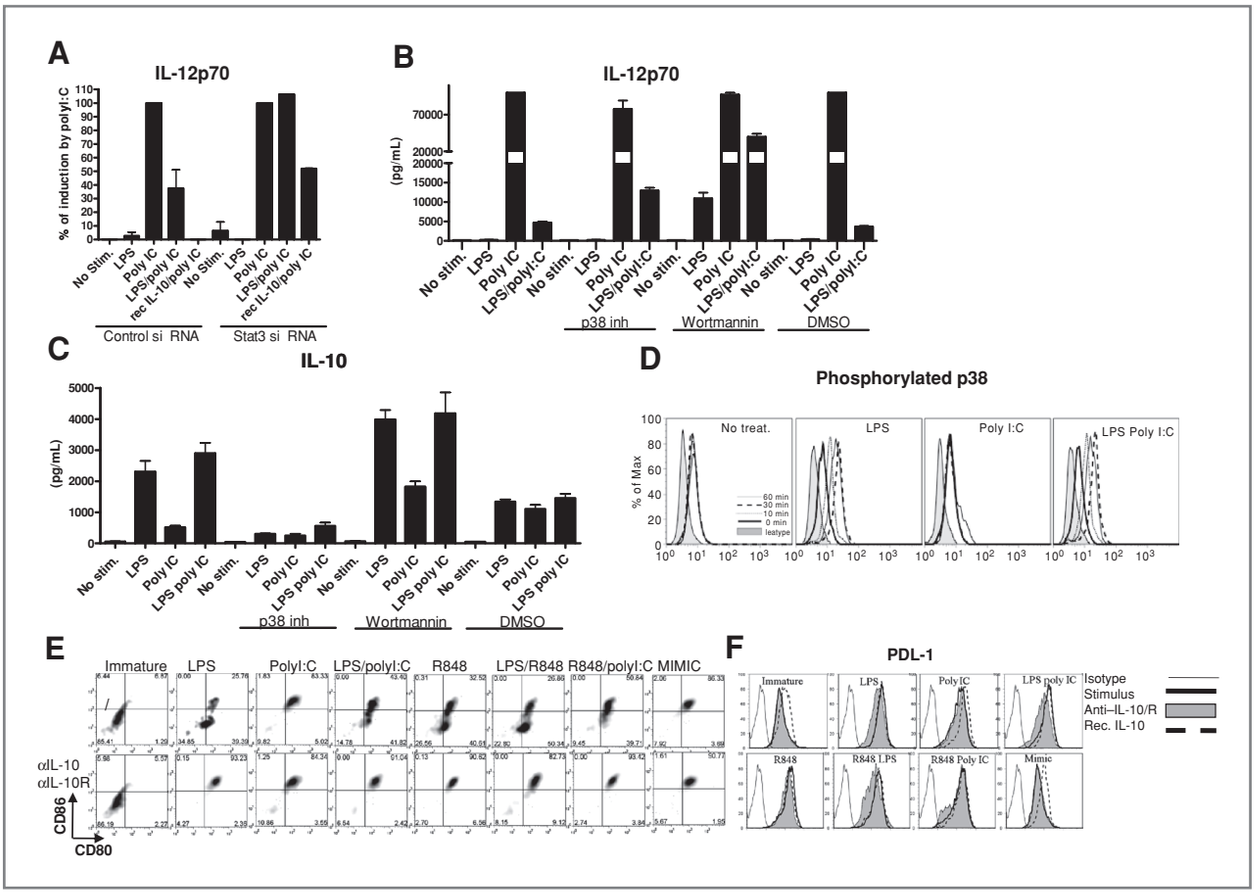

Figure 2. Inhibition of polyI:C–induced responses by LPS is mediated by IL-10, Stat3, and partially by P38. mo-DCs were electroporated with STAT3 siRNA and the amount of total protein was measured by Western blot (A) and quantified using densitometry (B). These cells were treated with LPS, polyI:C, or their combination, and IL-12p70 was measured (A). mo-DCs prior to exposure to LPS, polyI:C, or their combination were pretreated with a P38 inhibitor 10 ng/mL or wortmannin 100 nmol/L/mL, and IL-12p70, and IL-10 was quantified (B and C, respectively). Phosphorylation of P38 was monitored over time upon ligation of TLR4, TLR3, or their combination (D). mo-DCs were exposed to 100 ng/mL of LPS, 5ug/mL of polyI:C, 10 μmol/L of R848, and/or their combination as well as mimic cytokine cocktail or left untreated with or without blocking antibodies to IL-10 and -10R with or without recombinant IL-10. Cells were then stained with anti-CD80 and anti-CD86 (E), anti–PDL-1 antibody (F), or with the isotype control.

