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TLR Ligands Differentially Modulate T Cell Responses to Acute and Chronic Antigen Presentation

Nevil J. Singh,2* Maureen Cox,† and Ronald H. Schwartz*

The outcome of peripheral T cell activation is thought to be largely determined by the context in which the cognate Ag is initially presented. In this framework, microbial products that can activate APCs via TLRs are considered critical in converting an otherwise tolerogenic context to an immunogenic one. We examine this idea using a model system where naive T cells are stimulated in the periphery by a persistent self Ag. The addition of multiple TLR ligands to this context, acutely or chronically, failed to significantly alter the tolerogenic phenotype in the responding T cells. This contrasts with the ability of such adjuvants to improve T cell responses to soluble peptide immunizations. We reconcile this difference by revealing a hitherto poorly appreciated property of TLR ligands, which extends the duration of soluble Ag presentation in vivo by an additional two to three days. Finally, we could replace the requirement for TLR-mediated APC activation in soluble-Ag-induced T cell expansion and differentiation, by maintaining the Ag depot in vivo using repeated immunizations. These data suggest a novel process by which TLR ligands modulate T cell responses to acute Ags, without disrupting the induction of tolerance to persistent self Ags. The Journal of Immunology, 2007, 179: 7999–8008.

The adaptive immune system anticipates the diverse and rapidly mutating array of environmental pathogens that it is likely to encounter, by generating an equally diverse set of receptors clonally expressed on T and B cells. The relatively random process by which these receptors are generated also means that many of these cells would be able to recognize and respond to antigenic determinants encoded by the animal’s own genome. The heavy cost of such autoreactivity has driven the evolution of multiple mechanisms capable of regulating such responses (1). However, the recruitment of any of these mechanisms requires the lymphocyte to first discern whether the ligand it encounters represents a pathogenic insult. The logic behind this discrimination, especially for helper T cells, has been the subject of significant debate over the past four decades. Early experiments that demonstrated “immunological paralysis” instead of a measurable Ab response to immunizations with purified protein preparations led to the appreciation of the role of a second signal in helping the immune system to make this discrimination (2, 3). Although these original models dealt with T-B collaboration, subsequent studies on allogeneic responses suggested that an Ag-nonspecific signal was critical for activating T cells as well (4). The demonstration that APCs incapable of providing the second signal induced a tolerance phenotype in T cells highlighted the utility of such signals in determining T cell fate (5). In light of these studies, the concept of adjuvanticity that Dresser originally proposed was formulated as a cellular model for self/nonself discrimination by Charles Janeway. In this framework, the presence of pattern recognition receptors on APCs allowed them to specifically recognize pathogen-associated molecular patterns (PAMPs)3 and trigger their own transition from a resting to activated state (6). This allowed Ags associated with pathogens to be presented only by cells with an activated phenotype, which were uniquely capable of providing the critical second signal to T cells. In an alternate model, the initial activation of the APCs was proposed to be driven by endogenous alarm signals that do not necessarily require the immune system to make a self/nonself discrimination (7). Subsequently, both pathogen-derived and endogenous signals have been shown to activate dendritic cells in a variety of experimental models (8, 9). In elegant studies that characterized the microbial components responsible for such an activation, Medzhitov and Janeway (10) demonstrated that ligands of TLRs activate APCs and confer adjuvanticity to purified Ags. The expanding families of TLR ligands are currently thought to satisfy the Janeway postulate for PAMPs. Several experiments have subsequently validated the ability of such molecules to act as the “immunologist’s dirty little secret” (11) by amplifying Ag-specific effector responses to tumors, viral infections, etc.

This model is also quite relevant to autoreactivity and self tolerance. In this case, the PAMP model would predict that tolerance results from the presentation of Ag by cells that have not engaged a TLR ligand in vivo. Conversely, the administration of TLR ligands to the context of a self-reactive T cell engaging its cognate Ag in vivo, should now alter the outcome to a virulent effector response. Experimental evidence for this postulate, however, has been rather scarce.

Nevertheless, the administration of TLR ligands such as LPS has been shown to improve the expansion, differentiation, and survival of T cells responding to “tolerogenic” stimuli, such as immunizations with soluble peptides and superantigens (12, 13). Similarly, in the setting of tissue transplantation such molecules reduced the efficiency of costimulatory blockade as a tolerance induction strategy (14). In models of B cell-mediated autoimmunity, engagement of TLR9 on B cells has been shown to favor enhanced activation of self-Ag-specific cells (15). Recent reports

1Abbreviations used in this paper: PAMP, pathogen-associated molecular pattern; PCC, pigeon cytochrome C.

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(16) have also argued that LPS-stimulated dendritic cells may allow effector responses to be released from the control of regulatory T cells—another postulated mechanism for the maintenance of peripheral tolerance. Although the ability of TLR ligands to prevent the induction of peripheral tolerance to natural and model self Ags remains to be tested extensively, initial studies on CD8+ T cells suggest that this may be more complex than predicted by the PAMP model. Using CD8+ T cells specific for a pancreatic islet-expressed Ag, Redmond et al. (34) demonstrated that activation of APCs with an anti-CD40 treatment or a viral infection (with viral PAMPS) was insufficient to prevent the induction of tolerance. Nevertheless, effector responses against a tissue-specific Ag could be amplified sufficiently to cause pathology, in some cases by using similar treatments (17).

