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The Adjuvant Effects of *Mycobacterium tuberculosis* Heat Shock Protein 70 Result from the Rapid and Prolonged Activation of Antigen-Specific CD8⁺ T Cells In Vivo

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Heat shock protein 70 (hsp70) is a potent adjuvant that links innate and adaptive immune responses. To study how hsp70 activates naïve CD8⁺ T cells in vivo, we tracked Ag-specific CD8⁺ T cells in mice immunized with a fusion protein containing chicken OVA linked to hsp70 derived from *Mycobacterium tuberculosis* (OVA.TBhsp70). On a molar basis, OVA.TBhsp70 was several hundred times more effective than OVA peptide plus CFA in eliciting specific CD8⁺ T cell responses. Immunization with OVA.TBhsp70 activated >90% of detectable OVA-specific CD8⁺ T cells within 3 days and led to the persistence of cytotoxic effectors for at least 17 days. These studies demonstrate that the potent adjuvant effect of *M. tuberculosis* hsp70 results from the relatively complete, rapid, and durable activation of Ag-specific CD8⁺ T cells. *The Journal of Immunology*, 2002, 169: 5622–5629.

Heat shock protein 70 (hsp70) is a highly conserved superfamily of intracellular chaperones called stress proteins (1). Constitutively expressed stress proteins participate in protein synthesis, folding, trafficking, and degradation, while inducible stress proteins typically protect cells from environmental damage resulting from heat shock, free oxygen radicals, and other forms of stress (1–3). Almost 2 decades ago Srivastava and his colleagues (4) discovered that some inducible stress proteins, such as hsp70, potently stimulated Ag-specific T cell responses. Subsequent studies have revealed that these stress proteins were immunogenic because they simultaneously activated professional APCs and delivered peptides for presentation to MHC class I-restricted CD8⁺ and MHC class II-restricted CD4⁺ T cells (4–13). Inducible stress proteins such as hsp70 therefore link innate and adaptive immune responses and as such are the only known mammalian adjuvants (9).

Professional APCs such as dendritic cells (DCs) and macrophages express CD91 and the CD14/Toll-like receptor 4 complex, known mammalian adjuvants (9). Receptor-mediated binding of stress proteins initiates signal cascades in immature DCs that cause them to differentiate and migrate from the periphery to lymph nodes (LN), express costimulatory molecules, increase cell surface expression of MHC class I and II molecules, produce NO, and secrete proinflammatory cytokines (4, 10–22). Binding of TBhsp70 by CD40 also causes human DCs to release CC chemokines such as RANTES (15). Little is known about how these stress protein receptors signal APC to differentiate, except that two or more pathways exist, and MyD88/IRAK/NF-κB is required for at least one of them (13, 19–22). Regardless of how these signals are transduced, stress proteins are potent adjuvants that can elicit protective CTL-mediated immunity in viral and tumor animal models (4, 17, 23, 24). Success in these models has led to clinical trials to assess the safety and efficacy of Ag/stress protein complexes as cancer vaccines (25).

To maximize their potential as cancer vaccines, it is crucial to understand how stress proteins affect CTLs, since the immunological destruction of tumor cells is mediated primarily by this cell type. The effects stress proteins have on CTLs have been measured directly in vitro and ex vivo in terms of CTL phenotype and cytolytic activity (26–29) and indirectly in vivo by the enhanced survival of immunized animals upon tumor challenge (17, 23, 24). However, direct assessments of the quantitative and qualitative effects that stress proteins have on Ag-specific CD8⁺ T cells in vivo are lacking. To begin to address this, Young and colleagues (29, 30) constructed a fusion protein containing 115 aa of chicken OVA fused to TBhsp70 (OVA.TBhsp70) and found that immunization with this protein elicited a CD4⁺ T cell-independent, OVA-specific CTL response in mice. To quantitate this CTL response we have now used an adoptive cell transfer system to track OVA-specific donor CD8⁺ T cells in immunized recipients. Immunization with the OVA.TBhsp70 fusion protein resulted in the rapid and persistent activation of essentially all the transferred CD8⁺ T cells. This quick, relatively durable, and complete response can account for the potent adjuvant effects of TBhsp70.

