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**References**

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Cutting Edge: Granulocyte-Macrophage Colony-Stimulating Factor Is the Major CD8+ T Cell-Derived Licensing Factor for Dendritic Cell Activation

Lin Min, Siti Aminah Bte Mohammad Isa, Wang Shuai, Cher Boon Piang, Fam Wee Nih, Masayo Kotaka, and Christiane Ruedl

During priming, CD8+ T lymphocytes can induce robust maturation of dendritic cells (DCs) in a CD40-independent manner by secreting licensing factor(s). In this study, we isolate this so-far elusive licensing factor and identify it, surprisingly, as GM-CSF. This provides a new face for an old factor with a well-known supporting role in DC development and recruitment. Signaling through the GM-CSFR in ex vivo-purified DCs upregulated the expression of costimulatory molecules more efficiently than did any tested TLR agonist and provided a positive feedback loop in the stimulation of CD8+ T cell proliferation. Combined with a variety of microbial stimuli, GM-CSF supports the formation of potent “effector” DCs capable of secreting a variety of proinflammatory cytokines that guide the differentiation of T cells during the immune response. The Journal of Immunology, 2010, 184: 4625–4629.

Dendritic cells (DCs) are the key regulators of immune responses linking innate and adaptive components of the immune system. As sentinels of the mammalian immune system, they are among the first to detect, capture, and process invading infectious agents for the scrutiny of T cells (1, 2). Hence, the optimal “collaboration” of DCs and T cells is absolutely crucial for successful immunity.

The hallmark of DC life is a complex maturation process that involves the upregulation of MHC and costimulatory molecules and the secretion of several proinflammatory cytokines. This phenotypic shift dramatically increases the immunostimulatory potential of DCs, rendering them the most potent APCs of the immune system (3). Therefore, mature DCs can be considered “super-vaccines” that are being developed for this purpose to fight against infections and tumors.

Materials and Methods

Mice
C57BL/6, B6.129S1-Csf2rbtm1Cgb/J (GM-CSFR2/2), and C57BL/6-Tg(TcraTcrb)1100Mjb/J (OT-1) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All animal experiments were carried out within institutional guidelines.

DCs and T cell preparations
DCs were prepared from spleens, as described previously (5). FLT3 ligand (FLT3L)-derived bone marrow (BM) DCs were generated by incubating BM cells for 8 d with 100 ng/ml FLT3L. DC and T cell subsets were isolated from naive mice and purified by cell sorting (FACSviva, BD Biosciences, San Jose, CA).

The online version of this article contains supplemental material.

Abbreviations used in this paper: BM, bone marrow; DC, dendritic cell; FLT3L, FLT3 ligand; MFI, mean fluorescence intensity; PAMP, pathogen-associated molecular pattern; poly(I:C), polyinosinic-polycytidylic acid; TCF, T cell factor; WT, wild-type.

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Measurement of T cell and DC activation

T cells were stimulated on α-CD3/CD28-coated plates or with 10^{-5} M SIINFEKL OVA-pulsed DCs. FLT3L-derived BM DCs were stimulated with T cell factor (TCF)-containing supernatant, α-GM-CSF (Biologend, San Diego, CA), LPS, polyinosinic-polycytidylic acid (poly(I:C)), zymosan (all from Sigma-Aldrich, St. Louis, MO), and CpG (ODN 1668, TLRgrade, Alexis [Enzo Life Sciences, Farmingdale, NY]) at concentrations indicated in the respective figure legends. All were tested at a range of concentrations in initial experiments and subsequently were used at concentrations that caused maximal DC phenotypic differentiation and cytokine release, without increased cell death.

Every reagent was resuspended in endotoxin-free water (Sigma-Aldrich), and endotoxin-free OVA was purchased from Hyglos (Regensburg, Germany). Abs specific for CD80 and CD86 (Biologend) were used to monitor the maturation state of DCs. Mean fluorescence intensity (MFI) was assessed by flow cytometry (FACSCalibur, BD Biosciences).

For the coculture experiment, a 50:50 mixture of FLT3L-derived DCs (Ly5.1 wild-type [WT] and Ly5.1 GM-CSF-/-) was pulsed with 10^{-6} M SIINFEKL peptide, washed, and subsequently incubated with OVA-specific CD8+ T cells. Cells were triple-stained with Ly5.1, CD11b, and CD80 and SIINFEKL peptide, washed, and subsequently incubated with OVA-specific (Ly5.1+ wild-type [WT] and Ly5.1-/-) T cells. Twenty-four hours later, upregulation of CD80 and CD86 was detectable only on WT DCs, whereas the CD80 and CD86 expression was increased cell death.