We have previously reported a double transgenic model system that allows us to study the fate of naive peripheral T cells that encounter a self Ag either in the presence or absence of endogenous T cells (18). In both contexts, the induction of anergy (adaptive tolerance) is similar, although in the lymphopenic hosts, the autoreactive T cells differentiate and expand to greater numbers before they complete adaptation. This results in the manifestation of mild immunopathology. The simultaneous analysis of lymphopenic and nonlymphopenic hosts, therefore, allows us to study a variety of T cell fate decisions within the same experimental construct. In this report, we use this paired system to examine the ability of acute and chronic TLR-ligand stimulation to modulate T cell responses to a chronic self Ag. Interestingly, neither the induction of tolerance nor the long term survival of autoreactive T cells could be stably altered by the presence of such adjuvants. We further reconcile these observations with the studies on soluble Ag challenges, by demonstrating that TLR ligands can increase the window of Ag stimulation afforded by soluble Ag administration. These data suggest that although the temporal consequences of Ag presentation can be subtly altered by TLR ligands, the qualitative decision between T cell tolerance and immunity is not necessarily governed by the corecognition of PAMPs alone.

Materials and Methods

Mice and cells

All the mice used were obtained from the NIAID contract facility at Tac- tonic Farms, an American Association for the Accreditation of Laboratory Animal Care accredited facility. The B10.A, 5C.C7 (B10.A,5C.C7Tg, Rag2−/−), B10.A, pigeon cytochrome C (membrane PCC), B10.A, membrane PCC, CD3e−/−, and B10.A, CD3e−/− mice have been described in previous publications (18, 19). B6.CD28−/− (N10) mice were crossed twice to B10.A,5C.C7Tg, Rag2−/− mice, intracrossed and homozygous CD28-deficient, B10.A,5C.C7Tg, Rag2−/− mice selected for further breeding by a test cross. B10.D2, Rag2−/− mice were developed by crossing the ho- mozygous Rag deficiency onto the B10.D2 background 11 times, from a B6/129, Rag2−/− mouse. The DO11.10 TCR transgenic (a gift from Dr. K. M. Murphy, Washington University, St. Louis, MO) on a BALB/c back- ground were crossed onto the B10.D2, Rag2−/− background 8 times, in- tracrossed and homozygous transgenics selected from a test cross. For adoptive transfer experiments involving T cells from naive 5C.C7 or DO11.10 TCR transgenic mice, lymph node suspensions containing >90% CD4+ T cells (one or three million cells, as indicated in the text) were injected without further purification into recipients by the suborbital route.

TLR ligands

All TLR ligands used were dissolved in 1×PBS (Quality Biologicals) and injected i.p. unless otherwise stated. LPS from E. coli serotype O127:B8 (Sigma-Aldrich catalog no. L-3129) and poly(I:C) (Sigma-Aldrich catalog no. P-4929 or Fluka Biochemicals catalog no. 81349) were purchased com- mercially. CpG oligonucleotides were synthesized commercially (Invitro- gen) with the following sequence—TCCATGACGTTCCTGACGTT.

Cell isolation and analysis

Cells suspensions were prepared from transfer recipients by dispersing spleen and lymph nodes (cervical, axillary, brachial, inguinal, mesenteric, pancreatic, and peri-aortic) in sterile 1×PBS containing 5% FBS. FACS analysis was performed using a FACS Calibur (BD Biosciences) or FACS- SORT (BD Biosciences, with optical modifications by Cytek). Abs used were labeled with various fluorochromes and obtained from BD Bio- sciences, Caltag Laboratories, or eBioscience. The clones used were CD4 (RM4–5 or GK1.5), TCR-Vβ3 (KJ-25), CD11c (N418), MHC-class II (M5/114), CD80 (16-10A1), B220 (RA3–6B2), and CD45.1 (A20). For in vitro proliferation and cytokine analysis, T cells were enriched by negative selection as previously described (20) or stained with CD45.1 and CD4 before focusing on a TCS-Vantage or Aria (BD Biosciences) sorters (Na- tional Institute of Allergy and Infectious Diseases Flow Facility operated by Calvin Eigsti or Carol Henry). A total of 10,000 T cells were stimulated in vitro with titrations of MCC.88−103 peptide (ANERADILHYLKQATK) and a 25-fold excess of irradiated splenocytes from B10.A, CD3e−/− mice. Supernatants collected at 60 h after the initiation of culture were assayed for cytokines including IL-2, IL-4, and IFN-γ using the Quantikine calo- rimetric sandwich ELISAs (R&D Systems) as per manufacturer’s instruc- tions. In vivo injections were performed with the PCC.4−103 peptide (IFAGIKKKAERADILHYLKQATK) that also stimulates the 5C.C7 T cells to a similar level. Peptides for in vivo injection were tested to contain <0.01EU/ml endotoxin contamination. The OVA323−339 pep- tide was commercially purchased from AnaSpec.

Analysis of Ab and pathoblogy

Serum isolated from transfer recipients were assayed for total serum IgG levels using a calorimetric sandwich ELISA as previously described (18). In brief, plates coated with anti-κ Abs were incubated with dilutions of serum and the bound Ab detected with isotype specific, HRP-labeled sec- ondary Abs. The extent of limb deformation (arthritis pathology) was scored as previously described (18). Briefly, the presence of swelling in individual digits, curling of the digits and the extent of flexibility of the elbow or knee joints were scored individually. The maximum possible score in this system is 56 for all four limbs.

