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A Role for Toll-Like Receptor 4 in Dendritic Cell Activation and Cytolytic CD8⁺ T Cell Differentiation in Response to a Recombinant Heat Shock Fusion Protein

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Recombinant heat shock fusion proteins (Hsfp) injected into mice without added adjuvants can stimulate production of CD8 cytolytic T cells. Because initiation of productive immune responses generally requires dendritic cell (DC) activation, the question arises as to whether the Hsfp can activate DC independently of contaminating LPS. Using microarray analyses of DC from LPS-insensitive mice having a point mutation in Toll-like receptor 4 (Tlr4) (C3H/HeJ), or lacking Tlr4 (B10.Sc/Ncr), we show here that unlike a LPS standard, Hsfp activated DC from HeJ mice almost as well as DC from wild-type mice. Consistent with the microarray analysis, the Hsfp’s ability to activate DC was not eliminated by polymyxin B but was destroyed by proteinase K. The Hsfp did not, however, stimulate DC from mice lacking Tlr4. In vivo the CD8 T cell response to the Hsfp in mice lacking Tlr4 was impaired: the responding CD8 cells initially proliferated vigorously but their development into cytolytic effector cells was diminished. Overall, the results indicate that this Hsfp can activate DC independently of LPS but still requires Tlr4 for an optimal CD8 T cell response. The Journal of Immunology, 2004, 172: 2885–2893.
SDS-PAGE, circular dichroism, and two-dimensional gel electrophoresis (data not shown). Their LPS levels, however, varied from 2.3 to 48 endotoxin units (EU)/mg protein. We are grateful to A. Keating (MIT, Cambridge, MA) for the circular dichroism measurements, to G. Taccioli (Boston University, Boston, MA) for the two-dimensional gel electrophoresis, and to E. Wiebe for determining the endotoxin content of the proteins by the Limulus amebocyte lysate assay, Cape Cod Associates, Falmouth, MA. The LPS (endotoxin) used as a standard in this study was obtained from Sigma-Aldrich (E. coli 055:B5, lot 127H8614).

Mice
C57BL/6 (H-2^b ), C3H/HeJ (H-2^d ), and C57BL/10 (H-2^d ) mice were obtained from The Jackson Laboratory (Bar Harbor, ME), C57BL/10Sc/Ncr (H-2^b ) were from the National Cancer Institute (National Institutes of Health, Bethesda, MD), and C3H/HeJ (H-2^d ) were from Taconic Farms (Germantown, NY). Tlr4^-/- mice were generated by Dr. S. Akira (Osaka University, Osaka, Japan) and were a kind gift from Dr. D. Golenbock (13) (University of Massachusetts, Worcester, MA). 2C TCR-transgenic mice (H-2^b ) were from the National Institutes of Health, Bethesda, MD), and C3H/HeJ (H-2^k ) were from Taconic Farms (Bridgeport, MA) for the circular dichroism measurements, to G. Taccioli (Boston, MA). The LPS (endotoxin) used as a standard in this study was obtained from Sigma-Aldrich (E. coli 055:B5, Sigma-Aldrich) for 18 h in a 96-well plate. The cells were then stained with the anti-MHC class I Ab, Y3 (American Type Culture Collection, Manassas, VA) using increased expression of K^b as an indicator of DC activation.

Immunization protocols
The ability of 65-P1 to stimulate anti-SIY-K^b CD8 T cells in mice was determined in two ways.

Polyclonal response. Mice were injected twice, 1 wk apart, with 50 µg 65-P1 in PBS. The injections were made s.c. in the scruff of the neck and base of the tail. To determine whether the injected protein primed anti-SIY-K^b CD8 T cells, 1 wk following the second injection, cells from draining lymph node and spleen were pooled (from individual mice) and restimulated by culturing them in the presence of the SIY peptide (1 µM) without adding IL-2 or other growth factors. Six days later, they were analyzed for cytolytic activity and frequency of SIY-K^b tetramer-binding cells.