Materials and Methods

Mice and cell lines

Six- to 8-wk-old C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME), Charles River (Wilmington, MA), or Harlan...
proteins were purified to the gene encoding chicken OVA (33). EL4 was cultured in Alloclone medium (RP10 1640 supplemented with 10% FCS, 50 nM 2-ME, 4 mM L-glutamine, 100 μg/ml of penicillin, 100 μg/ml of streptomycin, 10 mM HEPES, 100 μM nonessential amino acids, and 1 mM sodium pyruvate). EG.7 was cultured in Alloclone plus 400 μg/ml of Genetecin (Life Technologies, Gaithersburg, MD). Both cell lines were maintained at 37°C in a humidified atmosphere containing 5% CO2.

Proteins, Abs, and reagents

The OVA (pKS28), TBHsp70 (pKS74), and OVA.TBhsp70 (pKS76) expression constructs were gifts from Drs. J. Richmond and R. Young (Massachusetts Institute of Technology, Cambridge, MA) (28, 30). To express these recombinant proteins, Escherichia coli BL21(DE3)pLysS transformants were grown at room temperature in Luria-Bertoni medium containing 100 μg/ml of carbenicillin and 35 μg/ml of chloramphenicol to an OD600 of ~0.4. Protein expression was then induced with 0.5 mM isopropylthigalactoside. TBHsp70 was expressed as a soluble protein. OVA.TBhsp70 and OVA were both expressed as inclusion bodies and refolded sequentially in 5, 2, 1, and 0.5 M guanidine-HCl before dialysis against the appropriate buffer for subsequent purification by column chromatography. The OVA.TBhsp70 and TBHsp70 proteins were purified by ATP affinity chromatography as previously described (28). The recombinant OVA fragment contained an N-terminal six-histidine tag and was purified on an NiSO4-charged chelating column according to the manufacturer’s instructions (Amersham Pharmacia Biotech, Arlington Heights, IL). Column fractions containing protein were pooled and dialyzed extensively against PBS. Protein purity was assessed by SDS-PAGE. Protein concentrations were determined with the Coomassie blue staining method according to the manufacturer.

Adoptive transfer and immunization

The CD8+ T cell response to a defined Ag was tracked using the adoptive transfer technique pioneered by Kearney et al. (34) and modified by Mescher and colleagues (35–37). In this system nonadherent LN cells from TCR transgenic mice are transferred into congenic recipients such that the final donor of the cells in the recipients is ~0.5% of the lymphocyte total. Donor lymphocytes were prepared by homogenizing LN from OT-I/PL or OT-LSL mice. Adoptively transferred OT-I/PL and OT-LSL cells responded indistinguishably to challenge with Ag (our unpublished observations), and so the choice of the Thy1.1 or CD45.1 allelic gene identity. Because LPS has adjuvant properties similar to those attributed to stress proteins, LPS levels in the purified recombinant protein preparations were quantified and functionally assessed. Each protein was endotoxin-free within the limits of the Limulus amebocyte lysate assay (<0.125 endotoxin units/ml). As a functional control, each protein preparation was compared with LPS for its ability to increase the expression of the costimulatory molecules CD80 and CD86 on DCs (38). Murine DCs were incubated in vitro with 100 ng of LPS with or without the LPS inhibitor polymyxin B or with 100 μg of each protein with or without polymyxin B. CD80 and CD86 expression was only enhanced following incubation with LPS alone (our unpublished observations). Together these results demonstrated that the three immunogens were essentially devoid of LPS.

