

Development of T_H1 $CD4^+$ T Cells Through IL-12 Produced by *Listeria*-Induced Macrophages

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Development of the appropriate $CD4^+$ T helper (T_H) subset during an immune response is important for disease resolution. With the use of naïve, ovalbumin-specific $\alpha\beta$ T cell receptor transgenic T cells, it was found that heat-killed *Listeria monocytogenes* induced T_H1 development in vitro through macrophage production of interleukin-12 (IL-12). Moreover, inhibition of macrophage production of IL-12 may explain the ability of IL-10 to suppress T_H1 development. Murine immune responses to *L. monocytogenes* in vivo are of the appropriate T_H1 phenotype. Therefore, this regulatory pathway may have evolved to enable innate immune cells, through interactions with microbial pathogens, to direct development of specific immunity toward the appropriate T_H phenotype.

Cytokine production by subsets of $CD4^+$ T cells regulates the effector responses to infectious organisms (1). The T_H1 subset produces IL-2, interferon- γ (IFN- γ), and lymphotoxin, facilitating cell-mediated immunity appropriate for intracellular and viral pathogens. The T_H2 subset produces IL-4, IL-5, and IL-6, which favors humoral immunity. Development of the appropriate T_H subset during an immune response is important because certain pathogens are most effectively controlled by either a predominantly T_H1 - or T_H2 -type immune response (2, 3). Because the host interacts with pathogens before the development of specific immunity, the directed development of the T_H phenotype may involve innate immune cells such as macrophages, natural killer (NK) cells, or mast cells (4). Thus, we examined whether discrete mechanisms regulate T_H phenotype development by host interactions with pathogens.

We employed an in vitro system using $\alpha\beta$ T cell receptor (TCR) transgenic mice in which the majority of T cells are $CD4^+$, ovalbumin (OVA)-specific, I-A^d-restricted, and phenotypically naïve (5, 6). Thus, antigen-dependent naïve T cell activation and short-term differentiation can be studied in vitro without the use of polyclonal activators or long-term culture techniques (6). This system allows for experimental control over the antigen-presenting cell (APC), cytokine environment, and exposure to pathogens during primary T cell activation. Previously, it was found that both IL-4 (6–8) and IL-10 (6) inhibited T_H1 development in this system. IL-4 acted directly on T cells to promote T_H2 devel-

opment, whereas IL-10 acted indirectly by suppressing the capacity of certain accessory cells to promote T_H1 development (6, 9). However, the relation of these and other in vitro, cytokine-mediated effects (10) to the physiologic regulation of T_H phenotype development by host interactions with pathogens was unclear.

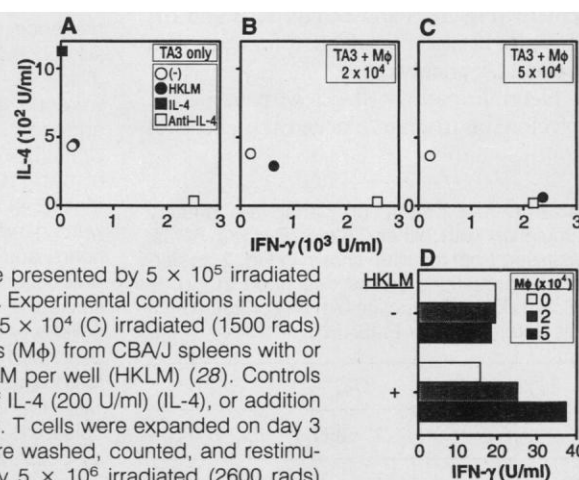
Here, we present an explanation for the ability of a host to direct the T_H phenotype of a developing $CD4^+$ T cell response against a pathogen. *Listeria monocytogenes*, an intracellular Gram-positive bacterium that induces strong cellular immunity in vivo (11), also promotes the in vitro development of the T_H1 phenotype in OVA-specific $CD4^+$ transgenic T cells (Fig. 1) (12). In this report, we show that the mechanism of *Listeria*-induced T_H1 development involves an interaction between *Listeria* and host macrophages that results in

IL-12 production. IL-12, then, directly induces T_H1 development in naïve $CD4^+$ T cells undergoing primary activation.