To ensure that the priming effects of the Hspf injections were essential for the anti-SIY T cell response to restimulation in culture, some mice were injected with a mixture of Hsp65 plus P1 (in amounts equimolar with the injected Hspf) and their spleen and lymph node cells were isolated and cultured with 1 µM SIY as above. For this control, we could have used mice that were un.injected or injected with PBS, but chose the Hsp65 plus P1 mixture as a more stringent control.

Adoptive transfer. Naive 2C T cells were isolated from spleen and lymph nodes of 2C/RAG mice. The cells were depleted of macrophages and DC by magnetic sorting according to the manufacturer’s instructions (Miltenyi Biotec, Auburn, CA) using anti-CD11c- and anti-CD11b-coated microbeads. Purity was in the range of 80–85% (1B2^+ CD11c^+ cells) as judged by FACS analysis using a clonotypic Ab (1B2) specific for the 2C αβ TCR. Briefly, 5 x 10^6 2C cells were injected retro-orbitally and 3 days later mice were injected s.c. with 65-P1 as described above. After 3 days, the frequency of 2C cells (percentage of total PI-negative cells that were 1B2^+) and their CD44 expression were determined by flow cytometry. These cells were also tested for cytolytic activity (see below). For some experiments, the adoptively transferred 2C cells were labeled with CFSE to determine whether they underwent proliferation in vivo.

Cytolytic assays
For these assays, target cells were ^51Cr-labeled T2-K^b cells. E:T ratios were 100:1, 33:1, and 11:1, and the SIY octapeptide was added at 1 µM. T2-K^b cells were a generous gift from P. Cresswell (Yale University, New Haven, CT) and after 4 h, supernatants were counted in a gamma spectrometer and percent specific lysis was calculated as ((experimental counts – spontaneous counts)/total counts – spontaneous counts) x 100. Control wells, from which the SIY peptide was omitted, had background specific lysis of <10%; the background values were subtracted from the results shown.

BMDC
The protocol used to prepare these cells was a modification of that described by Inaba et al. (17). Bone marrow was flushed from the femur and tibia, RBC were lysed, and cells were plated at 1 x 10^5/ml (1 ml/well) in 24-well plates in “complete” medium (RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 10 mM HEPES, 50 µM 2-ME, 100 U/ml penicillin, and 100 µg/ml streptomycin). The medium was supplemented with 20 ng/ml murine GM-CSF (R&D Systems, Minneapolis, MN). Cells were fed with fresh medium and GM-CSF on days 2 and 4. On day 6, the DC (immature) were harvested and purified over a SuperMACS column, according to manufacturer’s instructions, using anti-CD11c microbeads (Miltenyi Biotec) and used in assays the same day. Their purity, assessed with anti-CD11c and anti-CD11b Abs, was typically >95%.

For the LPS activation assay, 4 x 10^5 purified, day 6, BMDC were incubated with various concentrations of a LPS standard (E. coli 055:B5; Sigma-Aldrich) for 18 h in a 96-well plate. The cells were then stained with the anti-MHC class I Ab, Y3 (American Type Culture Collection, Manassas, VA) using increased expression of K^b as an indicator of DC activation.

Ag processing assay
A total of 4 x 10^5 purified, day 6, BMDC were incubated with various concentrations of 65-P1 for 2 h in a 96-well plate. The cells were then fixed with 0.5% paraformaldehyde solution for 15 min. Following fixation, the cells were washed extensively to remove paraformaldehyde and 1 x 10^6 naive 2C cells obtained from lymph nodes and spleen of 2C (RAG^-/-) mice were injected s.c. with 65-P1 as described above. After 3 days, the frequency of T2-K^b tetramer-binding cells that bind SIY-K^b tetramers. FACS analyses, gating on CD8^+ cells, were analyzed for cytolytic activity and frequency of SIY-K^b-tetramer-binding cells.

Each symbol represents an individual mouse (total sample size = 9–12/group). Horizontal bars represent mean values. Values of p (unpaired two-tailed t test) comparing each group with the LPS-low group are shown above each column. C. Titration of DC with the LPS standard; y-axis is K^b level normalized for the maximum K^b level.