Results

TLR ligand administration minimally affects clonal expansion to a self Ag

The consequence of a naïve T cell encountering its agnostic as a peripheral self Ag has been modeled in our laboratory by i.v. in- jecting PCC-specific T cells (5C.C7 TCR+−, Rag2−/−) into PCC transgenic mice. The transferred cells typically undergo a clonal expansion for 3−4 days (open squares in Fig. 1C and Ref. 19). If the transfer recipients do not have endogenous T cells (PCC+−, CD3e−/−), the expansion results in a 100-fold increase in cell number during this time. In contrast, a T replete host (PCC+−, CD3e−/−) allows only for a 5− to 10-fold expansion of the trans- ferred 5C.C7 T cells (open circles in Fig. 1D and Ref. 18). Fur- thermore, the transferred cells survive indefinitely in the lympho- pephic PCC transgenic host, whereas they are gradually eliminated in the T replete PCC transgenic host (open symbols in Fig. 1, C vs D and Ref. 18). This model allows us to study in parallel the processes of anergy induction (adaptive tolerance) as well as the disappearance (deletion) of autoreactive helper T cells following chronic self-Ag encounter in the periphery. Because either phenotype can be interpreted as being a conse- quence of encountering Ag on “resting” APCs, we activated the innate immune system by using model adjuvants of the TLR ligand family at the same time as transferring the self-Ag-specific T cells. Administration of 3 µg of bacterial LPS was sufficient to activate CD11c+ dendritic cells in this system as measured by the up- regulation of surface markers such as CD80 (Fig. 1A) or MHC class II (Fig. 1B) on splenocytes. In this experiment, CD80 levels increased ~2-fold (MFI of 103 to 221 in the CD3e−/− mice and 108 to 263 in the CD3e−/− mice) on the CD11c high splenocytes (Fig. 1A). The levels of MHC on the MHCIi positive populations in these spleens also increased (MFI of 270 to 509 in the CD3e−/−/.
mice and 308 to 385 in the CD3e+/- mice; Fig. 2B) confirming the general immune activation by this dose of LPS. The transgenic PCC is primarily expressed by hemopoietic cells and presented on dendritic cells and B cells (20). Therefore, TLR administration does activate the cells that critically present the self-Ag in this model system.

Administration of LPS before the transfer of 5C.C7 T cells to a lymphopenic PCC transgenic host resulted in a T cell expansion pattern similar to that of PBS injected hosts, up to 4 days after transfer (closed squares in Fig. 1C). Subsequently, in the plateau phase between days 10–25 after transfer, the LPS treated recipients had slightly more cells on average (1.7-fold greater, n = 6, p = 0.01). In a T replete host, the initial expansion of 5C.C7 T cells up to 3 days in T replete PCC transgenic mice was similar in the presence (closed circles in Fig. 1D) or absence (open circles) of LPS (n = 4, p = 0.42). However, the recovery of T cells on day 4 was ~3-fold greater in hosts that were injected with LPS (n = 3, p = 0.006) relative to the PBS injected group. This could result from a greater expansion of the transferred T cells between the third and fourth day, in the LPS-treated mice. Alternatively, a reduced death of the expanded T cells in such hosts could account for the enhanced recoveries on day 4. Nevertheless, the pattern of expansion, survival, and contraction of PCC-reactive T cells transferred to PCC transgenic mice, was not greatly altered by the administration of LPS.

We also tracked the dynamics of the 5C.C7 T cells in the presence of repeated LPS injections (every other day), which would modulate chronically the context of the persistent PCC presentation (closed triangles, Fig. 1, C and D). In the T-deficient host (Fig. 1C), this treatment did not greatly alter the overall pattern of the 5C.C7 response.

Nevertheless, in the T-sufficient PCC transgenic recipients, the LPS treatments did increase the initial clonal burst of the transferred 5C.C7 T cells. Subsequently, a larger number of T cells were maintained in the mice receiving repeated LPS injections, but only for an additional 3–4 days. Five days after transfer, we could see, on average a 4.5-fold (n = 3) greater number of cells in the multiple LPS-treated group, relative to those receiving a single shot of LPS. However, despite the chronic introduction of the TLR ligand, 5C.C7 T cells continued to disappear from the secondary lymphoid organs of the T-sufficient host after this period. The loss of cells was not correlated with any increase of such cells in other organs such as the lung, liver, gut, or bone marrow (data not shown), consistent with a deletional elimination of the autoreactive cohort.

Adaptive tolerance to the self Ag is induced despite the coadministration of PAMPs

The expanded PCC-specific T cells encountering continued antigenic stimulation enter a state of in vivo anergy known as adaptive tolerance (21). T cells in this state have impairments in TCR proximal signal transduction and can be assayed in vitro by a profound loss (~90%) in the T cell’s ability to make IL-2 (22). Extrapolating in vitro models of anergy induction to this state (23), one might argue that poor costimulatory signals afforded by resting APCs in vivo, similarly allows for the induction of adaptive tolerance. Accordingly, the PAMP model for immune activation would predict that the TLR ligand-mediated activation of APCs in vivo would prevent the induction of such tolerance. We examined adaptive tolerance in PAMP-treated transfer recipients, to test this postulate. T cells recovered from PCC transgenic, lymphopenic mice 4 days after transfer down-regulated IL-2 production to a similar extent (~30- and 40-fold in the two experiments averaged in Fig. 2A) irrespective of the absence (open squares) or presence of acute (closed squares) or chronic (closed triangles) LPS administration. Relative to the same naive T cell controls (asterisk symbols in Fig. 2, A and B), T cells recovered from T replete PCC transgenic mice (Fig. 2B) also down-regulated IL-2 secretion ~8–10-fold despite the absence (open squares) or presence (closed squares or triangles) of the TLR ligand. In the lymphopenic host, we also examined whether greater doses of the adjuvant would rescue the 5C.C7 T cells from adaptive tolerance induction (Fig. 2C). Increasing the dose of LPS (bars 3–5 in Fig. 2C) did not reverse the loss of responsiveness in the T cells. In fact, the opposite phenotype was
observed, where the IL-2 production decreased modestly with increasing concentrations of administered LPS ($p = 0.04$ in a paired two-tailed $t$ test between the 0.25 μg and 25 μg treatments in Fig. 2C, $n = 3$). This modest effect, however, dissipated after longer periods of residence in the PCC transgenic host. We then asked whether the failure of a PAMP to prevent anergy induction was unique to LPS. Similar experiments using Cpg, Poly(I:C), or combinations of LPS, Cpg, and Poly(I:C) (Fig. 2D) also failed to find any abrogation of anergy in these cells. The timing of adjuvant administration also did not significantly impinge on this process because LPS injections 6 h before or after T cell infusion induced similar down-regulation of the IL-2 phenotype in the 5C.C7 T cells (Fig. 2E).