The addition of heat-killed *L. monocytogenes* (HKLM) during primary in vitro activation of OVA-specific T cells resulted in enhanced IFN- γ secretion in primary cultures and rapid development of the T_H1 phenotype (Fig. 1) (12). The effects of *Listeria* on T cells were not direct but were accessory cell-dependent (12, 13). When the B cell hybridoma TA3 (6) was the exclusive APC used for activation of transgenic T cells by OVA, the addition of *Listeria* had no effect either on IFN- γ production during the primary activation or on subsequent T_H phenotype development (Fig. 1, A and D) (12). By using TA3 as the exclusive APC to present antigen, we could test whether other cells added to TA3-stimulated cultures were able to mediate the effects of *Listeria* on the T_H phenotype independently of their ability to present antigen (12, 13). This was necessary because many cell types, including macrophages and resting B cells, are poor activators of naïve T cell proliferation (6, 13, 14), so that their use as APCs results in insufficient T cell recoveries and prevents evaluation of the T_H phenotype.

The addition of macrophages purified by cell sorting from *H-2^k* (a nonpresenting haplotype) spleen to TA3-primed T cell cultures reconstituted the effects of *Listeria* observed in BALB/c (*H-2^d*) spleen-primed T cell cultures (Fig. 1, B to D; Fig. 2, E and F) (12). Full restoration of the effect occurred with 5×10^4 macrophages in which the *Listeria*-induced T_H1 phenotype was as pronounced as that produced by addition of

Fig. 1. Cellular requirements for the effects of *Listeria*. (A through C) Effects of HKLM on T_H phenotype development. T cell cultures were maintained as described (12). Briefly, $CD4^+$ T cells (2.5×10^5 from unimmunized transgenic mice) sorted by fluorescence-activated cell sorting (FACS) were activated under the indicated conditions with $0.3 \mu M$ OVA-peptide presented by 5×10^5 irradiated (10,000 rads) TA3 B hybridoma cells. Experimental conditions included the addition of 0 (A), 2×10^4 (B), or 5×10^4 (C) irradiated (1500 rads) bystander FACS-sorted Mac-1⁺ cells (M Φ) from CBA/J spleens with or without the addition of 2×10^7 HKLM per well (HKLM) (28). Controls included no addition (–), addition of IL-4 (200 U/ml) (IL-4), or addition of 11B11 mAb (10 μg /ml) (anti-IL-4). T cells were expanded on day 3 and harvested on day 7. T cells were washed, counted, and restimulated at 2.5×10^5 cells per well by 5×10^6 irradiated (2600 rads) BALB/c splenocytes with $0.3 \mu M$ OVA-peptide in the absence of experimental conditions to assess the effects of the indicated condition on T_H phenotype development. Supernatants were harvested after 48 hours and assayed for IL-4 and IFN- γ by enzyme-linked immunosorbent assay (ELISA) (6). (D) Effects of HKLM on IFN- γ production from naïve T cells. Supernatants were harvested on day 2 of the primary stimulation and analyzed for IFN- γ production by ELISA (6). The experiment was done three times with similar results. The reagents used and the method of isolation of T cells and macrophages have been described (12). Maximal effects in this system were consistently achieved with 5×10^4 macrophages.



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the monoclonal antibody (mAb) 11B11 (anti-IL-4) (Fig. 1C) (6, 8). Importantly, this restoration requires macrophage activation because addition of macrophages without *Listeria* had no effect either on IFN- γ production in primary cultures or on T_H1 phenotype development (Fig. 1). Other cell types, such as B cells, NK cells, and dendritic cells, are poor mediators of the effects of *Listeria* on T_H1 phenotype development (13). Thus, the macrophage is a critical cell for mediating the induction by *Listeria* of the T_H1 phenotype.

Macrophages could regulate the T_H1 phenotype either through direct cell-cell interactions or through soluble mediators. The ability of H-2^k haplotype macrophages to mediate the induction by *Listeria* of T_H1 development in OVA-specific, I-A^d-restricted T cells indicates that the effects of *Listeria* do not require direct major histocompatibility complex-TCR interactions. Additionally, *Listeria*-treated macrophages can promote T_H1 development through a semipermeable membrane, which suggests that a soluble factor mediates the effects of *Listeria* (12).

