Influence of Dendritic Cells on the in Vitro Allogeneic Cytotoxic Reaction of Lymphoid Cells Derived from Normal or *Listeria innocua*-Infected BALB/c Mice

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**Abstract.** The role of lymphoid dendritic cells (DCs) in the development of an allogeneic cytotoxic reaction *in vitro* was examined. The T+B and T cell subsets originating from the spleens or lymph nodes of normal and *Listeria innocua*-infected BALB/c mice were used as the effector cells. Their cytototoxicity to 51Cr-labeled C3H fibroblasts was determined after removal of DCs and replacing them again. Moreover, the influence of exogenous mIL-12 on the potency of DCs in the allogeneic reaction developed *in vitro* was checked. It was found that the DC-deprived T+B or T subsets of splenocytes, regardless of their origin, exhibited 27–38% lower cytototoxicity than those accompanied by natural DCs. The cytototoxicity of these subsets from normal lymph nodes decreased by 22%, while the activity of bacteria-primed cells dropped by 38%. Replenishing effector cells with isolated DCs restored their cytototoxicity. Pulsation of normal DCs with IL-12 had no effect on the recovery of normal cell cytotoxicity. However, the IL-12-pulsed DCs were able to intensify the cytototoxicity of T+B subsets derived from the spleens or lymph nodes of *L. innocua*-infected mice. The results suggest that the alloantigen presentation by DCs to cytotoxic lymphocytes also takes place in the reaction developed *in vitro*, regardless of effector cell origin.

**Key words:** dendritic cells; cytototoxicity; allogeneic reaction; *Listeria innocua*.

**Introduction**

Dendritic cells (DCs) are professional antigen-presenting cells, very efficient in the activation of naïve and resting T cells and in the restimulation of memory T cells. In addition, they can tolerate T cells to self antigens in the thymus; however, their tolerogenic activity can be modulated by CD40 ligation.

DCs present antigen to Th lymphocytes and regulate the function of effector cytotoxic T lymphocytes (CTL) as well as activate B lymphocytes to proliferate and mature into plasma cells. They are a phenotypic and functional heterogeneous population. In the mouse, lymphoid and myeloid lineages of them are distinguished. The lymphoid CD8+ DCs in mouse spleen and lymph nodes are characterized by the surface expression of the CD8α marker and a high level of surface MHC II antigen. They are suggested to direct the differentiation of Th1 cells and produce IFN-γ and IL-12, while CD8α- DCs, of myeloid origin, promote the Th2 type response.

Immature DCs stimulated with CD40L or bacteria

**Abbreviations used:** DCs – dendritic cells, CTL – cytotoxic T lymphocytes, mIL-12 – mouse recombinant interleukin-12, DTH – delayed T hypersensitivity, MLR – mixed leukocyte response.

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are able to secrete more IL-12 than mature cells. They express receptors for β chemokines and can be attracted to the site of inflammation. Their maturation process is stimulated by bacterial or viral products or by proinflammatory cytokines (TNF-α, IL-1β, IFNs). However, it is most effectively induced by their interaction with T lymphocytes, mediated by adhesion and co-stimulatory molecules.

The ability of DCs to activate the cytotoxic T lymphocytes prompted us to examine their role in the cytotoxic reaction developed in vitro to alloantigens by cell subsets of spleen and lymph node cells derived from normal BALB/c mice or from the mice injected with live Listeria innocua. In the latter case, the effect of stimulation in vivo of DC activity as well as the cytotoxicity of effector cells on the in vitro reaction was checked. Moreover, the influence of exogenous mrIL-12 on DC potency was determined.

### Materials and Methods

**Animals.** 10 to 12-week old BALB/c (haplotype H-2d) mice of both sexes, purchased from Laboratory Experimental Animals (Velaz), bred in the conventional conditions, were used for the experiments. Each experimental group consisted of 6–10 mice.

**Bacteria.** L. innocua, an unpathogenic strain from Welshimer’s collection, serovar 6a, was grown on triplicate agar enriched in vitamin B1 (5 μg/ml) at 37°C for 18–24 h.