The induction of adaptive tolerance in this model system is accompanied by significant differentiation toward effector cytokine production, but only in the lymphopenic context (18). Administration of LPS (Fig. 2F (L)), Cpg (Fig. 2F (C)), Poly(I:C) (Fig. 2F (P)), or a combination of all three (Fig. 2F) yielded only a modest increase in the IFN-γ assayed upon restimulation of such cells. For example, this addition resulted in a two-fold increase ($n = 3$) in IFN-γ production by T cells recovered from hosts treated with LPS as opposed to those that were not.

More importantly, in the T replete host where the transferred T cells fail to significantly maintain effector differentiation, the impact of LPS treatment would be expected to be more dramatic. Surprisingly, neither acute nor chronic injections of LPS allowed the 5C.C7 T cells to acquire a significant ability to secrete IFN-γ in these hosts (Fig. 2G, bars 5 and 6). Similar results were obtained for IL-4 production (data not shown).

**Autoimmune pathology is not exacerbated by TLR ligand administration**

The transfer of 5C.C7 T cells to PCC transgenic, T cell-deficient (CD3ε−/−) mice results in a chronic state of B cell activation in these mice, despite the induction of anergy in the T cells (18). This is marked by serum hypergammaglobulinemia, autoantibodies, and eventually a mild form of arthritis. All of these are greatly reduced in the T-sufficient PCC transgenic animal and no scorable arthritis pathology develops in such recipients. We therefore examined whether the coadministration of LPS could precipitate such autoimmune pathology in the latter hosts. The total serum levels of immunoglobulins IgG2a and IgG2b (Fig. 3, A and B) were elevated in T cell-deficient hosts in the absence of LPS (open symbols). The addition of acute (shaded symbols) or chronic (closed symbols) LPS did not enhance this phenotype significantly. This was also the case for the clinical score of arthritis pathology as described in Materials and Methods in Singh et al. (18).

The adoptive transfer of 5C.C7 T cells to the T-replete PCC transgenic mice does not result in measurable immunopathology (18). The addition of acute or chronic LPS to this environment did not allow for the development of hypergammaglobulinemia (Fig. 3C).
FIGURE 4. Impact of LPS on the response to acute Ag administration. 
A, Recovery of Ly5.1+ 5C.C7 T cells from the nodes and spleen of syngeneic Ly5.2+ mice, after peptide (open squares) or peptide plus LPS (filled squares) administration. A representative experiment of three is shown. B, Secretion of IL-2 after restimulation in vitro with (+) or without (−) agonist peptide by naive T cells (N) is compared with T cells recovered four days after challenge with peptide alone (P) or peptide plus LPS (P+L). 
C, Secretion of IFN-γ by the same T cell populations as in B.

3, A and B, triangles) or arthritic pathology (Fig. 3C, triangles). These animals also remained viable, even after chronic LPS treatment, suggesting that other T cells present in these mice also were not being activated to trigger lethal autoimmunity.

FIGURE 5. Modulation of the strength and duration of Ag presentation by LPS. A, Adoptively transferred CFSE-labeled 5C.C7 T cells were challenged with different doses of PCC (labels on the left) in the absence (left panels) or presence (right panels) of LPS. CFSE dilution is displayed after gating on the TCR "CD4+" population recovered 60 h later. B and C, The CFSE dilution of T cells in A is quantitated in terms of T cells entering cell division (A) or the mean divisions in the responding population (B) as described in the Materials and Methods. D, Mice injected with 30 μg of peptide in the absence (left panels) or presence (right panels) of LPS were infused with CFSE-labeled T cells 48 h later (top) or at the same time. The dilution of CFSE in the stimulated T cells was assayed 60 h later. E, The mean division number of T cells responding to PCC injected at various previous days is compared in the case of PCC injections alone (open squares) or in combination with LPS (closed squares).

LPS administration enhances clonal expansion to a soluble peptide stimulus

Our observations on the impact of TLR ligands on the response to a self-Ag contrasts with previous reports using soluble peptides, where these molecules are thought to improve clonal expansion, differentiation, and cell survival. These differences could result from disparities between the model systems (because the Ag and T cell combinations used in our experiment were different from previous studies) or a fundamental difference in the way acute and chronic Ags are treated by the immune system. We first examined in this model system, the validity of the TLR ligand effect on a soluble Ag stimulus. Three million adoptively transferred 5C.C7 T cells in mice that do not express PCC were challenged with soluble PCC81–104 peptide i.p. in the presence or absence of LPS (Fig. 4). In a T replete animal, the presence of LPS (Fig. 4A, closed squares) effected a 2- to 3-fold enhancement of the clonal expansion to the peptide (Fig. 4A, open squares). Both populations of cells subsequently declined in frequency from the secondary lymphoid organs. Although low numbers of peptide plus LPS expanded cells could still be detected after four weeks in this model, the T cells that responded to soluble peptide alone, were below the limit of our detection within two weeks. T cells recovered from the transfer recipients were restimulated in vitro with 10 μM concentrations of the MCC88–103 peptide (Fig. 4B). Similar to previous reports, peptide stimulation in the absence of adjuvant resulted in a significant (~4-fold, n = 3, p = 0.006) decrease in the T cell's ability to make IL-2. Priming in the presence of LPS had the opposite effect, enhancing IL-2 production by ~3-fold. Both stimulated populations of T cells gained the ability to secrete an effector cytokine (IFN-γ; Fig. 4C). However, this differentiation was more robust (~10-fold...
greater) if LPS was available in the immunogen. Thus, the magnitudes of the differences observed with an acute peptide stimulus were more significantly modulated by a TLR ligand, than those observed under chronic stimulation conditions.