FIGURE 1. LPS-low 65-P1 supplemented with a LPS standard becomes as effective as LPS-high 65-P1 in stimulating CD8 T cell production in B6 mice. Mice were injected twice, 1 wk apart, with 50 µg 65-P1 preparations that differed in LPS levels: LPS-low (2.3 EU/µg), LPS-high (48 EU/µg protein), and the LPS-low preparation coinjected with 2.4 EU of the LPS standard. Control mice were injected with a mixture of 48 µg Hsp65 (0.3 EU/µg protein) plus 2 µg P1 polypeptide. Seven days after the second injection, spleen and draining lymph node cells were restimulated in culture for 6 days with 1 µM SIY peptide, without adding IL-2, and then analyzed as in A and B. A, Cytolytic activity. The restimulated (“effector”) cells and ^51Cr-labeled T2-K^b target cells were at an E:T ratio of 100:1; 4-h assay in the presence of 1 µM SIY. B, Frequency (percent) of cells that bind SIY-K^b tetramers. FACS analyses, gating on CD8^+ cells, were analyzed for cytolytic activity and frequency of SIY-K^b-tetramer-binding cells.
mice were added to the culture. Eighteen hours later, activation of 2C cells was assayed by staining them with 1B2, anti-CD8/H9251, and anti-CD69 Abs. Oligonucleotide microarray analyses DC, prepared as described above, were plated out at 1/H1100310^7 cells in 8 ml in a 100-mm culture dish in RPMI 1640 complete medium (see above). After incubation at 37°C for 60 min to allow the cells to adhere to the dish, the 65-P1 protein was added to a final concentration of 100 g/ml and incubation was continued for various times. The 65-P1 preparation contained 7 EU/mg protein. By adding 5.6 EU of the LPS standard to some dishes, the DC were incubated with equivalent levels of endotoxin (0.7 EU/ml), introduced either in the Hsfp preparation or as the LPS standard. To another set of DC, unmodified Hsp65 was added at 100 g/ml. This protein had only 0.3 EU/mg protein and no additional LPS was added. To a control reference set of DC, only medium was added (“medium-only control”). After various incubation times, cells were harvested, lysed with TRIzol (Molecular Research Center, Cincinnati, OH), and total RNA was isolated, labeled, and prepared for hybridization to murine U74 oligonucleotide arrays (Affymetrix, Santa Clara, CA) using standard methods (18). The results were analyzed as described previously (19). Briefly, the fluorescence value for each gene at each time point (R_i) was given a score, S_i = (R_i - \mu_i)/\sigma_i, where \mu_i is the mean of all time points for the medium-only control and \sigma_i is the SD of the control time course. This scoring system corrected for genes having high noise in the control sample. A gene was considered to be up-regulated if the score was >1.8 for two or more consecutive time points. If the score was <1.2 for all time points, the gene was considered to be no more affected by the stimulus than by medium-only control.

The microarray results for individual genes up-regulated under any condition at any time point are recorded in Supplement Table I.

**FIGURE 2.** Gene expression profile induced in BMDC. Oligonucleotide microarray analyses of RNA after 0, 2, 6, 10, and 22 h of incubation of DC with the LPS standard or Hsp65 or 65-P1. The proteins were added to a final concentration of 1.6 nmol/ml. LPS (as the standard or as contaminant in the Hsfp) was present at 0.7 EU/ml, except for Hsp65 where it was present at 0.3 EU/ml. Each gene is represented by a single row and each time point by a single column. Genes are grouped according to their functional annotation. Color bars represent the ratio of hybridization measurements between the test samples and medium-only controls according to the scale shown (lower right). A, Comparison of DC from HeJ mice with the corresponding wild-type mice (HeN). B, Comparison of DC from ScNCr mice with the corresponding wild-type mice (B10). Ratios for individual genes up-regulated at any time point are shown in Supplement Table I.