We next measured levels of IL-10 produced under these various stimulation conditions. Significant IL-10 secretion was observed upon LPS stimulation, and synergistic induction of IL-10 upon R848/LPS and R848/polyI:C combined stimulation (Fig. 3B). Exogenously added rhIL-10 was used as a control. Strikingly, addition of only 300 pg/mL of recombinant IL-10 before stimulation induced far greater suppression of IL-12 production than that induced by LPS or R848-induced IL-10 (Figs. 3A and B) in combination with polyI:C. This suggested that the timing of IL-10 secretion, with respect to that of proinflammatory cytokine secretion, is important for its suppressive effect. We therefore measured IL-12p70 and -10 secretion kinetics in response to R848, polyI:C, and LPS. When secreted, IL-12p70 was measurable in supernatants at 5 to 6 hours in all conditions (Fig. 3C). The timing of IL-10 secretion, however, was dependent on the agonist used. Namely, if LPS was used alone or in combination with other agonists, IL-10 was measurable between 3 and 4 hours, before any IL-12 is significantly secreted. In contrast, R848 used alone or in a combination with any agonist other than LPS, induced IL-10 between 6 and 9 hours (Fig. 3D). We carried out a statistical test on the results of kinetics of IL-10 production at 5 and 6 hours, and found a significant difference ($P < 0.001$) in LPS versus R848/polyI:C or any other condition, provided it did not contain LPS. When LPS was compared with another condition that also included LPS, the significance at the time points of 5 and 6 hours was lost. This difference in timing explains how the addition of 5- to 10-fold lower amounts of recombinant IL-10 prior to TLR agonist stimulation (Fig. 2A) caused superior suppression of IL-12p70 compared with combinations of LPS/polyI:C and LPS/R848, which induce higher amounts of IL-10, but later. It also explains why TLR7/8 and TLR3 coligation resulted in significant inflammatory cytokine production, given the relatively delayed IL-10 secretion. To confirm these findings and to explain why R848 despite inducing later secretion of IL-10, does not produce much IL-12p70, but does so if blocking antibodies to IL-10/IL10R are introduced, we carried out a similar kinetic experiment but this time also

