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A Synthetic Peptide Administered with IL-12 Elicits Immunity to *Listeria monocytogenes*[1]

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IL-12 is a pivotal cytokine signal for the development of Th1-type cellular responses that are required for control of intracellular pathogens. We previously demonstrated that coinjection of IL-12 with heat-killed *Listeria monocytogenes*, which was not immunogenic when injected alone, elicited intense Ag-specific T cell responses that conferred protection against subsequent challenge with *Listeria*. Herein we describe the remarkable finding that a nonimmunogenic synthetic peptide corresponding to a dominant MHC class II (H-2^k)-restricted listerial determinant, when coinjected i.p. with murine IL-12, elicited potent Ag-specific immune responses that conferred protective immunity against *Listeria*. *The Journal of Immunology*, 1997, 159: 3675–3679.

IL-12 is a cytokine produced primarily by macrophages and B cells that promotes the development of Th1-type responses. In general, Th1 responses are required for the control of intracellular pathogens such as viruses, intracellular parasites (*Leishmania major*, *Toxoplasma gondii*), or intracellular bacteria such as *Listeria monocytogenes*. In intracellular parasite models, therapeutic administration of IL-12 induces a shift from a nonprotective Th2-type response to a protective Th1-type response that correlates with successful clearance of the parasites (1–6). Our lab (7) and others (4, 8, 9) have demonstrated that IL-12 also has potent adjuvant properties when coinjected with various Ag preparations.

The murine model of listeriosis is well characterized and thus is an ideal system for vaccine studies. Infection of mice with a sublethal dose of viable *Listeria* results in rapid clearance of bacteria and the development of long-lived T cell-mediated immunity (10, 11). In contrast, inoculation with high doses of viable *Listeria* leads to systemic infection characterized by uncontrolled replication of bacteria in the spleen and liver for 2 to 4 days, culminating in death 4 to 10 days postinfection (12). Because the level of bacterial replication is inversely related to the immune status of the infected mouse, enumeration of bacteria in the spleen or liver 2 to 4 days postinfection is a standard method for evaluating the efficacy of immunization strategies (11).

We previously demonstrated that heat-killed *L. monocytogenes* (HKLM),[3] a notoriously poor immunogen, conferred protective listerial immunity when injected in combination with IL-12 (7). To further evaluate the adjuvanticity of IL-12, a mixture of two synthetic peptides corresponding to known antigenic determinants of *Listeria* was evaluated for its ability to elicit specific immune responses when administered alone or when coinjected with rIL-12. The peptides used for these studies were homologous to residues 203–226 and residues 91–99 of LLO, an important virulence factor and antigenic target of *Listeria*. Residues 203–226 (LLOp203–226) contain two distinct overlapping MHC class II (an I-A^k and an I-E^k)-restricted epitopes (13), whereas region 91–99 (LLOp91–99) contains an MHC class I (H-2K^d)-restricted epitope (14). Two approaches were used to evaluate the immune responses of mice following multiple injections of LLO peptide(s) and IL-12: 1) measurement of Ag-specific responsiveness of peritoneal lymphocytes in vitro, and 2) enumeration of bacterial burden in the spleen after challenge with a large dose of viable *Listeria*. The data show that coinjection of IL-12 with the nonimmunogenic synthetic peptide LLOp203–226 gives rise to protective type-1 cellular immune responses.

Materials and Methods

Mice

Female C3HeB/FeJ (H-2^k) mice obtained from The Jackson Laboratory (Bar Harbor, ME) were used at 8 to 12 wk of age. Mice were housed in microisolator cages with laboratory chow and water available ad libitum.

Antibodies

Specificities and sources are as follows: anti-IFN-γ hybridomas R4–6A2 (obtained from American Type Culture Collection, (ATCC), Rockville, MD) (15) and XMG1.2 provided by Dr. Tim Mosmann, DNAX Inc., Palo Alto, CA (16); anti-mouse IL-12 hybridomas C15.6.7.6 and C17.8.20.15 provided by Dr. G. Trinchieri, Wistar Institute, Philadelphia, PA. mAb generated from cell lines in the laboratory were purified from culture supernatants by protein A or protein G affinity chromatography (17). Purified Abs from XMG1.2 and C17.8.20.15 were directly conjugated to biotin using standard techniques (17).