Flow cytometric analyses

LN and spleen cell suspensions were homogenized in PBS containing 2% FCS and 0.02% sodium azide and were incubated with anti-FcyRIIb to block Fc receptors. OT-I/PL and OT-LSL cells were then identified by flow cytometry with anti-CD8α-allophycocyanin and anti-Thy-1.1-PE, or with anti-CD8α- allophycocyanin and anti-CD45.1-PE, respectively. For analysis of CD44 or CD69 expression levels, cells were also incubated with CD44-FITC (for OT-I/PL) or anti-CD69-FITC (for OT-I.SJL) cells. Cells were incubated with Abs at 4°C for the dark for 30 min. Cells were then washed in PBS with 2% FCS and 0.02% sodium azide and were either analyzed directly or fixed in 1% paraformaldehyde in PBS. Events were acquired on a FACSCalibur (BD Biosciences, Mountain View, CA) and were analyzed using FlowJo software version 3.4 (Tree Star, San Carlos, CA).

CTL assays

Cytotoxicity was assessed in a standard 4-h 51Cr release assay. LN and spleen cells from immunized recipients were used as effectors against 1 × 105 31Cr-labeled EG.7 or EL4 target cells. After 4 h cells were pelleted by centrifugation, and the amount of 31Cr released into the supernatant was measured. Spontaneous release was determined by incubating target cells in the absence of effectors. Total release was determined by incubating target cells in medium containing Triton X-100. The percent lysis was calculated as: (experimental mean − spontaneous release)/total release − spontaneous release. Assays were performed in triplicate.

IFN-γ intracellular staining

Draining LN or spleen cells from immunized OT-I/PL recipients were homogenized and incubated overnight in Alloclone medium containing 200 μM SINIFEKL. The next day 1 × 106 cells were incubated with anti-FcyRIIb, followed by anti-CD8α-allophycocyanin and anti-Thy-1.1-PE to identify OT-I cells. To determine IFN-γ production, cells were additionally labeled with anti-mouse IFN-γ-FITC or FITC-labeled isotype-matched control after treatment with the Cytofix/Cytoperm Plus (with GolgiStop) kit according to the manufacturer’s instructions (BD Pharmingen). Events were acquired on a FACSCalibur (BD Biosciences) and were analyzed using FlowJo software version 3.4 (Tree Star).

Statistical analyses

The one-tailed Student’s t test was used to determine the significance of the results.

Results

Recombinant proteins used as immunogens are free of detectable LPS

OVA.TBhsp70, OVA161–276, and TBHsp70 were expressed as recombinant proteins in E. coli and were purified to ≥95% homogeneity. Because LPS has adjuvant properties similar to those attributed to stress proteins, LPS levels in the purified recombinant protein preparations were quantified and functionally assessed. Each protein was endotoxin-free within the limits of the Limulus amebocyte lysate assay (<0.125 endotoxin units/ml). As a functional control, each protein preparation was compared with LPS for its ability to increase the expression of the costimulatory molecules CD80 and CD86 on DCs (38). Murine DCs were incubated in vitro with 100 ng of LPS with or without the LPS inhibitor polymyxin B or with 100 μg of each protein with or without polymyxin B. CD80 and CD86 expression was only enhanced following incubation with LPS alone (our unpublished observations). Together these results demonstrated that the three immunogens were essentially devoid of LPS.
OVA.TBhsp70 is more immunogenic than SIINFEKL/CFA