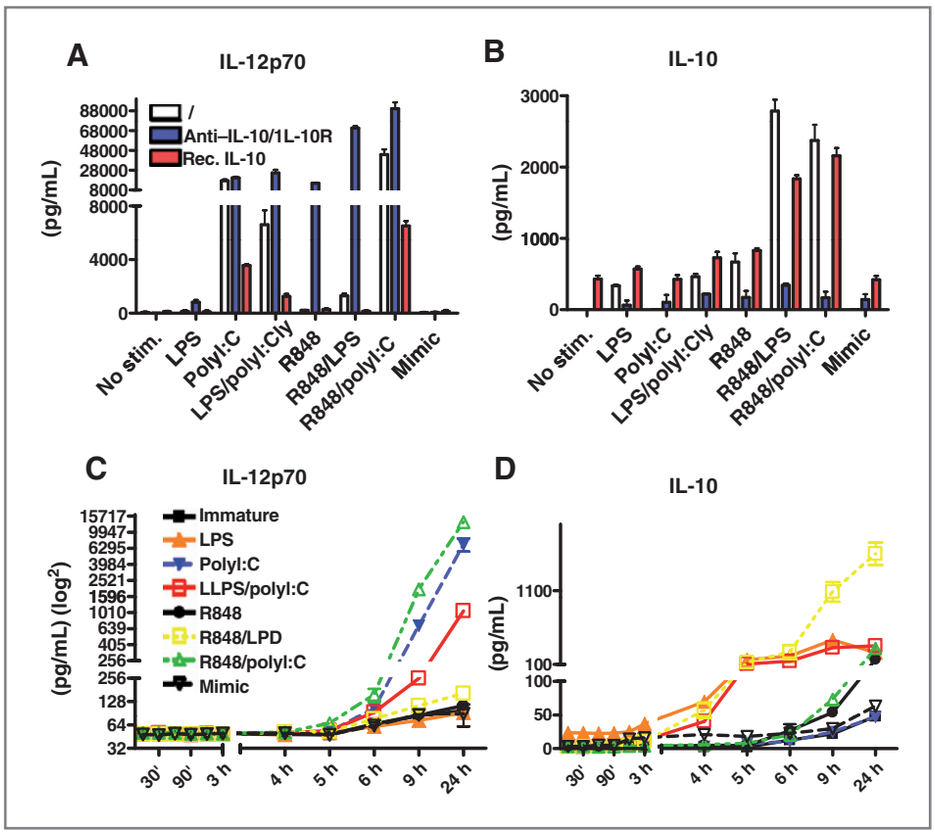

Figure 3. Kinetics of IL-10 secretion explains inflammatory capacity of TLR agonists. Mo-DCs were monitored for the secretion of IL-12p70 and IL-10 when either untreated or stimulated with LPS, polyI:C, LPS/polyI:C, R848, R848/LPS, R848/polyI:C, and Mimic. IL-12p70 and IL-10 was also monitored using the same conditions with or without blocking IL-10 and IL-10R, and with or without pretreatment with recombinant IL-10 (A, B respectively). The same experiment was repeated by monitoring IL-12p70 and IL-10 in supernatants collected over time (C, D respectively). Each experiment was carried out in triplicates and repeated at least with 3 different donors.

adding rHuIL-10 or αIL-10/IL-10R antibodies at 9 hours after stimulation (Fig. 4A–H). Early addition of rhIL-10 resulted in complete abrogation of IL12 production in all conditions tested. We noted that if IL-10 was blocked at 0 hours when mo-DCs were stimulated with R848 (Fig. 4E), the majority of production of IL-12 did not occur until after 9 hours, well after IL-10 is normally induced by this agonist without blocking IL-10 (Fig. 2D). R848 thus induces IL-12 relatively late allowing for also late induction of IL-10 to suppress further secretion of IL-12. These data explain the paucity of IL-12 in the supernatant of mo-DCs stimulated with R848. In addition, we show that addition of rhIL-10 at 9 hours in polyI:C-stimulated DCs only had a partial effect with suppressive capacity being much lower than any condition where there was IL-10 early (Fig. 4C). Addition of rHuIL-10 at 9 hours to R848/polyI:C-stimulated DCs had little effect as there was enough IL-10 by that time point (Fig. 4G). We observed similar temporal regulation of TNF-α by IL-10 in the same experimental setting (Supplementary Fig. S1E). In addition to cytokine control, we tested whether blocking IL-10/IL-10R would have an impact on the migratory capacity of DCs toward CCL21. We observed no differences in CCR7 surface expression (data not shown) or DCs' migration capacity (Supplementary Fig. S1H), suggesting that this process is independent of IL-10.

IL-10 has a well-established anti-inflammatory role in TLR signaling, however, we show for the first time that the kinetics of its secretion by mo-DCs ultimately affects the induction of inflammatory cytokines by TLR3, TLR4, and TLR7/8 agonists or their combinations.

Increased levels of inflammation by mo-DCs translate into higher and stronger specific CD8 T-cell responses

To test whether these differences in cytokine secretion actually translate into functionally different T-cell responses, we analyzed $CD8^+$ T-cell priming against the melanoma-associated antigen Melan-A/Mart-1 using its modified HLA-A2–restricted Melan-A/Mart-1 (ELAGIGILTV) epitope as an immunogen. PolyI:C-matured mo-DCs consistently induced higher frequencies of specific T-cell responses in comparison with LPS or LPS/polyI:C matured mo-DCs (Fig. 5A and Supplementary Fig. S2A). The combinations of R848/LPS and R848/polyI:C resulted in levels of primed $CD8^+$ T cells similar to polyI:C-matured DC alone, although the trend was higher in the latter (Fig. 5A and Supplementary Fig. S2A). When the quality of these tetramer-positive polyclonal populations was evaluated, immature and mimic-matured mo-DCs were found to induce $CD8^+$ T cells that secreted IL-13 and -5 (Fig. 5B and C, respectively), typical of Tc2-like $CD8^+$ T cells. On the other hand, TLR agonist ligation of mo-DCs induced Tc1 $CD8^+$ cells, regardless of the agonist, but with varying quality. polyI:C, R848, and the combination of LPS/R848 and R848/polyI:C were able to induce Melan-A/MART-1 $CD8^+$ T cells capable of secreting higher amounts of IFN-γ, TNF-α, and IL-2 upon restimulation (Fig. 5D–F,