Synthetic peptides

LLOp91–99 (NH_2-GYKDGNEYI-$CONH_2$) and LLOp203–226 (NH_2-KIDYDDEMAYSESQLIAKFGTAFK-$CONH_2$) were synthesized using F-moc chemical strategies using an automated Applied Biosystems (Foster

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[3] Abbreviations used in this paper: HKLM, heat-killed *Listeria monocytogenes*; LLO, listeriolysin-O; LLOp91–99, synthetic peptide homologue of residues 91–99 of LLO; LLOp203–226, synthetic peptide homologue of residues 203–226 of LLO; PEC, peritoneal exudate cells; PNA, plastic nonadherent peritoneal cells (lymphocyte enriched); BHI, brain-heart infusion.

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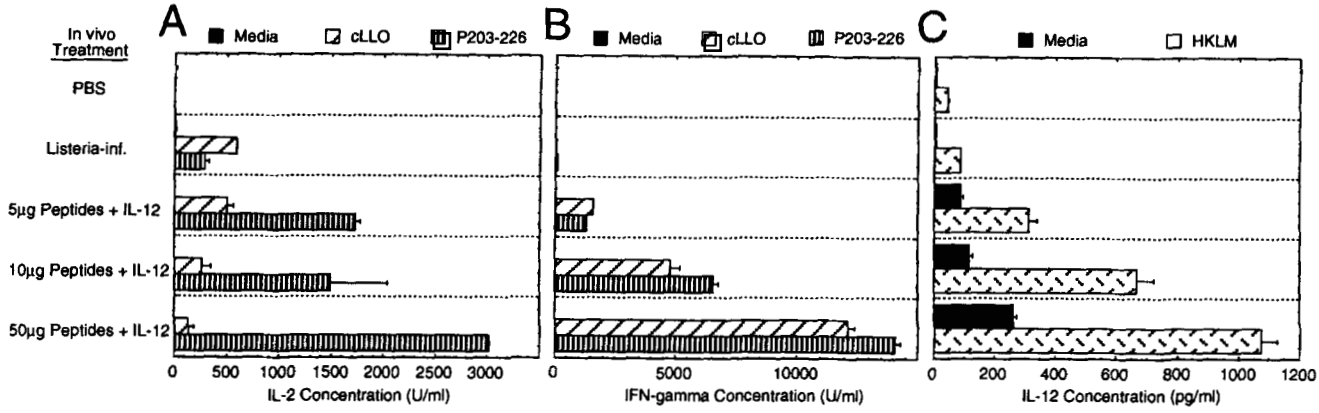

FIGURE 1. Immune responsiveness of peritoneal exudate cells from peptide + IL-12-immunized mice. C3HeB/FeJ mice (5/group) were immunized on days 0, 5, and 25 with either PBS, 0.5 μg of IL-12 in PBS, or mixtures containing either 5 μg, 10 μg, or 50 μg of both LLOp203–226 and LLOp91–99 along with 0.5 μg of rIL-12 as indicated. Mice were killed on day 31, peritoneal cells were collected by lavage, pooled, and PNA or adherent (macrophage) cell populations were prepared. PNA (1.5×10^6/ml) were restimulated in vitro (24 h at 37°C) with predetermined optimal concentrations of either LLO (13 μg/ml), LLOp203–226 (1 μg/ml), or culture medium alone as indicated. Production of (*A*) IL-2 or (*B*) IFN-γ by restimulated PNA cultures was quantitated as a measure of Ag-specific T cell responsiveness. *C*, IL-12 (p40/p70) production by macrophages following culture for 24 h at 37°C either with culture medium (no further stimulation) or with HKLM (10^8/ml) was also quantitated. All assays were performed in triplicate, and results are expressed as mean ± SD.

City, CA) 430A peptide synthesizer. Peptides were purified via HPLC, and composition analysis was performed.

Injections

All injection doses were prepared in a total volume of 1 ml, using endotoxin-free PBS, and were administered i.p. The amount of rIL-12 used for these studies (0.5 μg/mouse/dose; three doses) was much lower than the amount typically used in other in vivo IL-12 studies (1–4).

Listeria (strain 43251; ATCC) were grown overnight in brain-heart infusion (BHI) broth (Difco Laboratories, Detroit, MI) at 37°C with aeration, washed three times in PBS, and concentrations were determined by optical density with confirmation by colony counts on BHI agar plates.