**Infection of mice.** Effector cell donors were injected in 2 foot pads and in 4 points (2 near the head and 2 near the tail) of the back skin with 2 × 10^7 live bacteria suspended in 0.2 ml of saline 7 days before the experiment.

**Effector cell preparation.** Effector cells for the direct cytotoxicity test were isolated from both normal and L. innocua-infected BALB/c mice. The following cell suspensions were used: a) full splenocytes or lymph node cells; b) T+B cells, the splenocyte or lymph node cell subsets deprived of adherent cells by incubating on plastic plates (Nunc) at 37°C in a humidified atmosphere of 5% CO2 for 90 min; c) T+B cell subsets deprived of DCs by spinning in 14.5% metrizamide gradient (Sigma) at 600 × g for 10 min at room temperature. The method of DC isolation is described in14; d) DC-deprived T+B cells again replenished with normal DCs or DCs pulsed with mrIL-12 (5 ng/10^6 cells) (a gift from Dr. M. Gately, Hoffmann-La Roche Inc.) for 60 min at 37°C in a humidified atmosphere of 5% CO2; e) T cell subsets, the suspensions deprived of B cells by adsorption to nylon wool fibers (Polysciences); f) T cells deprived of DCs; g) DC-deprived T cells again replenished with normal DCs or IL-12-pulsed DCs (as above).

**Target cell preparation.** The fibroblasts were isolated from the cartilage of 16 to 18-day old fetuses of C3H normal mice. They were cultured in RPMI 1640 medium enriched in 10% FCS, glutamine (200 μmol/ml) and the antibiotics penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin (2.5 μg/ml) at 37°C in a humidified atmosphere of 5% CO2 for 7 days. 5 × 10^6 cells suspended in 1 ml of the medium (enriched only in 5% FCS) containing 200 μCl 51-sodium chromate ([51Cr], Polatom) were incubated for 6 h in conditions as above.

**Direct cytotoxicity test.** 2 × 10^6/100 μl effector cells were incubated with 2 × 10^6/100 μl [51Cr]-labeled fibroblasts (a cell ratio of 100:1) in flat-bottom microwell plates (Nunclon) for 24 h in conditions as above. Effector cell cytotoxicity was expressed as the specific [51Cr] release from fibroblasts, which was calculated:

\[
\text{radioactivity (cpm)} = \frac{\text{radioactivity (cpm)} + \text{radioactivity (cpm)}}{\text{radioactivity (cpm)} + \text{radioactivity (cpm)}} \times 100.
\]

The radioactivity was measured for 60 s in Mini-Gamma Counter 1275 (LKB-Wallac).

**Statistical analysis.** Values are expressed as mean ± standard deviation (SD). Statistical significance of differences was determined with the Student’s t-test for independent samples.

### Results

In order to check the role of dendritic cells in the allogeneic cytotoxic reaction developed in vitro by BALB/c spleen or lymph node cells to C3H fibroblasts, the cell subsets derived from normal or L. innocua-infected animals were deprived of DCs. Next, their cytotoxicity was compared with the activity of the cell subsets naturally containing DCs or replenished with DCs after their removal.

The number of DCs isolated from adherent cell-deprived spleen or lymph node cells ranged, regardless of their origin, from 0.97 to 1.58%. Then, in the experiments an intermediate value of 1.3% of DCs was used.
In the experiments cells are isolated from mice infected with DCs. Pulsation of normal DCs restored their cytotoxic activity. However, in the case of the cells derived from normal D. L. innocua-infected mice, the DC completion increased their cytotoxicity by 45% (p<0.05). When DCs were pulsed with IL-12, the cytotoxic activity of the effector cells increased by 60% (p<0.001).

An effect of the presence of DCs on the cytotoxic activity in vitro of tested cells, isolated from normal or bacteria-infected mice, is well seen in the calculated index values when the cytotoxicity level of effector cells naturally accompanied by DCs is expressed as a value of 1 (Fig. 1).