**LPS affects the strength and duration of soluble Ag presentation**

To understand this differential effect, we next examined the cellular mechanisms by which LPS might modulate the T cell response to acute Ags. Many consequences of LPS administration, such as the induction of proinflammatory cytokines, up-regulation of costimulation, etc. should be comparably available to T cells responding to both chronic and acute Ags. In addition to these parameters, however, the proliferation and differentiation of T cells in vivo has also been proposed to be driven by the strength and duration of the initial antigenic stimulation (24). TLR ligand-mediated enhancement of class II levels, Ag presentation, etc. (strength of stimulus) should affect both the display of chronic and acute Ags. However, effects of these adjuvants that mostly act at the level of increasing the duration of Ag presentation in vivo, are likely to have less of an impact on an Ag that is already being presented chronically.

We attempted to separately examine the consequences of LPS on the strength and duration of acute PCC peptide presentation using the same adoptive transfer approach. To quantitate the effective increase in the strength of initial antigenic signal that is caused by the coadministration of LPS, T cells that were adoptively transferred into CD3ε−/− hosts were challenged with titrations of the PCC ε1−104 peptide, with or without LPS (Fig. 5A). The choice of the CD3ε−/− host minimizes confounding contributions to the assay stemming from regulatory T cells, bystander responses, etc. that might otherwise affect the readout (proliferation of transferred cells). The CFSE-labeled 5C.C7 T cells initiate CFSE dilution between 0.1 and 0.3 μg of injected peptide (Fig. 5A). The subsequent response (at the 60 h time point) can be represented by the entry into cell cycle (Fig. 5B) or the number of subsequent divisions (Fig. 5C). By both measures, the presence of LPS greatly enhanced the response to the lowest dose that triggered proliferation (0.1–0.3 μg). However, there was no measurable advantage to LPS administration at any higher dose of Ag. These data suggest that a direct impact of LPS on the strength of the initial acute antigenic signal is relevant only to the lowest levels of Ag perceived by T cells. Quantitatively, this yields at best a 3-fold shift in the dose-response curve to Ag (2.8-fold in Fig. 5B and 3.3-fold in another determination). Furthermore, using doses of Ag that are saturating in this assay (>10 μg) allowed us to examine additional effects of the TLR ligand on Ag presentation.

Next, we assayed the potential for LPS to prolong the availability of Ag presentation in vivo, by first injecting a fixed dose of peptide (30 μg, in Fig. 5D) with or without LPS mixed in. T cells were transferred to CD3ε−/− mice that were so treated 48 h previously or a few minutes earlier (0 h). Once again, the use of a T cell-deficient host allowed us to monitor this process in the absence of any bystander response that could impact additionally on the clearance of Ag and/or the measurement of the response. T cells transferred to mice that were treated with the PCC ε1−104 peptide 48 h previously were triggered to proliferate weakly (Fig. 5D, top left panel) compared with T cells that received a similar dose of peptide immediately after transfer (Fig. 5D, bottom left panel). However, combining the peptide with LPS at the time of injection allowed recipient mice to retain much larger stimulation even after 48 h (Fig. 5D, top right panel). The proliferation from this retained peptide was only minimally reduced compared with that elicited by a peptide plus LPS administration soon after T cell transfer (Fig. 5D, bottom right panel). The decrease in the potency of the peptide during the 48-h period, in the absence of LPS can be estimated from dose-response curves such as those in Fig. 5A to be up to 30-fold (data not shown). The effect of LPS on stabilizing the duration of peptide presentation in vivo was further quantified by analyzing the mean division number of T cells (Fig. 5E). In the absence of LPS, PCC peptide elicits a division number of around 6 (6 ± 0.4; n = 4). If the T cells were transferred one day after the peptide administration, this number fell to around 3 (3 ± 0.2; n = 4), similar to that elicited from a 3-μg dose of peptide in Fig. 5B. This 10-fold decay is not evident if LPS was present in the first immunization with peptide. Even a day after transfer only a small drop in stimulation was observed (6.6 ± 0.3 to 6.1 ± 0.4; n = 4). Two days afterward the PCC plus LPS immunized mice also begin to lose their Ag depot, although the stimulation available in these mice is still much higher than that in the PCC alone treatment group (4.9 ± 0.4, n = 4). These data suggest that the availability of LPS prolongs the duration of Ag presentation in vivo.

We also validated this effect with a second TCR transgenic model involving an OVA-derived peptide that is recognized by DO11.10 TCR transgenic T cells. If the peptide was injected by itself 48 h before T cell transfer, only 2–5% (n = 4) more of the transferred T cells proliferated (Fig. 6A, middle panel) compared with mice that were only injected previously with PBS (Fig. 6A, top panel). However, if the OVA peptide was coinjected with LPS 48 h before T cell introduction, 88–96% (n = 4) of the transferred T cells responded by diluting CFSE (Fig. 6A, bottom panel). The effect could also be observed with TLR agonists other than LPS. The injection of PCC peptide together with Poly(I:C) (Fig. 6B, middle panel) or CpG (Fig. 6B, bottom panel) allowed for the injected PCC peptide to be presented more robustly to T cells transferred 48 h later. The adjuvanticity of LPS in some models has been shown to be dominated by its effect on up-regulating B7 family members on dendritic cells that engage CD28 on T cells (13). We therefore examined whether CD28−/− 5C.C7 T cells would be unable to sense the extended Ag presentation afforded by LPS (Fig. 6C). The deficiency of CD28 makes the 5C.C7 T cells about 10-fold less sensitive to Ag, making a direct comparison
with the CD28 sufficient cells difficult (our unpublished observations). Nevertheless, despite the deficiency of CD28, 5C.C7 T cells responded more robustly if the PCC peptide (100 μg) was introduced 48 h earlier with LPS (Fig. 6C, bottom panel) than without it (Fig. 6C, middle panel). In the experiment shown, the PCC alone injected mice only allowed for 8% of the transferred CD28−/− T cells to dilute CFSE, whereas 22% of them divided if LPS was also mixed with PCC during the initial injection. Similar results were obtained in one other experiment.