In vitro restimulation of peritoneal lymphocytes/macrophages

PEC were obtained from mice by peritoneal lavage. Macrophages were isolated by incubating PEC (2.5×10^6/ml/well) in 24-well tissue culture plates for 2 h at 37°C. Nonadherent cells were removed and pooled (designated PNA).

PNA (1.5×10^6/ml) were cultured with the indicated in vitro restimulants (see fig. legends) in 24-well plates (Corning Inc., Corning, NY) at 37°C for 24 h. Culture supernatants were frozen and saved for use in IL-2 and IFN-γ quantitation assays. LLO was prepared from *Listeria*-culture supernatants as described (18). Macrophage cultures were incubated at 37°C for 24 h in vitro with either culture medium (no stimulation) or 10^8 HKLM/ml. Supernatants were frozen and saved for use in IL-12 quantitation assays.

Cytokine quantitation assays

IL-2 production by PNA was measured by bioassay using the IL-2 dependent cell line HT-2 as described (7). IFN-γ production by PNA was measured by capture ELISA using the R4–6A2/XMG1.2 hybridoma pair as previously described (7). IL-12 (p40/p70) production by peritoneal macrophages was measured in cell-free culture supernatants by capture ELISA using the C17.8.20/C15.6.7.6 hybridoma pair as described (7).

Bacterial load in spleens of Listeria*-challenged mice*

Spleens from immunization/challenge recipients were homogenized and then disrupted by treatment with 0.5% Triton X-100 (Sigma Chemical Co., St. Louis, MO) in a total of 10 ml of PBS to release intracellular bacteria. Serial 10-fold dilutions of each sample were made, and 100 μl of each dilution was spread onto BHI agar plates to quantitate bacterial load.

Results and Discussion

To determine whether LLO peptides in combination with IL-12 could generate cell-mediated immunity, C3HeB/FeJ mice (5/group) were injected with the indicated (Fig. 1) Ag preparations on days 0, 5, and 25 and killed on day 30. As a benchmark for listerial immunity, one group was inoculated i.p. with a sublethal dose of viable *L. monocytogenes* on day 0 and received no further injections. Cell-free supernatants from peritoneal lymphocytes restimulated in vitro for 24 h with predetermined optimal doses of either LLOp203–226, LLO, or medium alone were analyzed to quantitate IL-2 (Fig. 1*A*) or IFN-γ (Fig. 1*B*) as an indication of Ag-specific T cell responsiveness.

Given the poor immunogenicity of synthetic peptides, the level of LLOp203–226-specific reactivity observed when the peptides were administered with IL-12 was remarkable. T cells from mice immunized with peptides + IL-12 were reactive with both LLOp203–226 and native LLO protein (Fig. 1, *A* and *B*). LLOp203–226-specific responses were generally more intense in peptide + IL-12 immunized mice than in those in which immunity was generated by infecting with *Listeria*. It should be noted, however, that these mice received only a single injection of *Listeria*. It is also important to note that no LLOp91–99-specific responses were observed (data not shown). As an additional measure of the overall immune status of these mice, IL-12 (p40/p70) production by peritoneal macrophages in response to in vitro restimulation was monitored. Treatment of mice with peptides + IL-12 increased the ability of macrophages to produce IL-12 upon restimulation in vitro (Fig. 1*C*). These results are consistent with previous studies using HKLM + IL-12 as the immunogen (7) and are indicative of a highly stimulated immune system in peptide + IL-12-treated mice.

To determine whether the potent LLOp203–226-specific immune responses generated by injecting mice with peptides + IL-12 would protect against subsequent challenge with a large dose of viable *Listeria*, C3HeB/FeJ mice (5/group) were immunized with the indicated concentrations of LLOp203–226 and LLOp91–99 in PBS or in combination with 0.5 μg of rIL-12 on days 0, 5, and 24. As a benchmark for acquired listerial immunity, one group of five mice was injected with a nonlethal dose of viable *Listeria* on day 0 and was boosted with a similar dose on day 25. Approximately 1 month later (day 52), the mice were challenged with viable *Listeria* ($6.5 \times LD_{50}$). CFU of *Listeria*