To assess the quantitative and qualitative aspects of the CTL response to OVA.TBhsp70, adoptively transferred OT-I cells in immunized recipients were tracked by flow cytometry. OT-I cells respond to OVA.TBhsp70 because it contains SIINFEKL (OVA residues 257–264), the peptide for which the OT-I TCR is specific (31). The adjuvant effects of TBhsp70 relative to CFA were assessed by comparing the responses of adoptively transferred OT-I cells in mice immunized with OVA.TBhsp70 vs SIINFEKL/CFA. The phenotypes and total numbers of OT-I cells were determined 3 and 17 days following immunization with 10 μg (0.1 nmol) of fusion protein or 50 μg (50 nmol) of SIINFEKL in CFA, amounts shown to stimulate optimal proliferation of OT-I cells (our unpublished observations). Significant numbers of OT-I cells were detected in the draining LN (p < 0.01) and spleens (p < 0.05) of mice 3 days after immunization with either OVA.TBhsp70 or SIINFEKL/CFA, but not with PBS or TBhsp70 alone (Fig. 1). OT-I cells in the former two groups also expressed high levels of CD44, indicating that they had undergone Ag-specific activation (our unpublished observations). Compared with PBS controls, immunization with OVA.TBhsp70 yielded 74- and 10-fold increases in OT-I cell numbers in LN and spleen, respectively, while SIINFEKL/CFA caused 126- and 18-fold increases. The responses to OVA.TBhsp70 and SIINFEKL/CFA were not significantly different. In contrast, only mice immunized with OVA.TBhsp70 had significant (p < 0.01) numbers of OT-I cells 17 days later (21- and 11-fold increases in the draining LN and spleen, respectively; Fig. 1). Because OVA.TBhsp70 induces a comparable proliferative response to 500-fold more peptide plus CFA by day 3 and induces a greater response by day 17, we conclude that TBhsp70 is a superior adjuvant to CFA.

OT-I cells proliferate more and persist longer in response to OVA.TBhsp70 than OVA/LPS

Responses to OVA.TBhsp70 were also compared with those elicited by OVA161–276 plus LPS, where comparable processing and presentation of the antigenic epitope are required and where neither adjuvant provides an Ag depot, as is the case for mineral oil in CFA. C57BL/6 mice were adoptively transferred with OT-LSJL LN cells and then immunized with 10 μg (0.1 nmol) of OVA.TBhsp70 or 1.5 μg (0.1 nmol) of OVA161–276, mixed with 10 μg of LPS (OVA/LPS) on days 3, 6, 12, 17, and 21 before analysis. OT-I cell numbers peaked in the draining LN 3 days postimmunization in both groups, but the frequency in the OVA.TBhsp70 group was about 6 times greater (p < 0.01) than that in the OVA/LPS controls (Fig. 2). A comparison with Fig. 1 reveals that the OT-I response to SIINFEKL/CFA is 10 times greater than the OVA/LPS response. This could result from the 500-fold molar excess of injected SIINFEKL vs OVA, more efficient loading of MHC class I molecules with peptide (i.e., processing is not required for SIINFEKL, while it is for OVA), or additional adjuvants in CFA vs LPS.

Immunization with OVA.TBhsp70 resulted in a more sustained elevation of OT-I cell numbers than SIINFEKL/CFA (Fig. 1) or OVA/LPS (Fig. 2). In the LN the number of OT-I cells in OVA.TBhsp70-immunized mice took longer to return to basal levels (21 days) than did the number of cells in the OVA/LPS group (6 days; Fig. 2). In the spleen the number of OT-I cells had not returned to basal levels 21–35 days postimmunization with OVA.TBhsp70 (Fig. 2 and our unpublished observations). This was not simply due to an increase in total cell numbers, because only the percentages of OT-I cells were high, while the numbers of total splenocytes remained comparable (our unpublished observations). Moreover, OT-I cell frequencies in the spleen displayed a reproducible periodicity in which two comparable peaks occurred 6 and 21–28 days postimmunization with OVA.TBhsp70 (Fig. 2 and our unpublished observations). We conclude that TBhsp70 is a superior adjuvant compared with SIINFEKL/CFA.
a more potent adjuvant than LPS in terms of both the magnitude and the duration of its proliferative effect on CD8⁺ T cells.