Interleukin-1, IL-6, tumor necrosis factor (TNF)- α , and transforming growth factor- β did not reproduce the induction by *Listeria* of the T_H1 phenotype (12, 15). The addition of IL-12 (16–18), however, skewed development of naïve CD4⁺ transgenic T cells activated by either TA3 cells or BALB/c splenocytes to the T_H1 phenotype (Fig. 2, B and D) and augmented primary IFN- γ production (18) (Fig. 2, A and C). The effects of IL-12 were comparable to the effects of *Listeria* when splenic APCs were used (Fig. 2, C and D). However, when TA3 was the exclusive APC used, only IL-12 could induce T_H1 development (Fig. 1, A and D; Fig. 2, A and B). Thus, IL-12 alone is sufficient for induction of the T_H1 phenotype.

Neutralization of IL-12 with a specific polyclonal antiserum blocked the effects of

Listeria on both IFN- γ production during the primary stimulation (Fig. 2E) and T_H1 phenotype development in the presence of splenic APCs (Fig. 2F). In contrast, neutralization of several other macrophage-derived cytokines had no effect on *Listeria*-induced T_H1 development (12, 19). Murine peritoneal macrophages (20) and human monocytes (16) can produce IL-12 in response to bacterial products, including *Listeria* (20). Thus, *Listeria*-induced T_H1 development appears to be the result of the production of IL-12 by macrophages.

Interleukin-10 inhibits T_H1 cytokine synthesis (9, 21) and T_H1 development in vitro (6, 12) and may suppress T_H1 responses in vivo (22). Interestingly, addition of IL-10 inhibited in vitro T_H1 development induced by *Listeria* (Fig. 2, E and F) (12) but not by IL-12 (Fig. 3). Because the effects of IL-10 are APC-dependent (6, 9, 23), these data suggest that IL-10 inhibits *Listeria*-induced IFN- γ production (9, 21) and T_H1 phenotype development (12) by blocking IL-12 production from macrophages.

Interleukin-12 increased primary IFN- γ

production during naïve T cell activation (Table 1). However, IL-12 did not enhance IL-2 production (Table 1), which suggests that the effects of IFN- γ production did not result simply from enhanced T cell activation. Also, both the addition of IL-10 and the neutralization on IL-12 inhibited the induction by *Listeria* of IFN- γ production, but neither condition altered IL-2 production (24). Thus, IL-2 and IFN- γ expression in CD4⁺ T cells involve separate activation pathways (23). Because IL-12 selectively enhances expression of IFN- γ during T cell activation, IL-12 might be considered an “IFN- γ costimulator.”

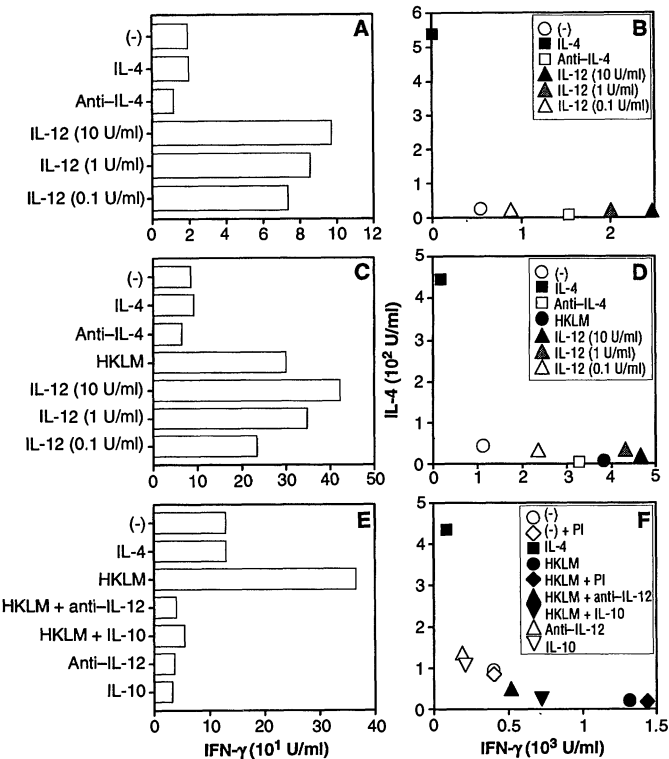
Moreover, because of this enhancement of IFN- γ production during primary T cell activation, IFN- γ may be the final mediator of T_H1 development (16, 25). Although we have found the presence of IFN- γ to be necessary for *Listeria*-induced T_H1 development, the addition of IFN- γ to primary T cell cultures was not sufficient for T_H1 development (12). Also, IL-12 could act by simply inhibiting endogenous IL-4 production (6, 8). Although neutralization of IL-4

Table 1. IL-2 production during the primary stimulation with either TA3 or BALB/c APCs, assessed from the data shown in Fig. 2. Experimental conditions are as described in Fig. 2. ND, not done. Production of IFN- γ or IL-2 (U/ml) was determined by ELISA (6).