**Abs and flow cytometry**

Flow cytometry was conducted using a FACSCalibur and CellQuest software (BD Biosciences, Mountain View, CA). The fluorophore-conjugated Abs to CD11c, CD11b, CD8a (clone 53-6.7), CD38, ICAM-1, CD69, and CD44 were purchased from BD PharMingen (San Diego, CA). The anti-CD8a Ab (clone CT-CD8a) was purchased from Caltag Laboratories (Burlingame, CA). 1B2, a clonotypic Ab specific for the 2C TCR (20), was isolated from supernatants of cultured 1B2 cells and purified by protein G affinity chromatography.

SIY-K^b oligomers (tetramers) were a kind gift from Drs. M. Maurice and H. Ploegh (Harvard Medical School, Cambridge, MA).

**Proteinase K digestion and PMB treatment**

Proteinase K. 65-P1 or LPS in PBS was incubated with 2 g/ml PK (Roche, Basel, Switzerland) at 37°C for 18 h and then dialyzed against PBS at 4°C for 18 h using a “slide-a-lyzer” with a 10,000 MWCO (Pierce, Rockford, IL). The samples were then added to the DC for another 18 h, at which point DC activation was measured by determining expression levels on the DC of CD38 and ICAM-1 (BD PharMingen).

Poliomyxin B. 65-P1 or LPS in PBS was incubated with 25 g/ml PMB (Sigma-Aldrich) at 37°C for 45 min and then added to DC. After 18 h, activation of the DC was assessed as noted above.

5 The on-line version of this article contains supplemental material.
Results

Low levels of LPS in 65-P1 significantly affect CTL activation

We showed previously that recombinant 65-P1 has the ability to stimulate mice to produce CD8^+ cytolytic T cells (CTL) that recognize a peptide-MHC complex (SIYRYYGL-Kb, called SIY-Kb) formed by proteolytic processing of the injected protein (3). The 65-P1 preparations we used contained low levels of LPS (7, 29, or 48 EU/mg protein), but a subsequently obtained fourth preparation contained the lowest level (2.3 EU/mg). The 65-P1 protein was indistinguishable in all four preparations (see Materials and Methods). To determine whether LPS at these levels affected the CTL response, we compared the responses to the preparation containing the lowest LPS level with the one having the highest level: these are referred to below as “LPS-low” and “LPS-high” 65-P1, respectively.

The polyclonal responses of B6 mice injected with LPS-low 65-P1 exhibited significantly lower levels of cytolytic activity and fewer SIY/Kb tetramer-binding cells than mice injected with LPS-high 65-P1 (Fig. 1, A and B, respectively). To determine whether the differences were due to LPS, we added enough LPS standard to the LPS-low preparation to bring the total level up to that of the LPS-high preparation. The result was that the CTL response of mice injected with the supplemented (“spiked”) LPS-low preparation increased to the same level as the LPS-high response.

To gain some idea whether the small amounts of LPS introduced into mice injected s.c. with 65-P1 could affect DC at the injection site, we exposed DC from B6 mice to various amounts of a LPS standard in vitro and used increased expression of Kb by DC to indicate a response. The response was half-maximal with LPS at ~1 EU/ml and reached a maximum at 5–10 EU/ml (Fig. 1C). Similar results were recently reported for mouse macrophages (21). Although these results cannot be extrapolated directly to DC in vivo, they give some indication that mice injected with amounts of a recombinant Hsfp, such as that designated LPS high, could receive sufficient LPS to stimulate DC maturation in vivo.

The CTL response to the 65-P1 LPS-low preparation raised the possibility that DC might be activated by the Hsfp itself and not by the small amount of LPS contaminating it. To evaluate this possibility, we used oligonucleotide microarrays to analyze DC from mice that are genetically unresponsive to LPS.

DC gene expression in response to Hsfp 65-P1

The oligonucleotide microarrays we used compared, on a genome-scale, the responses of BMDC from HeJ and ScNCr mice with BMDC from the corresponding wild-type mice (HeN and BL10 mice, respectively). Although BMDC may not mirror DC present at the site of Ag injection, we used these cells because a plentiful supply of highly purified, relatively homogenous DC was required for the microarray analysis. Not only would DC derived from the injection site be present in low numbers, but they would also consist of a mixture of activated and nonactivated cells: such a population would not be expected to yield meaningful microarray data.