Comparison of the cytotoxicity of the T cell subsets isolated from lymph nodes of normal or L. innocua-infected mice showed that they were pivotal cells in the in vitro allogeneic reaction. The cells derived from L. innocua-infected animals, i.e. when they were stimulated with bacterial antigens in vivo, exhibited in a few cases a higher cytotoxicity than normal cells. However, the contribution of DCs in developing their cytotoxic reaction was on a level (35–38%) similar to that of the reaction given by normal cells (22–34%). The only exception was the reaction of T+B subsets in the absence of DCs. It suggested that the presentation of target cell MHC I antigen to cytotoxic lymphocytes (CTL) by DCs also takes place in the reaction developed in vitro, regardless of the reacting cells origin.

### Table 1. Cytotoxicity of splenocytes and their subsets of the normal or L. innocua-infected BALB/c mice to allogeneic fibroblasts in the absence of DCs or presence of IL-12-pulsed DCs

<table>
<thead>
<tr>
<th>Effector cells</th>
<th>³¹Cr specific release (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>normal (mean±SD)</td>
</tr>
<tr>
<td>Splenocytes</td>
<td>10.1 ± 2.51</td>
</tr>
<tr>
<td>T+B cells</td>
<td>10.3 ± 2.16</td>
</tr>
<tr>
<td>T+B cells (+DCs)</td>
<td>7.5 ± 1.56</td>
</tr>
<tr>
<td>T+B cells (+IL-12-pulsed DCs)</td>
<td>10.1 ± 2.14</td>
</tr>
<tr>
<td>T cells</td>
<td>9.5 ± 1.93</td>
</tr>
<tr>
<td>T cells (+DCs)</td>
<td>6.3 ± 0.50</td>
</tr>
<tr>
<td>T cells (+IL-12-pulsed DCs)</td>
<td>7.7 ± 1.26*</td>
</tr>
</tbody>
</table>

*DCs isolated from normal or **DCs from L. innocua-infected mice were added.

### Table 2. Cytotoxicity of lymph node cells and their subsets of the normal or L. innocua-infected BALB/c mice to allogeneic fibroblasts in the absence of DCs or presence of IL-12-pulsed DCs

<table>
<thead>
<tr>
<th>Effector cells</th>
<th>³¹Cr specific release (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>normal (mean±SD)</td>
</tr>
<tr>
<td>Lymph node cells</td>
<td>9.7 ± 1.93</td>
</tr>
<tr>
<td>T+B cells</td>
<td>8.8 ± 0.09</td>
</tr>
<tr>
<td>T+B cells (+DCs)</td>
<td>7.6 ± 1.60</td>
</tr>
<tr>
<td>T+B cells (+IL-12-pulsed DCs)</td>
<td>9.4 ± 1.22*</td>
</tr>
<tr>
<td>T+B cells (+IL-12-pulsed DCs)</td>
<td>10.1 ± 1.61</td>
</tr>
<tr>
<td>T cells</td>
<td>9.5 ± 0.76</td>
</tr>
<tr>
<td>T cells (+DCs)</td>
<td>7.4 ± 1.52</td>
</tr>
<tr>
<td>T cells (+IL-12-pulsed DCs)</td>
<td>7.5 ± 1.49*</td>
</tr>
<tr>
<td>T cells (+IL-12-pulsed DCs)</td>
<td>10.1 ± 2.21</td>
</tr>
</tbody>
</table>

*DCs isolated from normal or **DCs from L. innocua-infected mice were added.
Discussion

Recently, two main subsets of DCs are thought to exist: DC1, secreting a large amount of IL-12 and promoting the development of Th1 cells, which play an essential role in the development of immunity to intracellular pathogens, and DC2, stimulating Th2 cells to the production of cytokines important in the immunity to extracellular infections. DCs activated by the interaction with Th1 cells enhance IL-12 secretion and stimulate the generation of cytotoxic T lymphocytes from naïve CD8+ cells. The activation process of DCs can also be induced by live bacteria, e.g. mycobacteria. Moreover, the cells of L. monocytogenes-attenuated mutants, able to persist in the organism for a long time, can activate DCs.