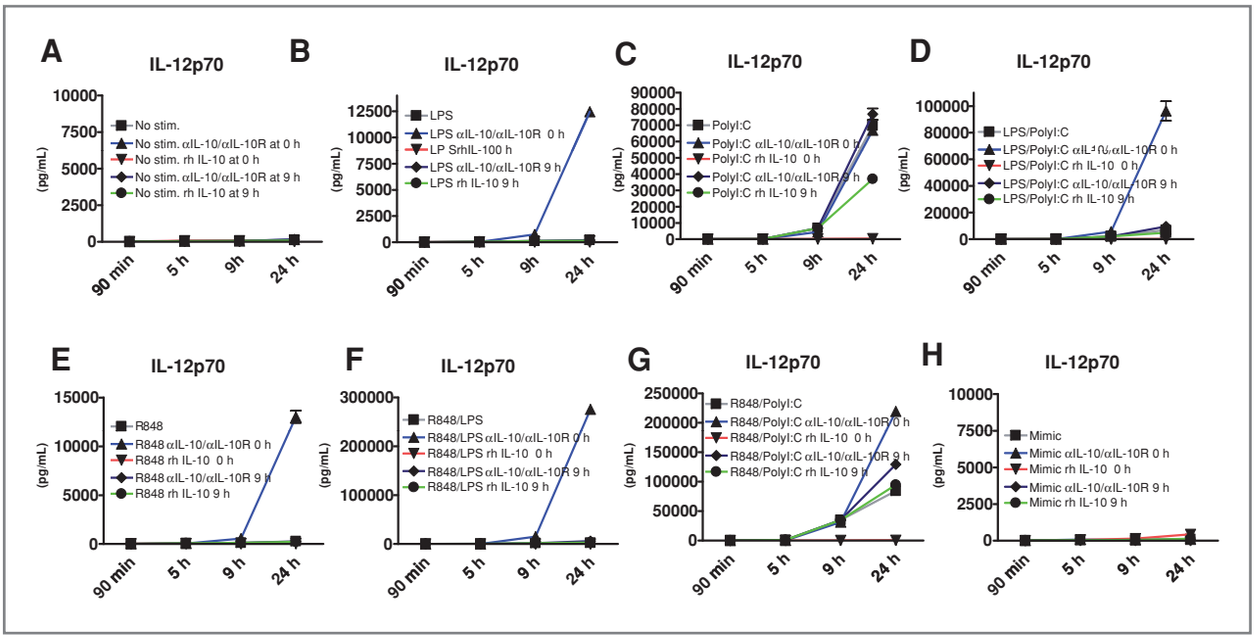

Figure 4. Delayed addition of rhIL-10 and blocking antibodies to IL-10/-10R further elucidates the mechanism of TLR agonists. Monocyte-derived DCs were exposed to 100 ng of LPS, 5 μg of polyI:C, 10 μmol/L of R848, and/or their combination as well as mimic cytokine cocktail or left untreated +/− rhIL-10 (300 ng/mL) at 0 and 9 hours and +/− anti–IL-10/-10R (2 μg/mL) at 0 or 9 hours; IL-12p70 was monitored over time in triplicate wells ($n = 2$) by CBA (A–H).

respectively). Thus, secretion of inflammatory cytokines by DC upon TLR triggering correlates with their T-cell stimulation capacity, inducing functionally differential antitumor responses.