Conditions	TA3		BALB/c	
	IL-2	IFN- γ	IL-2	IFN- γ
(–)	283	20	429	85
Anti-IL-4	252	12	353	65
IL-4	252	20	377	92
HKLM	ND		242	301
IL-12 (10 U/ml)	261	97	303	423
IL-12 (1 U/ml)	260	86	297	348
IL-12 (0.1 U/ml)	289	73	327	234
IL-12 (10 U/ml)*	ND		<1	5

*No antigen added.

Fig. 2. The effects of IL-12 on naïve T cell responses and phenotype development. (A and B) Effects of IL-12 when only TA3 B hybridoma cells are the initiating APC. Nylon wool-purified T cells (2.5×10^5) (12) from unimmunized transgenic mice were stimulated with 5×10^5 irradiated (10,000 rads) TA3 B hybridoma cells and 0.3 μ M OVA-peptide in the presence of the indicated concentrations of purified IL-12. Controls were no additions (–), addition of IL-4 (200 U/ml) (IL-4), and addition of mAb 11B11 (10 μ g/ml) (anti-IL-4). Supernatants were harvested after 48 hours, and primary IFN- γ production (A) was assessed by ELISA (6). T cells were cultured as described in Fig. 1 and restimulated on day 7 with irradiated BALB/c splenocytes without the addition of experimental conditions. The T_H1 phenotype (B) was determined by ELISA analysis of the amounts of IL-4 and IFN- γ in the 48-hour supernatants (6). (C and D) Effects of IL-12 when BALB/c splenocytes are the initiating APC. T cells were cultured as above, except 5×10^6 irradiated (2600 rads) BALB/c splenocytes were used as the initiating APCs. The addition of 2×10^7 HKLM (HKLM) was used as a control for induction of the T_H1 phenotype. (E and F) Effects of anti-IL-12 on *Listeria*-induced T_H1 responses. T cells were cultured with BALB/c splenocytes as APCs as described in (C) and (D). Experimental conditions included the addition of rabbit anti-IL-12 serum at 1:1250 dilution (anti-IL-12) or IL-10 (50 U/ml) (IL-10) with or without addition of 2×10^7 HKLM (HKLM). Controls included no additions (–), addition of IL-4 (200 U/ml) (IL-4), or addition of preimmune serum at 1:1250 dilution (PI). These experiments were repeated at least three times with similar results.



did not enhance IFN- γ production during primary T cell activation, the subsequent effects of IL-12 on T_H1 phenotype development could involve reduction of the amount of IL-4. Therefore, IL-12 appears to act directly on T cells to promote T_H1 development, but its mechanism may still involve IL-4 (26).

In vivo T cell responses, as well as individual T cell clones, generally exhibit either a T_H1- or T_H2-like phenotype (1–3). This could indicate either that the natural physiologic setting during T cell activation in vivo rarely involves concurrent IL-4 and IL-12 production or that T cells are inherently unable to simultaneously produce large amounts of IL-4 and IFN- γ . T cells activated in the presence of both IL-4 and IL-12 did not acquire the capacity to secrete high concentrations of both IL-4 and IFN- γ