Results and Discussion

**GM-CSF is the DC licensing factor secreted by CD8+ T cells**

We previously reported that CD8+ T cells stimulated via TCR/peptide-loaded MHC ligation or α-CD3/CD28 cross-linking secrete a potent factor (TCF herein) that is able to induce efficient upregulation of costimulatory molecules (5). In this study, we extended these observations and identified this activity molecularly. To this end, we first generated conditioned medium from a DC/T cell coculture or from α-CD3/CD28–stimulated T cells that was able to induce more robust upregulation of CD86 on FLT3-derived DCs than, for instance, an optimal concentration of CpG (8–11-fold versus 5-fold) (Fig. 1A).

To identify the potential of TCF, serum-free culture supernatant obtained after 16 h of α-CD3/CD28 stimulation of CD8+ T cells was concentrated and fractionated by preparative isoelectrofocusing electrophoresis followed by gel filtration (data not shown). Biological active fractions were analyzed further by mass spectroscopy. This analysis identified GM-CSF as a possible candidate. For validation, we stimulated FLT3L-derived BM DCs with different amounts of TCF-containing culture supernatants in the presence or absence of a neutralizing anti–GM-CSF Ab. The Ab neutralized the biological activity that induced DC maturation in a dose-dependent manner (Fig. 1B). To further corroborate the crucial role of GM-CSF as the key factor released by T cells in DC maturation, we analyzed the effect of GM-CSF blocking Ab in DC–T cell cocultures. OVA-specific CD8+ T cells and OVA-pulsed DCs were cocultured for 24 h in the presence of anti–GM-CSF, anti-CD40L, or a combination of both Abs for 24 h. The maturation of DCs was only upon neutralization of GM-CSF released during T cell activation but not by the blockade of the CD40–CD40L interaction. The combination of both Abs did not further increase the inhibition (Fig. 1C). To further confirm GM-CSF as the licensing factor secreted by CD8+ T cells, a 50:50 mixture of GM-CSF-/- and WT FLT3L-derived DCs, pulsed with OVA, were cocultured with OVA-specific CD8+ T cells. Twenty-four hours later, upregulation of CD80 and CD86 was detectable only on WT DCs, whereas the GM-CSF-/- DCs maintained the original expression pattern (Fig. 1D).

Previous observations suggested that CD40 signaling supports a full CTL priming by DCs (6). However, our results strongly imply that GM-CSF is the licensing factor released by CD8+ T cells during DC–T cell interaction.

**FIGURE 1.** GM-CSF secreted by CD8+ T cells is the licensing factor for DC maturation. A, FLT3L-derived DCs were incubated for 24 h with conditioned medium obtained from α-CD3/CD28–activated CD8+ T cells, as well as from DC/CD8+ T cell cocultures, and, as positive control, from cultures stimulated with 2.5 μM CpG. Upregulation of CD86 was measured by FACS. B, FLT3L-derived BM DCs were incubated with 50 or 25 μl TCF in the absence or presence of 10 or 1 μg/ml blocking α–GM-CSF. Upregulation of CD86 was measured by FACS. C, Anti–GM-CSF, but not α-CD40L, suppresses the activation of DCs. OVA-specific CD8+ T cells were cocultured for 24 h with OVA-pulsed DCs in the absence or presence of 10 μg/ml α–GM-CSF, α–CD40L, and a combination of both. Upregulation of costimulatory molecules was measured by double staining DCs with allophycocyanin-labeled α-CD11c and PE-labeled α-CD86 and α-CD80. D, WT, but not GM-CSF-/-, DCs mature upon 24 h of incubation with CD8+ T cells. A 50:50 coculture of FLT3L-stimulated Ly5.1+ WT and Ly5.1-/- GM-CSF-/- DCs were pulsed with 10^{-6} M SIINFEKL and incubated with OT-1 CD8+ T Cells for 24 h. CD80 and CD86 were measured on Ly5.1+CD11b+ and Ly5.1-/-CD11b- cells by FACS. Surface expression is indicated as MFI. All results are representative of three independent experiments.
interaction and that it can act independently from the CD40–CD40L axis.

**GM-CSF secretion profile of different naive and effector T cell subsets**

It is well known that T cells produce GM-CSF upon stimulation (7). We assessed in detail the capacity of different subsets of T cells to produce GM-CSF, in particular focusing on naive and effector T cells, using α-CD3/CD28 stimulations. Together with GM-CSF, IL-2 secretion was assessed as a positive control for a successful T cell activation. CD4+ and CD8+ effector T cells derived from peripheral lymph nodes produced high levels of GM-CSF as did naive CD8+ T cells. CD4+CD25+ regulatory T cells and γδ T cells and CD4+CD8+ T cells produced barely detectable levels of GM-CSF. Interestingly, specialized innate T cells, NKT cells, and dendritic epidermal T cells produced the most copious amounts of GM-CSF, suggesting that they may have super-regulatory roles in their specialized microenvironments (Fig. 2).