We next formally assessed the contribution of IL-12 in the priming of naive $CD8^+$ T cells by TLR agonist-matured DC, by repleting the cytokine through addition of rHuIL-12, or blocking endogenously produced IL-12. After 6 days of coculture, the ability of T cells to secrete IFN-γ was measured (Fig. 5G). We saw substantial increases in IFN-γ production with the addition of rHuIL-12 in all conditions tested, and we also observed complete abrogation of IFN-γ secretion by T cells after IL-12 was blocked suggesting a crucial role for IL-12 in our system.

Blocking IL-10 during mo-DC maturation increases the quantity and quality of CD8 T-cell responses

Given that IL-10 plays an important role in controlling inflammation induced by mo-DCs during TLR ligation, we tested if blocking it translated into differential magnitude and quality of CD8 T-cell priming. As shown in Figure 5H, inhibition of IL-10 during DC activation dramatically enhanced Melan-A–specific T-cell generation, except when IL-10 is not secreted, that is, upon polyI:C stimulation possibly through the combined effects of enhanced costimulation and dampened negative costimulation (Fig. 2E and F). We also tested whether neutralization of IL-10 improved stimulation of allogeneic T-cell responses by TLR agonist matured DCs. After 7 days, the ability of T cells to secrete IFN-γ was measured. Blockade of IL-10 significantly increased IFN-γ secretion by primed T cells (Fig. S2B). Lack of IL-10 secretion by DCs upon ligation with polyI:C explains why this adjuvant as a single agent is superior to others tested. The priming with combinations of LPS/R848 and R848/polyI:C can be further improved by blocking IL-10. Apart from polyI:C, all tested TLR agonists or their combinations, use IL-10 as a mechanism to control the priming of T cells. In immunocompromised melanoma patients blocking of IL-10 or usage of adjuvants which induce low levels of IL-10, like polyI:C may be beneficial in boosting their antimelanoma immunity.

Discussion

Using TLR agonists as adjuvants to mature DCs either *ex vivo* or *in situ* is an attractive method for initiating or boosting antitumor responses. Here, we show that certain combinations of TLR agonists are synergistic in their ability to mature DCs and induce T-cell responses, whereas others are immunosuppressive. Synergism was observed with LPS and R848 as previously described for cytokine production (16). We show for the first time that this also translates into more efficient $CD8^+$ T-cell priming. Inhibition of both inflammatory cytokine production and T-cell priming was observed when LPS and polyI:C were combined. Similar inhibition occurred when zymosan was added to polyI:C (Supplementary Fig. S3), which was recently confirmed in a study showing that TLR2 ligation controls inflammation induced by polyI:C (17). Altogether these findings suggest that general coligation of the TLR2/TLR4 with the TLR3