The DC were exposed in culture for various times to either 65-P1 or a LPS standard at a concentration (0.7 EU/ml) that matched the LPS level in the Hsfp. The DC were also exposed to unmodified Hsp65 or to culture medium alone to provide reference control values. RNA isolated from DC after 0, 2, 6, 10, and 22 h of culture was biotinylated and hybridized to Affymetrix U74A chips (~12,000 gene arrays). The criteria for considering whether or not a stimulus (LPS or Hsfp) caused increased expression of a gene are described in the study by Huang et al. (19) and more briefly in Materials and Methods. The results, described below, are represented by pseudocolors in Fig. 2 and summarized in Tables I and II. The values for individual genes that were up-regulated at any time point, under any condition, are given in Supplement Table I.

The Hsfp and LPS standard stimulated increased expression of ~700 genes (all categories) in DC from HeN mice, with an ~80% overlap of those stimulated by Hsfp and LPS. The corresponding numbers of genes up-regulated in DC derived from HeJ mice were ~380 and ~240, respectively. To determine whether the Hsfp and LPS standard stimulated increased expression of immune-related genes in DC derived from LPS unresponsive (HeJ) mice a number of immune-related genes up-regulated in DC by Hsfp65-P1 and LPS as determined by oligonucleotide array a

Table I. Number of immune-related genes up-regulated in DC by Hsp65-P1 and LPS as determined by oligonucleotide array a

<table>
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<th>Mice</th>
<th>Hsp65-P1</th>
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<td>BL10</td>
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<td>ScNCr</td>
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<td>HeN</td>
<td>74</td>
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<tr>
<td>HeJ</td>
<td>38</td>
<td>10</td>
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a Up-regulated genes were selected by criteria given in Materials and Methods and more fully in the study by Huang et al. (19). Immune-related genes are listed in Table II.

Table II. Hsp65-P1 stimulates increased expression of many immune-related genes in DC derived from LPS unresponsive (HeJ) mice a

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a DC from HeJ or HeN mice were incubated with 100 μg/ml 65-P1 or 0.7 EU/ml LPS. The genes are listed with commonly used symbols and GenBank accession number. Numbers are scores (s) for expression of the indicated genes.
overlap of genes that are known or inferred to be involved in innate and adaptive immunity (“immune genes”). In DC from HeJ mice, the response to LPS was negligible, indicating that the Tlr4 point mutation effectively blocked the LPS-induced changes seen with wild-type (HeN) DC. In contrast, 65-P1 was able to activate almost as many genes in HeJ DC as in HeN DC, indicating a LPS-independent effect of this Hsfp (Tables I and II). However, the induction of many of the genes was delayed in HeJ compared with HeN DC, and the expression profile induced by this protein differed somewhat in the DC derived from the two strains. For example, some of the immune genes induced in HeN DC, such as TNF-α and GRO1, were not induced by the Hsfp in the HeJ DC (see Fig. 2A, Table II, and Supplement Table I).

In an effort to validate the results of the oligonucleotide microarray analyses, we compared RNA levels with corresponding protein levels for a few selected genes. As seen in Fig. 3, A and B, when DC derived from an HeJ mouse were incubated with 65-P1, there was an increase in the surface protein expression of two representative immune-related genes, ICAM-1 and CD38. In addition, IL-1β secretion was also significantly higher in supernatants derived from HeJ DC that had been incubated with 65-P1 than from those that had been incubated with LPS (Fig. 3C). ELISA data for RANTES and TNF-α secretion were also in agreement with the microarray gene expression analyses (see Supplement Fig. 1).

The response of DC from B10 (LPS-sensitive) mice to the LPS standard and to the Hsfp was characterized by increased expression of a large number of genes, including many immune-related genes, with an ~80% overlap of up-regulated genes between these groups. As expected, ScNCr DC did not respond to LPS, but they made essentially no response to 65-P1 (Fig. 2B and Table I). Based on the assumption that the only significant difference between the HeJ and ScNCr DC is the point mutation in Tlr4 of the HeJ DC and the absence of Tlr4 from ScNCr DC, these results suggest that Tlr4 is required for the activation of immune-related genes by 65-P1.