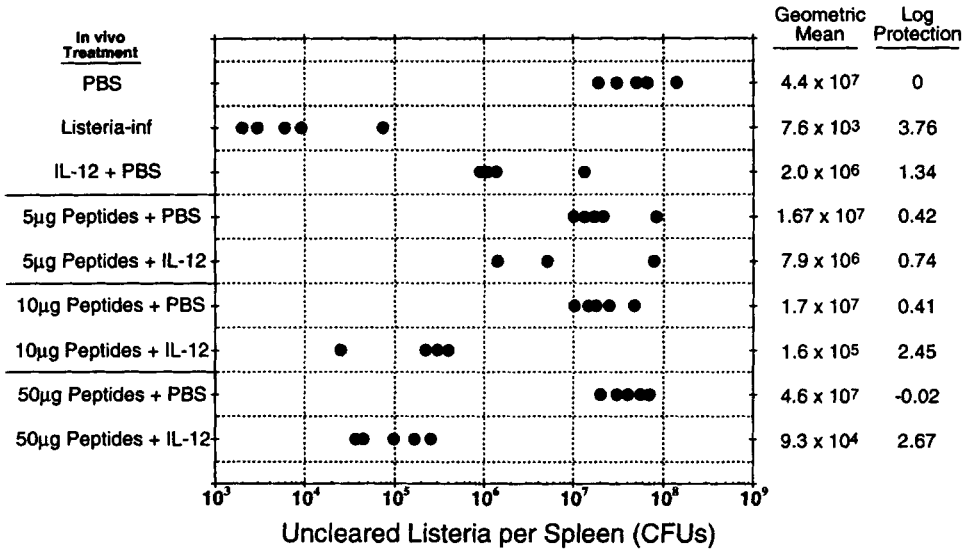

FIGURE 2. Mice immunized with LLOp203–226 and LLOp91–99 + IL-12 are resistant to subsequent challenge with *Listeria*. C3HeB/FeJ mice (5/group) were immunized on days 0, 5, and 24 with either PBS, 0.5 μg of rIL-12 in PBS, or mixtures containing either 5 μg, 10 μg, or 50 μg of both LLOp203–226 and LLOp91–99 in PBS alone or in combination with 0.5 μg of rIL-12 as indicated. An additional group of five mice was inoculated i.p. with a sublethal dose of viable *Listeria* on day 0 (8.2×10^3/mouse or $0.16 \times LD_{50}$) and again on day 24 (7.7×10^3/mouse or $0.15 \times LD_{50}$). On day 52, each mouse received i.p. a challenge dose of *Listeria* (3.2×10^5/mouse or $6.4 \times LD_{50}$). All mice were killed on day 56 (4 days after challenge), and bacterial load in each spleen was determined. Log protection represents the difference observed between treatment groups and mice treated with PBS alone.

per spleen were enumerated 4 days postchallenge (Fig. 2). As expected, the *Listeria*-infected/immune mice were well protected (3.76 log reduction in CFU as compared with unprotected mice injected with PBS only). Remarkably, the mice treated with either 50 or 10 μg of peptide + IL-12 were also protected (2.67 and 2.45 log reduction in CFU, respectively). The lowest dose of peptide + IL-12 failed to elicit protective immunity (0.74 log reduction in CFU). When administered without IL-12, peptides failed to confer immunity, even at the highest dose tested (Fig. 2). Moreover, the presence of Ag-specific T cells (as indicated by IL-2 and IFN-γ production by peritoneal lymphocytes in vitro) and activated macrophages (as indicated by IL-12 production in vitro) correlated well with the generation of protective immunity in these mice (data not shown).

Both $CD4^+$ and $CD8^+$ cell-mediated responses play a role in listerial immunity (19, 20). The protective immunity depicted in Figure 2 was generated by immunization with a synthetic peptide (LLOp203–226) homologous to a region of LLO that contains two MHC class II-restricted epitopes ($I\text{-}A^k$ and $I\text{-}E^k$) and a peptide homologue of an MHC class I-restricted ($H\text{-}2K^d$) epitope of LLO (LLOp91–99). However, due to the apparent differences in the binding grooves of $H\text{-}2^d$ and $H\text{-}2^k$ MHC molecules (21), it is unlikely that the K^d-binding peptide (LLOp91–99) serves as an Ag in mice of the $H\text{-}2^k$ haplotype. In fact, we have been unable to demonstrate presentation of this peptide by macrophages from C3HeB/FeJ mice (our unpublished observation), and no responsiveness to LLOp91–99 was observed upon restimulation of T cells (in vitro) obtained from the peptide + IL-12-immunized mice (data not shown).