Sethum et al. found that DCs were potent allograft antigen presenters for allograft rejection, while Macatonia et al. noted that DCs were effective in the stimulation of CD4+ or CD8+ T lymphocytes to proliferation and IFN-γ secretion in the primary allogeneic mixed leucocyte response (MLR). These findings confirmed the previous observations of Inaba et al. that DCs were essential for the activation of the CD4+ and CD8+ cell subsets in the primary MLR and IL-2 secretion, as well as in an efficient clustering of the responding cells of both subsets. Besides, they showed that the DCs were able to induce CD8+ cytotoxic T lymphocytes in the absence of CD4+ T cells.

In our experiments the relevance of DCs to the development in vitro of the cytotoxic reaction given by BALB/c spleen or lymph node T+B and T cell subsets to C3H fibroblasts was examined. Because the procedure of isolation of DCs required the removal of adherent cells, we could use only the T+B or T cell subsets for the experiments. The intensity of the cytotoxic reaction in the presence or absence of DCs was evaluated as the percentage of specific release of 51Cr from labeled allogeneic fibroblasts. In order to activate the DCs as well as to intensify the cytotoxicity of the effector cells, we infected cell donors with live, unpathogenic bacteria of L. innocua. In our previous works we found that L. innocua is able to increase the natural cytotoxicity of mouse lymphoid cells and their production of IL-2 or IL-12. As activated DCs can produce IL-12 we tried to check the effect of the exogenous cytokine on their potency in the stimulation of the allogeneic cytotoxic reaction. To achieve this, IL-12-pulsed DCs replaced natural DCs. Nagayama et al. suggest that IL-12 can act directly on DCs to induce their functional activation via 12Rβ1-mediated signaling events.

The results obtained showed that the spleen cell subsets derived both from normal and bacteria-injected animals developed from 27 to 38% a weaker reaction after removal of DCs. The T+B cell subset completed with isolated DCs recovered its cytotoxic activity. The reaction of normal cells returned to the normal level and was not changed even after the addition of IL-12-pulsed DCs. On the contrary, the cells from L. innocua-infected mice after addition of DCs or IL-12-pulsed DCs reacted to allogeneic target cells 23–33% more intensively. The T cell subset (the cell suspension depleted of B cells) isolated from the spleens of both animal groups exhibited a similar activity when it was freed from DCs and when it was completed again with DCs or IL-12-pulsed DCs. The marked enhancement of the cytotoxic reaction of the lymph node T+B cell sub-

![Graph A](image1.png)

**Fig. 1.** The cytotoxicity of DC-deprived and DC-completed effector cells derived from normal (A) or L. innocua-infected mice (B) compared with the cytotoxicity of naturally DC-containing cell subsets, assigned the value of 1.
set derived from L. innocua-injected mice to alloantigen caused by its replenishment with IL-12-pulsed DCs could be explained by the ability of IL-12 to increase the generation of an allospecific CTL response in mice\(^3\). The differences in cytotoxic potency of spleen and lymph node effector cells derived from normal and, particularly, from L. innocua-infected mice can result from the difference between lymph nodes and spleen cells in the production of lymphokine in vitro\(^2\). Serushago et al. \(^2\) noticed that in L. monocytogenes-injected mice the lymph node cells were able to develop a specific delayed T hypersensitivity (DTH), but never induced protection, while spleen cells evoked DTH and were protective. Spleen and lymph node T cells expressed CD4 and CD8 markers equally and DTH response was solely dependent on CD4\(^+\) cells.

The higher cytotoxicity in vitro of the T+B cell subset than the T cells suggested that B cells or B cell-derived cytokines could participate in this reaction.

The results of the paper showed the relevance of DC presence to the development of a cytotoxic reaction in vitro by normal or bacteria-primed spleen or lymph node effector cells to target cell alloantigens. They suggest that the process of target cell MHC I antigen presentation by DCs to CTL also occurs in the reaction in vitro.

References

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