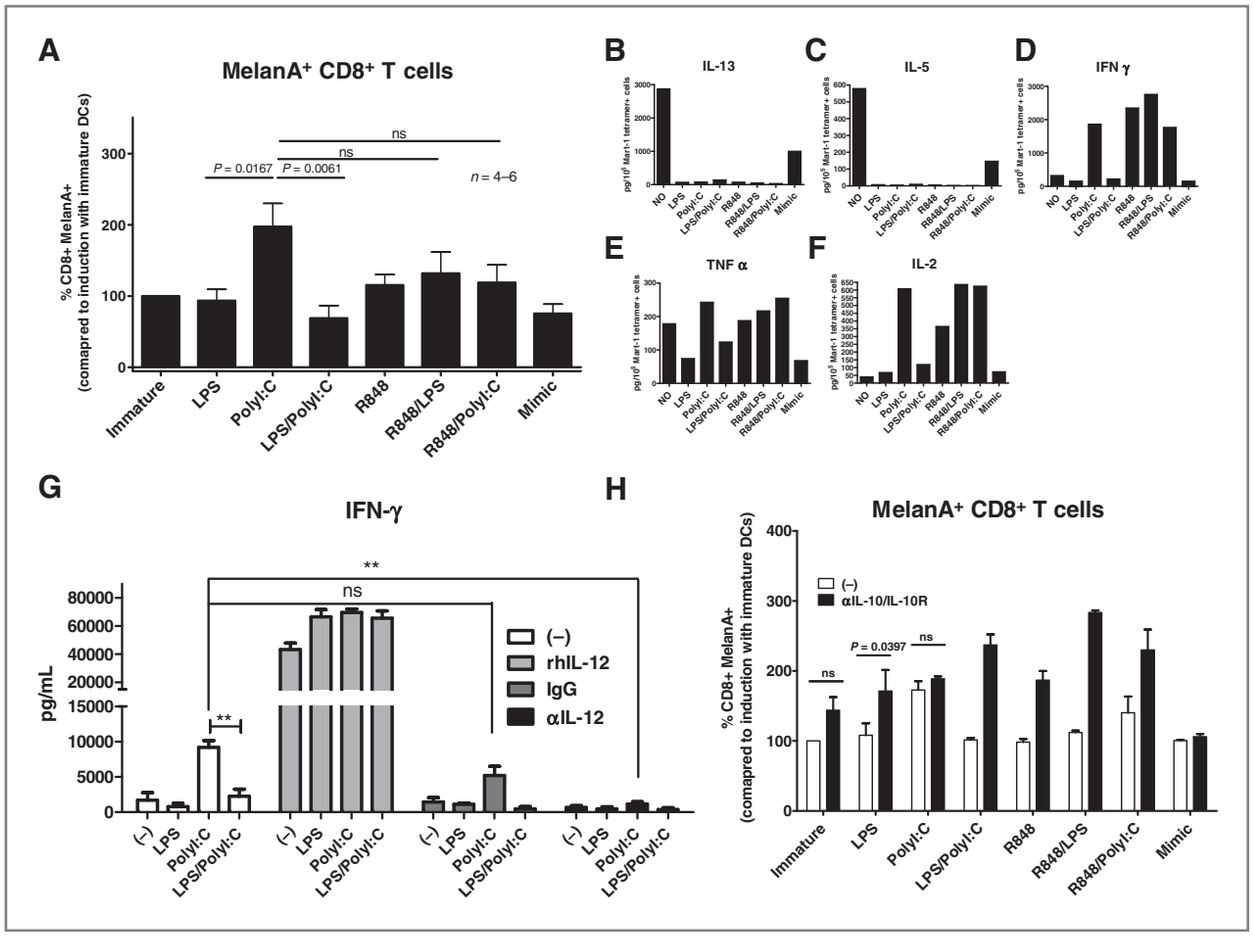

Figure 5. Increased levels of inflammation in mo-DCs translate into higher numbers of specific CD8 T-cell responses. Healthy donor naïve A2.1+ $CD8^+$ T cells were primed with peptide encoding for the HLA A2.1 restricted Mart-1/MelanA (ELAGIGILTV) epitope and the frequency of tetramer positive cells was monitored ($n = 6$; A). Tetramer positive cells from all conditions from a donor were expanded and examined functionally for secretion of IL-13, IL-5, IFNγ, TNFα, and IL-2 (B, C, D, E, and F, respectively). Differentially matured mo-DCs and allogeneic naïve $CD8^+$ T cells were cocultured for 6 days in a MLR reaction in presence of vehicle (–), rhIL-12, anti-IL-12, or IgG. Proliferated cells were stimulated overnight with αCD3/CD28 beads and evaluated for the production of IFN-γ. **, $P < 0.01$ when comparing PolyI:C to LPS (with vehicle) and when comparing PolyI:C (with vehicle) to PolyI:C when IL-12 was blocked. NS, not significant when comparing PolyI:C (with vehicle) to PolyI:C when IgG was used (G). Healthy donor naïve A2.1+ $CD8^+$ T cells were primed with peptide encoding for the HLA A2.1 restricted Mart-1/MelanA (ELAGIGILTV) epitope and the frequency of tetramer positive cells was monitored in 2 additional donors with a condition of αIL-10/IL10R Abs for each treatment, showing a representative donor (triplicate wells; H).

pathways will lead to suppressed levels of proinflammatory cytokines.

Our study shows that the antagonism of LPS on TLR ligation occurs through an IL-10–dependent mechanism. The involvement of IL-10 was shown by using blocking antibodies to IL-10 and -10R, which completely rescued the inhibitory profile elicited by LPS. Phosphorylation of p38 mitogen-activated protein (MAP) kinase was required for maximal IL-10 secretion as selective inhibition of p38 MAP kinase phosphorylation reduced IL-10 secretion. We postulate that the consistent partial rescue is because of the fact that p38 MAP kinase is also required for 12p70 production (18). IL-10 activity required STAT3 as verified by using siRNA specific for STAT3. Altogether, these results indicate that LPS activates p38 MAP kinase, leading to IL-10 (as well as IL-12) production. IL-10 subsequently mediates its inhibitory effects via IL-10R and STAT3, which exert the immunosuppressive activity. IL-10 has recently been shown to lead to ubiquitination of MyD88-associated signaling molecules (19), suggesting a possible additional mechanism of action in attenuating TLR-dependent responses.