In contrast to 65-P1, the effect of unmodified (“native”) Hsp65 on DC from any of the four mouse strains tested was negligible. This finding is illustrated in Fig. 2A and more fully documented in Supplement Table I.
Effects of PMB and PK

The microarray findings indicate that the 65-P1 protein, independently of LPS, can stimulate DC. To verify these findings, we determined whether the ability of 65-P1 to activate wild-type DC was affected by PMB and PK. After incubating 65-P1 or LPS for 45 min with PMB at 25 μg/ml, they were incubated with B6 DC for 18 h. Similarly, 65-P1 or LPS were incubated with 2 μg/ml PK for 18 h, followed by an 18-h dialysis against PBS (10,000 MWCO) and then incubated for 18 h with B6 DC. Activation of DC was assayed by cell surface expression of CD38. The PMB treatment virtually eliminated the effects of the LPS on 65-P1 to activate DC but had little effect on the ability of 65-P1 to stimulate the DC (Fig. 4A). Conversely, PK treatment of the LPS standard followed by extensive dialysis had no effect on its ability to affect CD38 expression. However, this treatment markedly reduced the ability of 65-P1 to activate DC (Fig. 4B). Taken together, these findings support the microarray data in showing that independently of LPS 65-P1 can stimulate DC. The ScNCr mouse has a large chromosomal deletion that includes not only the Tlr4 locus, but also two other as yet uncharacterized loci. To determine whether the absence of Tlr4 was responsible for the lack of response by ScNCr DC to 65-P1, we treated 65-P1 with PMB, as above, and added this protein to DC derived from mice with a targeted deletion of Tlr4 (Tlr4−/−). As seen in Fig. 4, C and D, the DC from the Tlr4−/− mice failed to be activated by 65-P1, just as we observed for the ScNCr-derived DC.

Cytolytic CD8 T cell responses in ScNCr mice injected with 65-P1

Since 65-P1 elicited no response in ScNCr DC, it was of interest to examine CD8 T cell responses to this protein in ScNCr mice. The SIY octapeptide can be excised from this Hsfp by DC ex vivo and presented by Kb as SIY-Kb complexes (D.P., E.G., and H.N.E., manuscript in preparation). Correspondingly, when injected into H-2b mice (B6 or B10), the 65-P1 protein stimulates production of CD8+ CTL that recognize the SIY-Kb complex. In this study, we used an adoptive transfer system to study the response of naive anti-SIY-Kb CD8 T cells in mice injected with 65-P1. Purified naive T cells from mice expressing a TCR transgene that recognizes SIY-Kb (the 2C TCR) were adoptively transferred into ScNCr mice and control wild-type (B10) mice. The recipients were then injected with 65-P1 in PBS (LPS-low 65-P1). Three days later, cells were isolated from the draining lymph nodes and scored directly for anti-SIY-Kb cytolytic activity and, with the aid of the clonotypic anti-2C TCR Ab (1B2), the abundance of 2C T cells and their CD44 expression was determined.

As seen in Fig. 5A, the CTL activity elicited was significantly lower in the ScNCr recipients than in the B10 mice or in other H-2b mice (B6; data not shown). In contrast to CTL activity, however, the frequency of the transferred 2C cells was as high in the ScNCr mice as in the wild-type (B10) mice (Fig. 5B). The similar frequency in ScNCr and wild-type mice implied that in response to the injected Hsfp, naive 2C cells proliferated as well in the absence as in the presence of Tlr4. This suggestion was confirmed with adoptive transfer of CFSE-labeled naive 2C cells: these cells were stimulated by the injected Hsfp to proliferate as vigorously in the ScNCr mice as in the B10 mice (Fig. 5D). Moreover, nearly all of the proliferating cells became CD44+, regardless of whether the mice were B10 or ScNCr (Fig. 5C). As shown in Fig. 5E, using activation of 2C T cells (CD69 up-regulation) as a readout of Ag processing, the DC from ScNCr mice were as effective as those from wild-type mice in processing 65-P1 and presenting SIY-Kb complexes on their cell surface. Taken together, these findings suggest that Tlr4 is required for the acquisition of optimal cytolytic activity in response to 65-P1, but not for the initial T cell activation and proliferative response to this Hsfp.