To determine whether immunization with a single peptide (the MHC class II-restricted LLOp203–226) and IL-12 would elicit protective antilisterial immunity, C3HeB/FeJ mice (5/group) were immunized with 50 μg of LLOp203–226 in PBS or in combination with 0.5 μg of rIL-12 on days 0, 5, and 24. To address the issue of whether IL-12 was required in the third immunization, additional groups of five mice were given identical 1° and 2° doses of peptide + IL-12, but received only 0.2 μg of or no IL-12 along with LLOp203–226 in the 3° immunizing dose. As a benchmark for acquired listerial immunity, one group of five mice was injected with a nonlethal dose of viable *Listeria* on day 0 and boosted with a similar dose on day 24. Approximately 1 mo later (day 51), the mice were challenged with viable *Listeria* (approximately $8 \times LD_{50}$). Four days later, the CFU of *Listeria* per spleen were enumerated (Fig. 3). As expected, the *Listeria*-infected/immune mice were well protected (2.87 log reduction in CFU as compared with unprotected mice injected with PBS). Mice that received LLOp203–226 + IL-12 in each immunization dose (3° doses contained LLOp203–226 and either 0.5 μg or 0.2 μg of IL-12) were also protected against challenge with viable *Listeria* (2.14 and 2.28 log reduction in CFU, respectively). Interestingly, mice that received a 3° dose of LLOp203–226 with no IL-12 did not produce protective antilisterial responses (0.12 log reduction in CFU). When administered in PBS (without IL-12) in each immunization dose, the peptide immunogen failed to elicit protective immunity (Fig. 3). The presence of LLOp203–226-specific T cells (as indicated by IL-2 and IFN-γ production by peritoneal lymphocytes in vitro; Fig. 4) and the ability of macrophages to become activated with regard to IL-12 production (data not shown) correlated well with the protective immunity observed in these groups.

This is the first demonstration that immunity to an intracellular bacterial pathogen can be achieved by immunizing with a synthetic peptide. In fact, this is the first report that immunization-derived (rather than adoptive) T cell responses against any single immunodominant protein segment of *Listeria* are capable of conferring host protection. Because of the class II MHC-binding ability of the active peptide, it is also apparent that $CD4^+$ T cell responses can be sufficient for immunity to *Listeria* infection. It is also important to note that listerial immunity could not be achieved by immunization with peptide in