We also verified the previous findings (13) that PI3K is a negative regulator of TLR signaling (18). PI3K has been implicated in the negative regulation of the p38 and NF-κB pathways that are crucial for full TLR signaling cascades (13). Although our data suggest an IL-10–independent activity of PI3K on TLR signaling, as PI3K blockade resulted in higher amounts of IL-10, it remains possible that PI3K regulates IL-10 uptake or its downstream effects.

Interestingly, in human DCs, other TLR agonist combinations such as R848/polyI:C or R848/LPS showed at least additive or synergistic effects in their ability to induce high levels of inflammatory cytokines *in vitro* despite high levels of IL-10. We explained these phenomena by showing that the

kinetics of IL-10 secretion is different among the TLR agonists used. Namely, LPS is capable of inducing IL-10 very early, at about 3 hours, whereas R848 does so at about 6 to 9 hours. This delay allows for synergism of R848/polyI:C to occur, that can be further augmented if blocking antibodies to IL-10 and -10R are present. The R848/LPS combination also displayed a weak synergistic profile especially in the induction of IL-12p70, despite early secretion of IL-10. Here, we postulate that the combined signals from TLR4 and TLR8 were sufficiently potent to allow for small amounts of IL-12 to be secreted, but which is still dependent on IL-10, as its blockade released several fold higher levels of all cytokines measured (e.g. IL-12p70, TNF-α, IL-6, and so on). These findings indicate that the timing of IL-10 secretion influences synergy between TLR agonists.

The levels of inflammation also translated into differential priming capability of DCs. IL-10 suppressed expression of CD80 and CD86, whereas it upregulated PDL-1. If mo-DCs were matured with polyI:C while pulsed with MelanA/Mart-1 (ELAGIGILTV) peptide, they yielded a higher frequency of tetramer positive cells compared with the LPS or LPS/polyI:C stimuli tested. Furthermore, $CD8^+$ MelanA/Mart-1^+ cells generated from conditions inducing strong inflammatory profile also secreted higher levels of IFN-γ, TNF-α, and IL-2. Altogether, blocking IL-10 augmented the frequency of $CD8^+$ MelanA/Mart-1^+ cells induced, in all conditions where IL-10 was initially secreted, most apparently through higher expression of IL-12. The higher levels of CD80 and CD86 and lower expression of PDL-1 also contribute to the amplification in priming. No significant change/enhancement was seen with polyI:C which induces little to no IL-10.

The implications of these findings are many. PolyI:C seems to be the most potent TLR adjuvant tested because of its induction of proinflammatory cytokines in the absence of IL-10, and maintenance of high levels of CD80 and CD86. PolyI:C is already approved for clinical testing in humans, and can be used to mature DC *ex vivo* prior to their administration *in vivo* or by *in situ* administration. The use of polyI:C in animal models has thus far yielded impressive results in terms of proinflammatory cytokines secretion by bone marrow-derived DCs and priming of polyvalent $CD4^+$ responses (9). Our results point to ways to enhance vaccines that engender immunity.

In summary, the ability of LPS to mediate IL-10–dependent suppression on the polyI:C-induced inflammation suggests complex interactions of TLR agonists with each other. It also unveils an important role temporal secretion of IL-10 has in the biologic system, although possibly allowing us to exploit it in vaccine design.

Disclosure of Potential Conflicts of Interest

A.M. Salazar is Chief Executive Officer and Scientific Director of Oncovir Inc., which provided poly-ICLC. The other authors disclosed no potential conflicts of interest.