**CD4⁺ T cells are not required for the OT-I response to OVA.TBhsp70**

C57BL/6.CD4⁻/⁻ gene-targeted recipients were adoptively transferred with enriched (>97%) CD8⁺ OT-I cells to determine whether CD4⁺ T cells were required for the OT-I response to OVA.TBhsp70. OT-I cell proliferation and differentiation in OVA.TBhsp70-immunized CD4-deficient recipients were indistinguishable from those in wild-type recipients (our unpublished observations). Our results support the conclusion of Huang et al. (29) that CD4⁺ T cells are not required for the TBhsp70 adjuvant effect on CTL responses.

**OVA.TBhsp70 rapidly activates the majority of OT-I cells**

The persistence of splenic OT-I cells induced by OVA.TBhsp70 could be residual from the original proliferative burst or result from the continuous division of a subset of cells in this initial burst. To distinguish between these possibilities, the kinetics of the OT-I proliferative and activation responses to OVA.TBhsp70 were compared with those to OVA/LPS. TBhsp70 alone was not examined as OT-I cells were previously shown to be unresponsive to it (Fig. 1 and our unpublished observations). OT-LSIL cells were labeled with CFSE before adoptive transfer and immunization with either 0.1 nmol OVA.TBhsp70 or OVA in LPS. Draining LN and spleen cells were harvested on days 1–5 after immunization, and OT-I cells were analyzed for proliferation, as measured by loss of CFSE intensity (39, 40), and for activation, as measured by a transient increase in CD69 expression.

Fig. 3A shows that OVA.TBhsp70 activates more OT-I cells in a shorter time than OVA/LPS. The frequency of CD69⁺ OT-I cells in the draining LN of mice in the OVA/LPS group peaked at 32% 1 day after immunization, but in OVA.TBhsp70-immunized mice it continued to rise until it peaked at 72% on day 2. OVA.TBhsp70 immunization activated more than twice as many OT-I cells by day

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**FIGURE 3.** OVA.TBhsp70 rapidly activates the majority of OT-I cells. C57BL/6 mice were adoptively transferred with CFSE-labeled OT-I.SJL LN cells and immunized s.c. with 0.1 nmol of OVA.TBhsp70 or OVA plus LPS. Mice were sacrificed on the indicated days postimmunization. A. OT-I cells were analyzed for CFSE content and CD69 expression. Dot plots are representative of three mice per group. B. The percentages of OT-I cells that had divided zero or one, two to four, or five or more times on the indicated days postimmunization with OVA.TBhsp70 (H) or OVA/LPS (L) are shown in the stacked bar graphs. These percentages were determined by drawing gates around OT-I events based on their CFSE intensities in A and dividing the numbers of gated events by the total number of events in the corresponding dot plot.
immunization led to a higher percentage of CD44\textsuperscript{high} OT-I cells increased CD44 expression on OT-I cells. However, OVA.TBhsp70 immunization with either OVA.TBhsp70 or OVA/LPS led to increased CD44 expression on OT-I cells. However, OVA.TBhsp70 immunization led to a higher percentage of CD44\textsuperscript{high} OT-I cells than did OVA/LPS. This was sustained throughout the course of the 21-day experiment, while CD44 levels dropped after day 6 in the OVA/LPS group.

The kinetics of OT-I proliferation in the spleen differed from those in the draining LN. Cells that had divided did not appear in the spleen until 3 days, coincident with the peak of OT-I expansion in the draining LN. The relatively low percentage of CD69\textsuperscript{+} OT-I cells in the spleen suggested that they were activated elsewhere, as did their expression of the activation marker CD44 (Fig. 4). Immunization with either OVA.TBhsp70 or OVA/LPS led to increased CD44 expression on OT-I cells. However, OVA.TBhsp70 immunization led to a more complete, rapid, and lasting activation of OT-I cells than does immunization with OVA/LPS. This conclusion is consistent with the uniformly higher and durable expression of the activation marker CD44 on OT-I cells in mice immunized with OVA.TBhsp70 vs OVA/LPS (Fig. 4). These data suggest that OT-I cells in the former group remain cytolytic effectors, while in the latter group they convert to a memory population (41, 42).