Can repeated injections of peptide mimic the effect of TLR coadministration?

The minimal effect of TLR ligands on immediate Ag presentation, but the dramatic enhancement of the duration of Ag availability in vivo, suggested an interesting new activity for these adjuvants that may be independent of their proinflammatory activity. If this were the case, repeated administration of the soluble peptide should, in the absence of LPS, mimic the activity of peptide plus LPS on T cell activation, by compensating for the decay of the peptide. We tested this possibility by adaptively transferring 5C.C7 T cells into B10.A mice and then challenging them twice daily with i.p. injections of PCC81−104 peptide. The number of T cells recovered from mice that received only a day of PCC challenge (Fig. 7, A (L)) were recovered at ~3.6-fold lower numbers compared with mice that were challenged with a single inoculation of peptide mixed with LPS (Fig. 7, A (L)). However, continued administration of peptide, in the absence of LPS, resulted in a concomitant increase in the number of T cells recovered up to 3 days and then plateaued (Fig. 7A). In addition, T cells recovered from mice that had been challenged for 2–3 days with repeated peptide administration differentiated to make amounts of IFN-γ that were comparable to or greater than those made by T cells primed with PCC plus LPS (Fig. 7B). These data suggest that even in the absence of inflammatory mediators available in the presence of a TLR ligand, continued presence of the Ag was sufficient to drive greater expansion and differentiation of the T cells.

The effect of repeated peptide injections on anergy induction (as measured by their ability to secrete IL-2) was, however, the opposite. Although a single day of PCC injection decreased the IL-2 production by only 3-fold (ratio of means in Fig. 7C, n = 3), continued injection for 4 days brought about a further decrease – resulting in an average difference of 27-fold relative to naive T cells. Therefore, unlike the enhanced expansion and effector cytokine production induced by repeated peptide immunizations in the absence of LPS, IL-2 production was instead further down-regulated. This is, however, consistent with a progressive differentiation of the cells to a deeper state of anergy. Thus, these data support a model in which three of the cellular processes measured here – expansion, differentiation, and anergy induction, were all driven to a greater extent by the continued presence of Ag.

Discussion

In this study, we investigated the ability of TLR ligands to modulate the response of a helper T cell to its agonist, presented as a chronic peripheral self Ag. In a variety of assays that measured the responsiveness of the T cells themselves (proliferation and cytokine production) or systemic phenotypes (Ab production and the manifestation of arthritic pathology), we failed to find much evidence for a significant impact of such adjuvants in this model system. Nevertheless, consistent with previous reports, the responses of the same TCR transgenic T cell to a soluble peptide immunization were enhanced by the coadministration of TLR ligands. We propose that the differential activity of LPS in these two contexts may be explained, at least in part, by the propensity of TLR ligands to extend the duration of Ag display in vivo, a characteristic that is unlikely to significantly affect an Ag that is already available persistently in the host. These findings, discussed below, are very relevant to furthering our understanding of the regulation of helper T cell responses to systemic self Ags and potentially also to persistent microbial infections, commensal organisms, etc.

TLR ligand-mediated APC activation is insufficient to prevent adaptive tolerance

In many in vivo model systems, T cells stimulated with soluble peptides or self Ags enter into a state of nondeleterious tolerance that shares some features with clonal anergy but is, in fact, a distinct biochemical phenomenon termed adaptive tolerance (21, 22, 25). Because clonal anergy was originally induced in cell culture models by using APCs that could not provide accessory signals (5), it has often been assumed that Ag engagement (signal 1) in the relative deficiency of costimulatory signals (signals 2 and 3) is also responsible for the induction of adaptive tolerance in vivo. In this framework, self Ags can be thought to be tolerogenic, as they are presented in contexts lacking the Ag-nonspecific, microbe-derived ligands needed to activate APCs. However, such a pathogenic “switch” that turns on the APC only in the presence of a microbial infection, also presents conceptual challenges. For example, the relative abundance of self Ags in the immediate context of an infected tissue makes it likely that an activated dendritic cell will then be able to turn on self-reactive T cells and potentially trigger autoimmunity. Several solutions have been proposed to circumvent this problem, including the ability of dendritic cells to discriminate different sources of Ag based on close association with microbial products (26). Nevertheless, the potential for self Ags to access the same compartments cannot always be excluded, especially if the Ag is also highly expressed in that local tissue. The same hurdle also applies to models where the utility of TLR ligands in self/nonself discrimination is dependent on their ability to overcome the activity of regulatory T cells (16). An alternate solution to this problem is the availability of tolerance mechanisms in T cells that are relatively insensitive to the state of activation of the APC. It is likely that adaptive tolerance represents one such process that exploits the ability of T cells to quantitatively adjust to the levels of Ag in the environment, in a manner originally proposed by Grossman and Paul (20, 27). Because these mechanisms rely on making T cells “functionally ignorant” of the amounts of self Ag, they can be robustly independent of inflammatory cues. This is in fact the principal conclusion of this study. It is particularly striking in the CD3ε−/− host, where adaptive tolerance (and not clonal deletion or suppression) is the exclusive
tolerance process in operation (18). In this model, neither the expansion to self Ag (Fig. 1C) nor the down-regulation of IL-2 production (Fig. 2) was affected by TLR ligands. The latter was also true for the induction of adaptive tolerance in the T cell sufficient host (Fig. 2B). In a broader sense, systemic tolerance, as measured by the presence of serum autoantibodies or arthritis pathology, was also not measurably affected by TLR ligands (Fig. 3). It is important to emphasize that the cells that present PCC in this model system are indeed targeted by the TLR ligands we administer (28).