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References

1. Bhardwaj N. Harnessing the immune system to treat cancer. J Clin Invest 2007;117:1130–6.
2. Gilboa E. DC-based cancer vaccines. J Clin Invest 2007;117:1195–203.
3. Lamm DL, Blumenstein BA, Crawford ED, Montie JE, Scardino P, Grossman HB, et al. A randomized trial of intravesical doxorubicin and immunotherapy with bacille Calmette-Guerin for transitional-cell carcinoma of the bladder. N Engl J Med 1991;325:1205–9.
4. Brichard VG, Lejeune D. GSK's antigen-specific cancer immunotherapy programme: pilot results leading to phase III clinical development. Vaccine 2007;25Suppl 2:B61–71.
5. Vansteenkiste J. Final results of a multi-center, double-blind, randomized, placebo-controlled phase II study to assess the efficacy of MAGE-A3 immunotherapeutic as adjuvant therapy in stage IB/II non-small cell lung cancer (NSCLC). Proc Am Soc Clin Oncol 2007;25.
6. Shackleton M, Davis ID, Hopkins W, Jackson H, Dimopoulos N, Tai T, et al. The impact of imiquimod, a Toll-like receptor-7 ligand (TLR7L), on the immunogenicity of melanoma peptide vaccination with adjuvant Flt3 ligand. Cancer Immun 2004;4:9.
7. Adams S, O'Neill DW, Nonaka D, Hardin E, Chiriboga L, Siu K, et al. Immunization of malignant melanoma patients with full-length NY-ESO-1 protein using TLR7 agonist imiquimod as vaccine adjuvant. J Immunol 2008;181:776–84.
8. Pufnock JS, Cigal M, Rolczynski LS, Andersen-Nissen E, Wolfl M, McElrath MJ, et al. Priming $CD8^+$ T cells with dendritic cells matured using TLR4 and TLR7/8 ligands together enhances generation of $CD8^+$ T cells retaining CD28. Blood 2010;117:6542–51. Epub 2011 Apr 14.
9. Longhi MP, Trumpfheller C, Idoyaga J, Caskey M, Matos I, Kluger C, et al. Dendritic cells require a systemic type I interferon response to mature and induce $CD4^+$ Th1 immunity with poly IC as adjuvant. J Exp Med 2009;206:1589–602.
10. O'Neill DW, Bhardwaj N. Differentiation of peripheral blood monocytes into dendritic cells. Curr Protoc Immunol 2005;Chapter 22: Unit 22F 4.
11. Vandepapelière P, Horsmans Y, Moris P, Van Mechelen M, Janssens M, Koutsoukos M, et al. Vaccine adjuvant systems containing monophosphoryl lipid A and QS21 induce strong and persistent humoral and T cell responses against hepatitis B surface antigen in healthy adult volunteers. Vaccine 2008;26:1375–86.
12. O'Garra A, Murphy KM. From IL-10 to IL-12: how pathogens and their products stimulate APCs to induce T(H)1 development. Nat Immunol 2009;10:929–32.
13. Fukao T, Koyasu S. PI3K and negative regulation of TLR signaling. Trends Immunol 2003;24:358–63.
14. Lee HK, Lund JM, Ramanathan B, Mizushima N, Iwasaki A. Autophagy-dependent viral recognition by plasmacytoid dendritic cells. Science 2007;315:1398–401.

15. Ma W, Lim W, Gee K, Aucoin S, Nandan D, Kozlowski M, et al. The p38 mitogen-activated kinase pathway regulates the human interleukin-10 promoter via the activation of Sp1 transcription factor in lipopolysaccharide-stimulated human macrophages. J Biol Chem 2001;276:13664–74.
16. Napolitani G, Rinaldi A, Bertoni F, Sallusto F, Lanzavecchia A. Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. Nat Immunol 2005;6:769–76.
17. Lai Y, Di Nardo A, Nakatsuji T, Leichtle A, Yang Y, Cogen AL, et al. Commensal bacteria regulate Toll-like receptor 3-dependent inflammation after skin injury. Nat Med 2009;15:1377–82.
18. Fukao T, Tanabe M, Terauchi Y, Ota T, Matsuda S, Asano T, et al. PI3K-mediated negative feedback regulation of IL-12 production in DCs. Nat Immunol 2002;3:875–81.
19. Chang J, Kunkel SL, Chang CH. Negative regulation of MyD88-dependent signaling by IL-10 in dendritic cells. Proc Natl Acad Sci U S A 2009;106:18327–32.