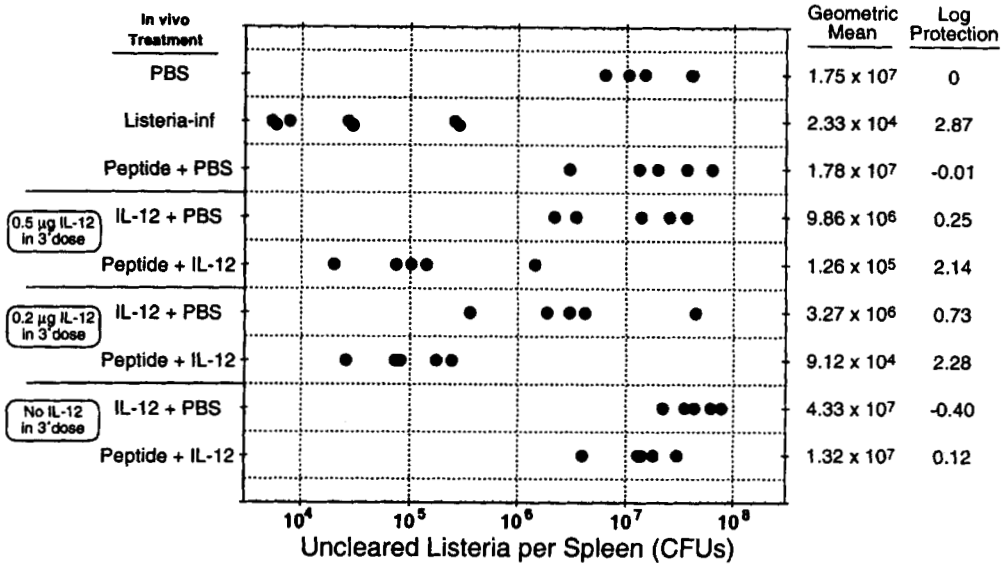

FIGURE 3. Mice immunized with a synthetic peptide corresponding to a class II-restricted determinant of LLO (LLOp203–226) + IL-12 are protected against challenge with *Listeria*. Inclusion of IL-12 in each injection is required to generate protective immunity. C3HeB/FeJ mice (5/group) were immunized on days 0, 5, and 24 with either PBS, 0.5 μg of rIL-12 in PBS, 50 μg of LLOp203–226 + PBS, or along with 0.5 μg of rIL-12 as indicated. Additional groups of mice received identical 1° and 2° doses of IL-12 + PBS or LLOp203–226 + IL-12, but received either 0.2 μg of IL-12, or no IL-12 along with PBS or LLOp203–226, respectively, in the 3° dose (day 24). Another group of five mice was inoculated i.p. with two sublethal doses of viable *L. monocytogenes* (1.7×10^4/mouse on day 0 and 9.5×10^3/mouse on day 24). On day 51, each mouse received i.p. a challenge dose of *Listeria* (4×10^5/mouse or $8 \times LD_{50}$). Mice were killed 4 days later (day 55), and bacterial load in each spleen was quantitated.

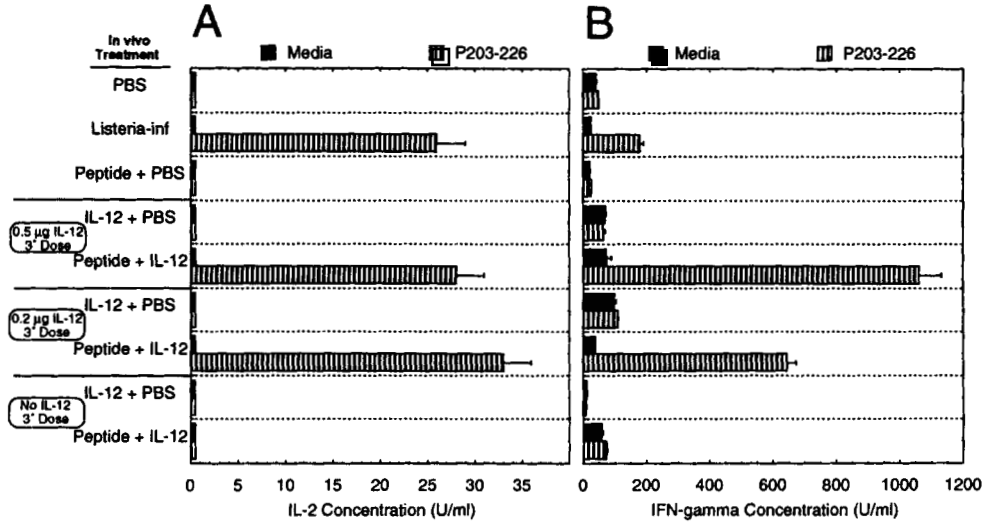

FIGURE 4. Listerial Ag-specific T cell responses from LLOp203–226 + IL-12-immunized mice correlated with generation of protective immunity. PNA isolated from mice in Figure 4 were restimulated in vitro (24 h at 37°C) with either culture medium (no restimulation) or LLOp203–226 (1 μg/ml). Production of (*A*) IL-2 or (*B*) IFN-γ was quantitated as a measure of Ag-specific T cell responsiveness. All assays were performed in triplicate, and results are expressed as mean ± SD.

CFA followed by multiple doses of peptide in IFA (our unpublished data). In documented studies with other infection models, protective immunity has been elicited with complex peptide immunogens (multiple Ag peptide (MAP) or peptide conjugated to carrier protein); however, these responses have typically required the use of highly inflammatory/toxic adjuvants (e.g., CFA) (22–26). The ability of a single cytokine (IL-12) to effect responsiveness to a synthetic peptide immunogen in the absence of conventional adjuvants may add a new dimension to the design of synthetic peptide-based vaccines for a variety of intracellular pathogens, including bacteria, viruses, and parasites. The robust adjuvanticity of IL-12 also leads to the speculation that IL-12 may be a central cytokine signal required for the T cell-stimulating properties of many adjuvants. The remarkable effectiveness of peptide/IL-12 vaccines in the *Listeria* model demands an evaluation of this strategy with other infection/immunity models. If the effectiveness of polypeptide/IL-12 vaccines proves to be generalizable, then the many advantages of synthetic peptide immunogens such as molecular precision and homogeneity, stability, safety, and cost effectiveness could be exploited.

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