In fact, the presentation of Ag by nonhemopoietic cells (that may not have been receptive to TLR stimulation) is not critical for the induction of tolerance in this model (Singh et al., manuscript in preparation). The failure of LPS, PolyIC, CpG, or Pam-3-Cys-mediated activation of APC to prevent this form of tolerance induction, however, does not necessarily rule out the possibility that other inducers of APC activation may indeed be successful in this regard (17, 29–33). But it is interesting that similar results have been obtained in some CD8⁺ T cell tolerance models, where the presenting cells were activated with viral infections, anti-CD40 treatment, or TLR ligands (34, 35).

Finally, it is tempting to argue that these conclusions will be widely relevant to many similar adoptive transfer models, and perhaps even to autoreactive T cells within an endogenous polyclonal repertoire. However, there is not a complete consensus in the literature about the fate of helper T cells recognizing a self Ag in experimental constructs similar to ours (36–43). In fact, in many instances, such cells can trigger chronic graft-vs-host disease or a variety of tissue specific pathologies (depending on the expression of the target Ag). This is further exacerbated in lymphopenic models, where “systemic tolerance” is often hard to demonstrate. We have, however, previously reported that in this model, the manifestation of mild autoimmune pathology is in fact possible even after stable induction of adaptive tolerance in the T cells. In a lymphopenic situation, this is probably due to the survival of a large number of autoreactive T cells that have undergone significant effector differentiation, despite the induction of anergy (18, 44). Such a phenomenon had been called “split anergy” in CD8⁺ T cell effector differentiation, despite the induction of anergy (18), but in fact may simply reflect the relative differences in the thresholds for eliciting different responses in T cells, i.e., the TCR proximal alterations that increase the activation threshold in adaptively tolerant T cells may not be sufficient to prevent low levels of effector responses. In such cases, unless secondary mechanisms (such as suppression or deletion) are recruited, systemic immunopathology may eventually result (36, 37). Of course, the quantitative and qualitative nature of the observed pathology will also depend on the amounts and distribution of the target Ag. A consensus then, at least with regards to the induction of cell intrinsic adaptation, could be that persistent self Ag presentation dominantly induces such a cellular tolerance. This study suggests that this can also operate in the face of strong TLR ligand-mediated APC activation.

This framework will have to be validated in other models by separately measuring parameters of adaptation and effector function. In graft-vs-host disease models where polyclonal T cells are used, such an analysis is complicated by the involvement of multiple Ags, T cell clones and tissues that may even vary during the progression of the response. Of course, a better understanding of the process of T cell adaptation and its relation to Ag persistence will allow us to eventually extend this framework to complex cell populations with heterogeneous antigenic targets.

The inflammatory adjuvanticity of LPS

The ability of LPS and other TLR agonists to up-regulate the expression of costimulatory molecules and proinflammatory cytokines by various cells is well documented. The consequence of these second and third signals on a T cell response can be segregated into three components: one that relates to the prevention of anergy (as measured by IL-2 production or proliferation), the second that relates to the enhancement of T cell differentiation (marked by secretion of effector cytokines, migratory properties etc.), and the third that allows for a greater survival of the activated cells subsequent to the expansion phase. The most prominent molecular cascade implicated in these processes has been the CD28 pathway, which is triggered by the B7 family ligands on activated dendritic cells. It influences cytokine production as well as the balance of pro- and anti-apoptotic proteins in activated T cells (46–48). In addition to CD28, several other T cell surface molecules sense the qualitative changes on APCs that have been activated appropriately. These include molecules such as OX40, ICOS, PD1, etc. which play important roles in the survival, differentiation, and responsiveness of the T cells (49, 50). In addition, the induction of IFNs in TLR ligand-activated cells as well as the triggering of NFKβ and bcl3 dependent pathways in T cells can enhance their survival (12, 51, 52).

The differentiation program in the activated T cells is also modulated by cytokines such as IL-12, IL-23, IL-1, IL-6, etc. up-regulated by microbial products (53). Not surprisingly, therefore, the “classical” view of TLR ligand-mediated adjuvanticity on a T cell response has focused in large part on these diverse and biologically potent products elicited by such compounds.

Despite this, the only classical activity that is evident in our experiments with persistently stimulated 5C.C7 T cells is an initial enhancement of T cell expansion; and that too only in an animal with an intact repertoire of T cells (CD3e⁻ in Fig. 1D). The effect is more striking in the groups treated with LPS chronically, but is also evident with a single infusion of the adjuvant. The failure to see such differences in the CD3e⁻/⁻ host (Fig. 1C) could be attributed to a saturation phenomenon, in which the robust expansion of T cells runs out of “space”, even if LPS is administered. Nevertheless, we have observed a 3- to 5-fold greater expansion of T cells even in such mice if a high dose of Ag was administered in the beginning (our unpublished observations) or if the T cells were relieved of CTLA4 (54). It is therefore possible that this effect may also reflect the subtle changes in Ag density resulting from LPS-induced up-regulation of MHC molecules or Ag presentation, rather than one of the “classical” inflammatory signals. Consistent with this idea, repeated injections of Ag in the absence of TLR ligands, lead to a greater expansion of T cells (Fig. 7A).

The minimal impact of the inflammatory milieu on enhancing the differentiation of chronically stimulated T cells toward effector cytokine production is also surprising. It has been shown that different TLR ligands may skew T cell differentiation toward distinct effector classes (55). In our model, neither the isotypes of serum Abs (Fig. 3) nor the preliminary analyses of IL-4 and IL-17 secretion (data not shown) support such a deviation. However, the important role that T cells themselves play in eliciting cytokines such as IL-12p75 and IL-4 in the early phases of an immune response has been discussed in the literature previously (56–58). Our data support the idea that upon availability of an abundant high affinity TCR agonist, accessory signals can take a backseat in driving differentiation as well. Intriguingly, the trans-inhibitory effect of endogenous T cells on this differentiation process (in the T sufficient host) is also not overcome significantly, by providing TLR stimulation.