To assess what effect, if any, the lack of Tlr4 had on secondary T cell responses, we immunized B6 and ScNCr mice with 65-P1, as in Fig. 1A, and evaluated CTL activity from spleen and draining lymph nodes following 6 days of restimulation in culture with SIY peptide. As seen in Fig. 6A, CTL activity was substantially lower in cells from the ScNCr mice than from B6 mice. In addition, the total number of cells in the restimulated cultures from the ScNCr mice was decreased significantly in comparison to B6 mice (Fig. 6B). This finding is in contrast to the adoptive transfer experiment in which the number of CD8 T cells was equivalent between the two groups of mice (Fig. 5B). These results suggest that in vivo the absence of Tlr4, the anti-SIY-Kb CD8 T cells were poorly primed, even though ScNCr DC are competent at cross-presenting 65-P1 to naive CD8 T cells (Fig. 5E).

FIGURE 4. Effects of PMB and PK on 65-P1 activity. CD38 or ICAM-1 protein levels were assessed on B6, ScNCr, or Tlr4−/− DC after 18-h incubation with either 65-P1 (100 μg/ml), LPS (1 EU/ml), or poly(I:C) (25 μg/ml) that had been preincubated with either PMB or PK. A, 65-P1 or LPS were incubated with PMB (25 μg/ml) in PBS for 45 min before addition to DC. B, 65-P1 or LPS were incubated for 18 h in PBS in the presence of PK (2 μg/ml), dialyzed against PBS for an additional 18 h, and then added to DC. C, As for A, Level of activation was determined by CD38 expression. D, As for A, Level of activation was determined by ICAM-1 expression. Results shown are representative of two to four experiments. Error bars are the SD obtained from triplicate samples. Background is subtracted.
**Discussion**

**DC activation by 65-P1 is LPS independent but requires Tlr4**

Our comparison of different preparations of a recombinant Hsfp indicate that LPS, present as a contaminant at levels previously considered inconsequential, contributes significantly to the cytolytic T cell response elicited by these proteins in mice. Nevertheless, the present findings also lead us to conclude that the 65-P1 protein itself can activate DC, independently of LPS. This conclusion is based on three findings: 1) the Hsfp, unlike LPS, stimulated increased expression of many immune-related genes in DC from HeJ mice; 2) the stimulation of wild-type DC by Hsfp was eliminated by digesting the protein with PK; and 3) Hsfp stimulation of DC was unaffected by incubating the protein with PMB under conditions that eliminated the effect of a LPS standard on these DC. Since this Hsfp failed to stimulate DC that lack Tlr4 (from ScNCr and targeted Tlr4<sup>−/−</sup> mice), it appears that the protein’s effect on DC is also Tlr4 mediated.

Can other non-LPS PAMPs be responsible for the findings attributed here to the 65-P1 protein? If this were the case, the hypothetical contaminant would have to exhibit the following properties: 1) it would have to be a protein, to account for the effect of PK; 2) it would have to act through Tlr4, because it is inactive on DC from ScNCr and Tlr4<sup>−/−</sup> mice; 3) it can, nevertheless, still act through Tlr4 having a point mutation, as in DC from HeJ mice; 4) it would have to be active at extremely low concentrations because of the protein’s purity (>95%; data not shown); and 5) it would have to fractionate consistently along with 65-P1 in size-exclusion chromatography (TSK3000 column). Given all of these requirements, we believe it most unlikely that the LPS-independent effects of 65-P1 on DC are due to an unknown protein that requires Tlr4 for its activity. In addition, the absence of a response by immune-related genes to this Hsfp by ScNCr DC, as seen in the microarray data (Fig. 2, Table I, and Supplement Table I), also supports this conclusion.