**OT-I cells present 17 days after OVA.TBhsp70 immunization are functional CTLs**

To determine whether immunization with OVA.TBhsp70 resulted in the development of CTL effectors, day 17 splenic and draining LN OT-I cells were tested for IFN-\(\gamma\) production and cytolytic activity (35–37). To measure IFN-\(\gamma\) levels, cell suspensions were pulsed overnight with SIINFEKL and then examined for intracellular IFN-\(\gamma\) production (Fig. 5). OT-I cells from both the draining LN and spleens of OVA.TBhsp70-immunized mice were capable of making IFN-\(\gamma\) on day 17 (81 and 96\%, respectively). This compares to 61 and 81\% of IFN-\(\gamma\)\textsuperscript{+} OT-I cells from the draining LN and spleens of OVA/LPS-immunized mice, respectively.

Cells from mice immunized with OVA.TBhsp70 were also specifically cytolytic. Cytolytic activity was measured directly ex vivo without Ag restimulation by mixing effector cells with either \(^{51}\text{Cr}\)-labeled E.G7 targets pulsed with SIINFEKL or \(^{51}\text{Cr}\)-labeled EL4 targets (negative controls). The effector cells were specifically cytolytic, as they only lysed the EG.7 (Fig. 6) and not the EL4 targets (our unpublished observations). The overwhelming majority of these effectors were OT-I cells; the others were endogenously derived. These data indicate that functional CTLs persist at least 17 days after immunization with OVA.TBhsp70.

**The duration of the response in OVA.TBhsp70-immunized mice is not due to Ag persistence**

One explanation for the persistence of effector CTLs is that naive OT-I cells are still being activated by Ag 2 wk postimmunization. This is unlikely because OVA.TBhsp70 activates almost all (94\%) detectable OT-I cells within 3 days, and the day 17 OT-I cells do not express CD69 and are not blasting (based on their low forward and side light scatters; our unpublished observations). To formally rule out the possibility that OVA.TBhsp70 formed an Ag depot that lasted for 2 wk, CFSE-labeled naive OT-I cells were transferred into mice that had been immunized 15 days previously but had not been adoptively transferred before immunization. Draining LN and spleens were harvested on the indicated days postimmunization, and CD44 expression on OT-I cells was determined by flow cytometry. A, A representative histogram showing CD44 expression on OT-I cells from mice immunized with OVA.TBhsp70 (■) or OVA/LPS (□). The gate defines the CD44\textsuperscript{high} population. B, Percentages of CD44\textsuperscript{high} OT-I cells from draining LN (top) or spleens (bottom). The data shown are the mean of three mice per group.

**FIGURE 4.** OT-I cells from immunized recipients express high levels of CD44. C57BL/6 mice were adoptively transferred with OT-I.SJL LN cells and immunized s.c. with 0.1 nM OVA.TBhsp70 or OVA plus LPS. Draining LN and spleens were harvested on the indicated days postimmunization, and CD44 expression on OT-I cells was determined by flow cytometry. A, A representative histogram showing CD44 expression on OT-I cells from mice immunized with OVA.TBhsp70 (■) or OVA/LPS (□). The gate defines the CD44\textsuperscript{high} population. B, Percentages of CD44\textsuperscript{high} OT-I cells from draining LN (top) or spleens (bottom). The data shown are the mean of three mice per group.