**TLR ligands modulate the duration of Ag presentation**

The novel perspective that this study offers, to explain the inability of TLR ligand stimulation to significantly modulate T cell responses to a chronic Ag is that a prominent function of such adjuvants, when coadministered with acute Ags, is to make such Ags more chronic. In addition to qualitative changes in context, the
strength and duration of the antigenic signal itself play important roles in determining T cell fate. CD4+ T cells that are stimulated only for a brief period of time, even with a strong stimulus, fail to subsequently differentiate as well as cells that continually contact Ag for a 2–3 day period (59). This may stem from the necessity for the progressive accumulation of TCR signals to trigger a complete differentiation program in the naïve T cell (60). Alternatively, the delay in the expression of receptors on T cells that allow them to respond to differentiation factors made by activated APCs may leave at a disadvantage those cells that loose contact with Ag after only a short duration. Therefore, it is not surprising that a 2–3 day extension of Ag presentation mediated by TLR ligands has a significant effect on the fate of the responding T cells.

The ability of LPS-activated DC to present Ag better to T cells has also been reported previously (61). It can therefore be argued that the assay we use to measure Ag retention is merely reading out a better response of the T cells to the same amounts of residual Ag, when the DC were previously activated by LPS. However, in our model, the administration of LPS serves to increase the effective Ag presentation only by ~3-fold and only at the lower doses of Ag (Fig. 5, A–C). The decay of Ag is, in fact, more pronounced. In titration experiments, 300 μg of injected peptide decays to the equivalent of 3 μg or less within one day (data not shown). Therefore, we favor a model where the Ag itself is maintained in the system longer, after TLR engagement.

This “depot” effect can be mediated either by extending the lifespan of the APCs that captured the Ag or by prolonging the maintenance of pMHC complexes on the cells. Intriguingly, several studies suggest that lifespan of dendritic cells is in fact reduced after activation by microbial stimuli, especially in the absence of T cell interactions (62, 63). However, because dendritic cell subsets undergo significant rearrangement in the lymphoid organs after activation by inflammatory stimuli (64), it is still possible that the lifespan of minor but critical subsets are in fact prolonged. The increase in the retention of peptide MHC complexes on DCs, however, has been documented quite extensively (65). The activation process is thought to stabilize such complexes either by improving their transport to the surface or reducing their degradation within the cell or both (66, 67). These properties may also synergize with the enhanced Ag capture and retention, mediated by actin rearrangement in DCs, induced by TLR ligands (68) to create the depot effect. These would also be at play in the course of a chronically available Ag, but less likely to significantly alter the T cell response to it (because new pMHC complexes will be generated on the APCs in these mice from the constitutively expressed Ag).

Finally, we also examined the components of LPS adjuvanticity that may result from the induction of Ag depots, by attempting to compensate for Ag decay with repeated injections of peptide. This strategy is admittedly only a surrogate, because neither the actual slope of the decay (as modulated by LPS) nor the cell type-specific distribution of Ag during the decay process, are likely to be perfectly replicated by this approach. Nevertheless, we could still substitute this “inflammation free” context to obtain a significant enhancement of expansion and differentiation of SC.C7 T cells (Fig. 7, A and B). The 2 days of peptide administration, which would most likely resemble the extension of Ag presentation afforded by LPS, result in expansion and differentiation to levels similar to that elicited by peptide plus LPS. Continued administration of peptide beyond this window drives these processes even farther. Expanding on the ideas of a progressive differentiation of T cells after Ag encounter (69) it can, therefore, be argued that a brief window of T cell stimulation (as with a single injection of peptide) leads only to weak/abortive activation, whereas a longer window of stimulation (due to the depot effect of LPS or multiple injections) is necessary for optimal T cell activation. The measurements of IL-2 production, however, present a minor contradiction. Consistent with the “windows of stimulation” idea, the peptide plus LPS treatment does result in an increased ability of the T cells to make IL-2 (relative to the peptide alone; Fig. 4B). In this line of reasoning, continued Ag stimulation beyond the “optimal window” would tolerate T cell function. Indeed, in the transgenic PCC model, IL-2 production is in fact transiently increased in the T cells, before being strongly down-regulated over a 4 day period (Fig. 4 in Ref. 20). Similarly, repeated injections of soluble peptide also lead to a further down-regulation of IL-2 (Fig. 7C). The contradiction stems from the failure of this treatment to initially increase IL-2 production (treatment up to day 2 in Fig. 7B) as would be expected if that duration of stimulation was sufficient to mimic the “optimal window” effect of LPS. The simplest explanation is the possibility that repeated injections do not precisely mirror the decay kinetics as modulated by LPS. Alternately, some of the classical activities of LPS that are not recapitulated by repeated peptide injections may in fact be critical for the up-regulation of IL-2 production.

The depot effect that we highlight here could also play an important role in other aspects of TLR ligand-modulated T cell differentiation that we have not examined. For example, the creation of local depots of Ag by the activity of TLR ligands could explain the enhanced homing and/or retention of activated effector/memory T cells in certain tissue locations (70). These could also underlie the longer maintenance of “memory” in animals primed with Ag in the presence of TLR ligands. The adjuvants that Janeway originally described as the “immunologists dirty little secret” (11) were long debated to improve immunizations either by acting as Ag depots (paraffin oil) or irritants (mycobacteria, alum etc). It is, therefore, quite intriguing that quantitative analysis of highly dendritic adjuvants such as LPS, CpG, or poly(I:C) reveals the same dichotomy in their mode of action. More importantly, however, this study suggests that such adjuvants in the context of acute immunizations, such as in a vaccination, are unlikely to trigger significant breakdown of steady state peripheral tolerance.

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