Recently, several unrelated proteins have also been reported to activate DC and macrophages via Tlr4. These include β-defensin, pneumococcal pneumolysin, the extra domain A of fibronectin, Hsp60, and Hsp70 (22–26). In these reports, various measures were taken, as in this study, to exclude the confounding effects of contaminating LPS. How can such diverse structures all require Tlr4 for their activity? It is unlikely that each of these proteins binds directly to Tlr4, and, indeed, we know that the 65-P1 protein, which binds to wild-type DC, binds virtually as well to ScNCr DC (D.P., E.G., and H.N.E., manuscript in preparation). Some Tlr can form heterodimers and thereby potentially expand the variety of ligands they bind; Tlr4, however, has...
not been found to form such structures (27, 28). The activation of DC via Tlr4 by LPS is regarded as the paradigm reaction for Tlr-mediated activation of DC by conserved microbial structures. It remains to be seen whether Tlr4 can bind diverse ligands or participate in the cross-presentation of peptide-MHC complexes in vivo by DC in the steady state (i.e., nonactivated) tolerize responding T cells (8, 13, 14).

Our results are also reminiscent of the CD8 cells described in several other situations, including, mice that have been immunized with heat-killed Listeria monocytogenes (as opposed to live Listeria) (32), in humans recently infected with HIV-1 or mice infected with lymphocytic choriomeningitis virus (33, 34). In all of these circumstances, the T cells proliferate extensively and up-regulate expression of CD44, but exhibit little cytolytic activity. Such T cells may be poised to become fully effective cytolytically if the required differentiation factors were to be supplied (35).

Besides showing that trace levels of LPS have a major impact on the cytolytic CD8 T cell response elicited by an Hsfp (65-P1), this study suggests that the 65-P1 protein itself, by stimulating DC, contributes to the development of CD8 cytolytic T cells. Tlr4 is of obvious importance for the LPS effect but, in addition, this receptor appears to be required for LPS-independent activation of DC by 65-P1. A role for signaling through a Toll-like receptor in the activation of Th1 cells has been previously reported (22, 36) and a role for Tlr9 has been described in the activation of autoreactive B cells (37). Whether ligation of other DC receptors can substitute for Tlr4 in the generation of an optimal cytolytic CD8 T cell response to this or other Hsfp remains to be seen.

Acknowledgments

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References


FIGURE 6. Secondary CTL responses to 65-P1 are diminished in ScNCr mice. B6 and ScNCr mice were injected twice, 1 wk week apart, each time with 50 μg 65-P1 LPS-low (2.3 EU/mg). Control mice were injected with a mixture of 48 μg Hsp65 (0.3 EU/mg protein) plus 2 μg P1 polypeptide; data for these controls are not shown as they did not differ from those controls in Fig. 1 A. Seven days after the second injection, spleen and draining lymph node cells were restimulated in culture for 6 days with 1 μM SIY peptide, without adding IL-2, and then analyzed as in A and B. A. Cytolytic activity. The restimulated (effector) cells and 51Cr-labeled T2-K b target cells were at E:T ratios of 100:1, 33:1, and 11:1; 4-h assay in the presence of 1 μM SIY. Values of p comparing B6 and ScNCr at each E:T are as follows: 100:1 = 0.0004, 33:1 = 0.0008, and 11:1 = 0.003. B. Total number of cells present following 6-day culture. Value of p comparing number of cells in B6 and ScNCr cultures = 0.04. A and B. Total sample size = 7–9/group. Each symbol is an individual mouse.


Supplement Figure 1. Concordance between mRNA and protein expression levels for representative chemokines.
Left panels are hybridization fluorescence levels from Affymetrix microarrays (relative fluorescence units: RFU). Right panels are standard ELISA measurements (R&D Systems) of protein levels from the supernatant of HeJ or HeN DC preparations incubated with different stimuli (see legend on right).
A. TNF
B. RANTES