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Discussion

Our studies provide a mechanistic explanation for the potent adjuvant effects of TBhsp70. Following immunization with OVA.TBhsp70, almost all OT-I cells are activated and undergo a massive proliferative burst within 3 days that results in the persistence of Ag-specific effector CTLs for at least 3 wk. In contrast, immunization with OVA plus LPS or CFA causes smaller, transient increases in OT-I cells that return to basal levels within 1–2 wk. Differential CD44 expression by OT-I cells also suggests that their encounter with Ag is distinct in the presence of TBhsp70 vs LPS. Mice immunized with OVA.TBhsp70 have a larger percentage of CD44high cells than the LPS controls. CD44 expression remains high in the OVA.TBhsp70-immunized mice and is consistent with their sustained OT-I effector response. The conversion of splenic and LN OT-I cells to a CD44int phenotype by day 6 in the LPS controls suggests that the activated OT-I cells are differentiating into central memory T cells (41) consonant with their ability to produce IFN-γ upon Ag rechallenge in vitro. Taken together, our data demonstrate that TBhsp70 is both qualitatively and quantitatively superior to CFA and LPS in eliciting Ag-specific CTL cell responses in vivo. We propose that these effects account for the success of TBhsp70 as a vaccine in animal models (4, 17, 23, 24) and speculate that other stress proteins act similarly.

Analogous to mammalian gp96 and hsp70, the adjuvanticity of TBhsp70 is most likely due to its ability to activate DCs (8, 10–13, 21). Because activated DCs can deliver all the signals (Ag, co-stimulation, and IL-12) required to fully stimulate naive CD8+ T cells (39), it is not surprising that the development of CTL responses is critically dependent on this professional APC (43). Mammalian hsp70 induces DC maturation through receptors such as the CD14/Toll-like receptor 4 complex and CD91 (13, 14). To our knowledge, it is not yet known whether TBhsp70 binds these two receptors. However, TBhsp70 specifically binds CD40 and through it induces human monocyte-derived DCs to produce CC chemokines (15). TBhsp70 binding to CD40 also causes the human myelomonocytic cell line KG1 to differentiate into cells with DC features (15). Finally, TBhsp70 can activate immature bovine DCs through an undefined receptor (44). Together these data strongly suggest that mammalian and TB hsp70 are potent adjuvants because they both activate DCs. Although they clearly can signal through different receptors, it remains to be seen whether they share signaling pathways.

![FIGURE 5](http://www.jimmunol.org/) OVA.TBhsp70 induces more OT-I cells to produce IFN-γ than OVA/LPS. C57BL/6 mice were adoptively transferred with OT-I/PL LN cells and immunized s.c. with 0.1 nM OVA.TBhsp70 or OVA plus LPS. Draining LN and spleens were harvested 3 or 17 days postimmunization, and single-cell suspensions were made and pulsed overnight with the SIINFEKL peptide. The cells were stained for intracellular IFN-γ (solid line). The percentage of cells producing IFN-γ is given in the upper right corner of each histogram and is based on gates set with the negative isotype control (dotted line). The data shown are representative of two experiments with two mice per group.

![FIGURE 6](http://www.jimmunol.org/) OVA.TBhsp70 induces naive OT-I cells to differentiate into cytolytic effectors. C57BL/6 mice were adoptively transferred with OT-I/PL LN cells and immunized s.c. with 0.1 nmol of OVA.TBhsp70 or OVA plus LPS. Draining LN and spleen cells were harvested 17 days later and used directly without restimulation as effectors in a cytolytic assay. The targets were 51Cr-labeled EG.7 cells pulsed with SIINFEKL. The E:T cell ratio corrected for the number of OT-I cells is shown in parentheses to the left of each group (the percentage of draining LN OT-I cells in OVA.TBhsp70-immunized mice was about 3 times higher than that in the OVA/LPS group). Data shown are an average of three wells from two mice per group. No lysis above background levels occurred when 51Cr-labeled EL4 targets were used (our unpublished observations).
DCs present peptides to both MHC class II-restricted CD4+ Th cells and MHC class I-restricted naive CD8+ CTLs. CD4+ T cells participate in CTL responses by supplying IL-2 and activating DCs (45). However, CD4+ T cells are not required to generate all CTL responses. Udono et al. (6) originally demonstrated that gp96-induced CTL responses were CD4+ T cell independent. This was later extended by Huang et al. (29) when they similarly showed that CD4+ T cells were not required for the CD8+ T cell response to TBshp70. In the presence of peptide-pulsed DCs, Wang et al. (46) found that CD8+ T cells can provide their own help in the form of endogenous IL-2 if they are present at sufficiently high precursor frequencies. Following this reasoning, we speculate that the proliferative burst induced by TBshp70 yields enough OVA-specific CD8+ T cells to produce “self-help.” This would account for the CD4+ T cell independence of the OVA.TBshp70-mediated OT-I response.