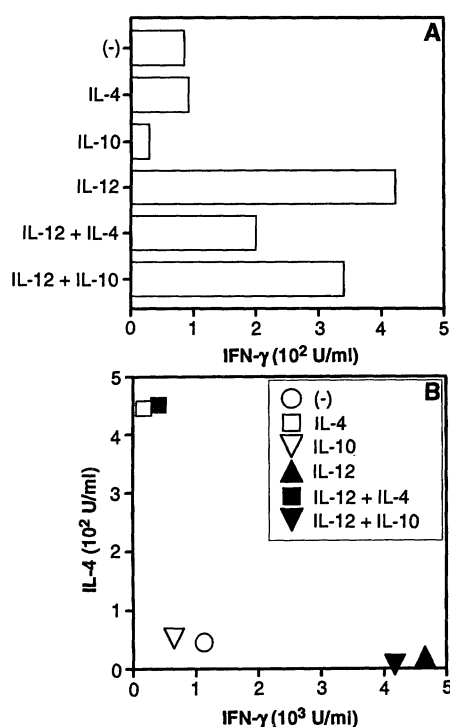


Fig. 3. Effects of IL-4 and IL-10 on IL-12-induced T_H1 development. **(A)** Effects on IFN- γ production from naïve T cells. Nylon wool-purified T cells were stimulated with OVA and irradiated BALB/c splenocytes as described in Fig. 2, C and D. Experimental conditions included additions of IL-4 (200 U/ml) (IL-4) or IL-10 (50 U/ml) (IL-10) in the absence or presence of IL-12 (10 U/ml) (IL-12) as indicated; (–) is as in Fig. 2. **(B)** Effects on T_H phenotype development. Cytokine profiles of developing transgenic T cells were assessed as described in Fig. 1. This experiment was repeated three times with similar results.

(Fig. 3B). Rather, the IL-4 effects were dominant over those of IL-12, which inhibited the induction by IL-12 of IFN- γ (Fig. 3A) and T_H1 development (Fig. 3B). Thus, the phenomena of distinct cytokine profiles for CD4⁺ T cells may result from an intrinsic, mutually exclusive regulation of T_H1 and T_H2 cytokine genes.

On the basis of these and other data (12, 13), we propose the following framework for naïve T cell activation and T_H phenotype differentiation. Macrophages are important mediators of *Listeria*-induced T_H1 development but are ineffective APCs for the stimulation of naïve T cell proliferation (6, 13, 14). Thus, other APCs, such as dendritic cells, are likely to be responsible for activating naïve T cells for proliferation and clonal expansion (13, 14, 27). Because dendritic cells are poor mediators of the effects of *Listeria* on T_H1 phenotype development (13), there appears to be a physiologic segregation of the ability to prime naïve T cells and the ability to skew T_H phenotype development (13). Future analysis of T_H phenotype development must, therefore, account for these distinct aspects of peripheral CD4⁺ T cell development.

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15. We have not yet addressed the potential role of other factors, such as IFN- α , steroids, or prostaglandins [F. D. Finkelman *et al.*, *ibid.* **174**, 1179 (1991); R. A. Daynes, B. A. Araneo, T. A. Dowell, K. Huang, D. Dudley, *ibid.* **171**, 979 (1990); T.-K. Li and B. S. Fox, *J. Immunol.* **150**, 1680 (1993)]. Although these factors may be involved in *Listeria*-induced T_H1 development, their contribution is likely to be small (Fig. 2F). However, they may be involved in T_H phenotype regulation of other host-pathogen interactions.
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19. Neutralization of TNF, which has been reported to block the effects of IL-12 on NK cell lysis [B. Naume, M. Gately, T. Espevik, *J. Immunol.* **148**, 2429 (1992)] and IFN- γ production (20), does not block the IL-12-dependent effects of *Listeria* (12). T cells and NK cells are distinct lineages and have different activation requirements. Thus, TNF may not be necessary for IL-12 effects on naïve T cells that undergo antigen activation but may be required for IL-12 stimulation of NK cells.
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26. Addition of IL-12 or anti-IL-12 with anti-IL-4 markedly enhances or inhibits, respectively, IFN- γ production during the secondary stimulation (24). IL-4 production, however, is unaffected by these conditions. These data suggest that the amount of IL-4 during the primary stimulation is critical for subsequent IL-4 expression by T cells and that IL-12 independently regulates the amount of IFN- γ produced. Previously, we have found that the T_H1 and T_H2 phenotypes produced by anti-IL-4 and IL-4 treatment are stable in vitro (24). However, we have not yet addressed the in vitro stability of T cell lines derived exclusively in the presence of IL-12. Because the events that regulate the T_H phenotype occur quite early (by day 3 during *Leishmania major* infection [P. Scott, *J. Immunol.* **147**, 3149 (1991)]), our principal interest was in the short-term effects of *Listeria* and IL-12.
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