**GM-CSF drives robust DC activation**

We next compared GM-CSF and a panel of classical DC activators for their ability to induce maturation in different DCs. CD11c+CD8+ and CD8− subsets, conventional CD11c+B220+, and plasmacytoid CD11c+B220− DCs derived from BM FLT3L cultures were tested as representative noninflammatory steady-state DC populations (reviewed in Ref. 8). Interestingly, GM-CSF was one of the strongest inducers among TLR-dependent [LPS, CpG, poly(I:C), and zymosan] and independent (anti-CD40) stimuli, when tested on FLT3L-derived CD11c+CD11b+ DCs as well as ex vivo-isolated spleen CD11c+CD8− and CD11c+CD8+ DC populations (Fig. 3A). Only plasmacytoid CD11c+B220+ DCs were unresponsive to GM-CSF because of the lack of GM-CSFR expression (data not shown).

Upregulation of costimulatory molecules on GM-CSF–licensed DCs correlated with an augmented ability to support T cell proliferation. In fact, both conventional spleen DC subsets (CD11c+CD8− and CD11c+CD8+) induced more efficient T cell proliferation upon 24-h stimulation with GM-CSF (Fig. 3B). In contrast, GM-CSFR−/− DCs showed a 20% reduced capacity in T cell activation compared with the WT counterparts (Supplemental Fig. 1A). When GM-CSF priming activity was compared with that of potent TLR agonists, such as CpG, similarly enhanced T cell stimulation was observed (Supplemental Fig. 1B).

**GM-CSF induces the synthesis of proinflammatory cytokines in synergy with TLR agonists**

The ability of mature DCs to stimulate naive T cells is mainly determined by the expression levels of MHC and costimulatory molecules. However, the production of proinflammatory cytokines can influence the outcome of Th cell polarization (e.g., IL-12 p70 for Th1 priming). We measured the effect of GM-CSF on the production of several proinflammatory cytokines, such as IL-12p70, IL-6, and TNF-α, and other DC-related cytokines, such as IL-2 and -10. However, we did not observe any modulation of the secretion of these specific cytokines when GM-CSF was the sole stimulus (Fig. 4A). The only cytokine clearly induced via GM-CSF triggering was MCP-1 (CCL2), a small cytokine belonging to the CC chemokine family and involved in the recruitment of T cells as well as other cells, including DCs and monocytes. Therefore, although GM-CSF strongly enhances DC maturation, which ultimately leads to increased T cell activation, it alone does not trigger the secretion of proinflammatory cytokines, such as IL-12, a strong promoter of Th1 and CTL responses (9).
It has been described that stimulation of DCs via CD40 requires microbial priming to induce an optimal inflammatory response (10). Furthermore, direct recognition of microbial components by DCs is essential for priming an appropriate T cell differentiation (11). Therefore, we tested whether GM-CSF was synergizing with TLR-dependent microbial stimuli in terms of the upregulation of proinflammatory cytokines. Interestingly, GM-CSF seems to have a strong facilitating effect on microbial stimuli-induced cytokine release. In fact, combinations of GM-CSF with different TLR agonists, such as LPS, CpG, and poly(I:C), clearly lead to augmented IL-12p70 secretion. However, the most dramatic synergism was observed between GM-CSF and zymosan, which culminated in a >10-fold increase in IL-12p70 production (Fig. 4B). Similar synergism was observed in TNF-α and IL-6 and -2 secretion (data not shown).

Recently, a coordinated interaction between NK cells (IFN-γ producer), pathogen-associated molecular patterns (PAMPs), and CD4+ T cells (CD40L) was suggested as a combinatorial code for effective IL-12p70 secretion (3). In this study, in the context of CD8+ T cell/DC cross-talk, we suggest GM-CSF as a major factor in DC activation during Th cell-independent antiviral responses. This process can be further regulated by viral PAMP (as well as by other microbial PAMPs) signaling, which effectively enhances proinflammatory cytokine release by activated DCs. The combination of T cell-released GM-CSF and microbial patterns then fully license DCs to prime CTLs efficiently.

In this study, we described a new functional facet for the “old” growth factor GM-CSF (12). T cell-derived GM-CSF clearly plays an important role in the mutually enforcing cross-talk between DCs and T cells that drives the subsequent immune response. This short-range effect of GM-CSF can be added to its well-known long-range effects that were shown to be important in DC differentiation from BM progenitors, DC survival, and DC recruitment to sites of GM-CSF secretion (13, 14). It is no wonder that GM-CSF was found to be the best adjuvant in the settings of tumor vaccination (15, 16).

Not surprisingly, GM-CSF has been implicated in the development of several autoimmune diseases (17), such as experimental autoimmune encephalitis (18) and collagen-induced arthritis (19), and recently it was recognized to be the key factor in promoting the formation and survival of inflammatory IL-6-dependent Th17 cells in experimental myocarditis (20).

Clearly, GM-CSF, in combination with appropriate microbial stimuli, should remain one of the targets for further development in our efforts to enhance immune responses. This extended knowledge may lead to increased understanding about how vaccinations or immune therapies should be tailored.

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Disclosures

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References