If TBshp70 indirectly induces CD8+ T cell effectors to produce endogenous IL-2, then this cytokine could continue to drive proliferation, and this could explain the persistence of CTL effectors 3 wk postimmunization when Ag can no longer activate naive CD8+ T cells. In a model of CTL-mediated tumor immunity, Shrikant and Mescher (36) found that lytic effectors failed to remain at the tumor site and control tumor cell growth in the absence of exogenous IL-2. They suggested that effective CD8-targeted immunotherapy may be critically dependent on sustaining the response and reactivating cells that have left the site of tumor growth and become nonresponsive. Schoenberger, Ahmed, and Pamer (47–50) have shown that once the CD8+ T cell developmental pathway is set into motion by antigenic stimulation under optimal conditions, it proceeds without any further need for Ag. That is, Ag-activated CD8+ T cells go through at least eight cell divisions, become cytolytic, and eventually revert to long-lived memory cells without further Ag stimulation. Bevan and Fink (51) suggest that this optimal early “hit” with APCs requires high Ag density and strong costimulation. We speculate that OVA.TBshp70 stimulates DCs to provide both high Ag density and strong costimulation to naive OT-I cells, and that this optimal encounter causes the OT-I cells to differentiate into relatively long-lived (i.e., 3 wk) effectors that persist in the absence of Ag. Our data showing that naive OT-I cells are not activated if transferred 15 days postimmunization support this.

These data also eliminate Ag restimulation as a cause of the increase in splenic OT-I cells seen 21–28 days after immunization with OVA.TBshp70. Therefore, this rebound probably results from the migration of CD44int/CD62LOT-I cells from non-lymphoid tissues (52). We speculate that a proportion of OVA.TBshp70-activated OT-I cells become effector memory T cells that migrate from lymphoid to non-lymphoid tissues 1–2 wk postimmunization, but return in 3–4 wk (52, 53). Because the frequencies of splenic OT-I cells on days 6 and 21–28 postimmunization are equivalently high, the return of these putative effector memory OT-I cells is apparently synchronous. The reasons for this are not yet clear.

The resurgence in cancer vaccine development over the last decade has resulted from identifying tumor-associated peptides and enhancing their presentation to T cells (50, 54). Kaech et al. (50) noted that effective vaccines must induce as large an effector T cell population as possible, since this burst size determines the number of subsequent memory T cells that form. By tracking a specific CD8+ T cell response to an Ag/stress protein complex in vivo, we have quantified how profoundly a stress protein stimulates CD8+ T cell proliferation and differentiation into a relatively long-lived effector population. We have also shown qualitatively how this adjuvant effect differs from the effects of LPS and CFA. Our results may have clinical relevance, as stress protein/Ag complexes are being evaluated as cancer therapeutics. Janetzki et al. (25) isolated gp96/peptide complexes from a variety of solid tumors (e.g., breast, colon, and pancreatic carcinomas), and in six of 12 patients tested, immunization led to CD8+ T cell responses specific for the autologous tumor. NK activity was also elicited in eight patients. No adverse toxicities or autoimmune responses occurred. Another recent report showed that hsp70/peptide complexes isolated from melanomas specifically activated melanoma-specific human CD8+ T cells in vitro by cross-priming APCs (55). Together these studies suggest that stress protein/peptide complexes hold promise for cancer vaccines, and our results help to reveal why.

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References

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