**Brief Definitive Report**

**High Level IL-12 Production by Murine Dendritic Cells: Upregulation via MHC Class II and CD40 Molecules and Downregulation by IL-4 and IL-10**

By Franz Koch,* Ursula Stanzl,* Patricia Jennewein,* Katrin Janke,* Christine Heufler,* Eckhart Kämpgen,* Nikolaus Romani,* and Gerold Schuler§

*From the Departments of Dermatology, University of Innsbruck, Innsbruck, Austria; University of Würzburg, Würzburg, Germany; and University of Erlangen-Nürnberg, Erlangen, Germany

**Summary**

We have shown previously that dendritic cells (DC) produce IL-12 upon interaction with CD4+ T cells. Here we ask how this IL-12 production is induced and regulated. Quantitative PCR, and in situ hybridization for IL-12 p40 and an ELISA specific for the p70 heterodimer were used to determine IL-12 production. We demonstrate that ligation of either CD40 or MHC class II molecules independently trigger IL-12 production in DC, and that IL-12 production is downregulated by IL-4 and IL-10. The levels of bioactive IL-12 that can be released by triggering with an anti-CD40 mAb or with a T cell hybridoma are high (range 260-4700 pg/ml from 1 × 10⁶ DC in 72 h). The CD40-mediated pathway indicates that IL-12 production is induced in DC upon interaction with activated, CD40 ligand-expressing helper T cells, even in the absence of cognate antigen recognition. Side-by-side comparison of IL-12 production, and blocking experiments employing an anti-CD40 ligand mAb, suggest that the CD40-mediated pathway is quantitatively more significant than induction via the MHC class II molecule. The importance of the CD40/CD40 ligand interaction for IL-12 induction in DC likely contributes to the recent finding that mice lacking the CD40 ligand are impaired in mounting Th1 type cell-mediated immune responses.

**Materials and Methods**

*Mice.* Specific pathogen-free C57BL/6, BALB/c, and C3H/He mice of both sexes were purchased from Charles River (Sulzfeld, Germany) and used at 6 to 8 wk of age.

*Media and Reagents for Cell Culture.* Culture medium was RPMI-1640 supplemented with 10% fetal calf serum, gentamycin, and 2-mercaptoethanol (Biological Industries, Kibbutz Beit Haemek, Israel).

*Dendritic Cells.* A previously described standard procedure involving overnight culture and final purification by rosetting with Ig-coated ox erythrocytes (EAIgG) was applied for the preparation of DC from spleen (6). Populations obtained in this manner contained consistently >90% DC.

*Reagents and Cells for the Stimulation of Dendritic Cells.* For stimulation of IL-12 production in murine DC we used the following...
mAbs: hamster anti–mouse CD40 ligand/gp39 (clone MR-1, IgG; PharMingen, San Diego, CA), rat anti–mouse CD40 (clone 3/23, IgG2a; Serotec, Oxford, UK), rat anti–mouse MHC class II/I-A<sup>d</sup> (clone B21-2, IgG2b; TIB229 from the American Type Culture Collection [ATCC], Rockville, MD), mouse anti-I-E<sup>d</sup> (clone 14-4-4S, IgG2a, HB32 from ATCC), and mouse anti-I-A<sup>k</sup> (clone 10-2.16, mouse IgG2a, TIB93 from ATCC). Hybridoma supernatants were used at a final dilution of 1:5, purified antibodies at final concentrations of 1–10 μg/ml. For inhibition experiments, mAb anti–CD40 ligand was applied at 50 μg/ml. mAb Y–Aε (7) and T cell hybridoma 1H3.1 (8), both specific for the L-Ext peptide 52-68 bound to I-A<sup>ε</sup> were kindly provided by Drs. R. Germain (Bethesda, MD) and A. Rudensky (Seattle, WA). Dr. Rudensky also supplied us with control peptides: pigeon cytochrome c (PCC 88-104, binds to I-E<sup>E</sup>); mouse cytochrome c (m-cyt-c 88-103, I-E<sup>c</sup>); undefined (I-A<sup>e</sup>). All peptides were used at 5–10 μM final concentration. Myoglobin peptide-specific T cell hybridomas 11.3.7 (A<sub>b</sub>E<sub>e</sub>-restricted) and 13.26.8 (I-E<sup>e</sup>-restricted) (9) and myoglobin peptide 132-147 (10 μg/ml final concentration) were gifts of Dr. A. Livingstone (London, UK). Finally, sodium periodate-modified primary naive murine CD4<sup>+</sup> T cells were used as a stimulus for DC in the oxidative mitogenesis assay. They were prepared from nylon wool non-adherent spleen and mesenteric lymph node cells by treatment with a cocktail of mAbs against MHC class II, B220, and CD8<sup>+</sup> plus complement. Periodization was performed as described (10). Regulation of IL-12 production by DC was studied using recombinant murine IL-4 (specific activity 1 x 10<sup>7</sup> U/mg) and IL-10 (specific activity 5 x 10<sup>5</sup> U/mg), both purchased from Genzyme Corporation (Cambridge, MA).

**Measurement of IL-12.** IL-12 was measured in 0–36 and 0–72 h supernatants of DC cultures (1 x 10<sup>6</sup> DC / ml 24 well) in a colored ELISA. Supernatants were analyzed after 36 and 72 h of coculture with periodate-modified T cells or T-T hybridomas. Murine IL-12 p70 heterodimer was detected by a two-site enzyme-linked immunosorbent assay (ELISA) (11) using rat anti–mouse IL-12 p75 mAbs that were generously provided by Drs. M.K. Gately and D.H. Presky (Hoffmann-La Roche, Nutley, NJ). 9A5 (anti–IL-12 p75) was used as capture antibody, SC3 (anti–IL-12 p40) as detection antibody. This was detected by a p70-specific ELISA (Table I, rows A vs. B) as well as by IFNγ induction in a bioassay (data not shown). In addition, we observed a strong upregulation of IL-12 p40 mRNA by in situ hybridization (Fig. 1). Our earlier data strongly suggested (5) that in these experiments the source of IL-12 is the DC.

**Results and Discussion**

**Dendritic Cells Produce IL-12 upon Interaction with T Cells.** When purified murine spleen DC sensitized naive helper T cells in an oxidative mitogenesis assay we found that substantial amounts (up to almost 5 ng/ml from 10<sup>6</sup> DC) of bioactive p70 heterodimer were released, yet only after 72 h. This was detected by a p70-specific ELISA (Table I, rows A vs. B) as well as by IFNγ induction in a bioassay (data not shown). In addition, we observed a strong upregulation of IL-12 p40 mRNA by in situ hybridization (Fig. 1). Our earlier data strongly suggested (5) that in these experiments the source of IL-12 is the DC.

The delayed onset of IL-12 production suggested that a molecule that is upregulated by T cells upon activation and could interact with a receptor on DC might be responsible for triggering IL-12 production in DC. A prime candidate was the CD40 ligand (CD40-L) as (a) CD40-L is expressed following T cell activation (13) and we confirmed this by flow cytometry (Fig. 2, A and B), (b) CD40-L is known to interact with the CD40 molecule on mature, immunostimulatory DC, and to induce several biological effects (e.g., upregulation of MHC class I and II, adhesion and costimulatory molecules) (14), and (c) CD40-L has been shown to induce release of IL-12, albeit at lower levels (<100 pg/ml) from human (15) and murine (15a) monocytes. Addition of blocking anti–CD40-L mAb greatly reduced IL-12 production (Table 1, rows B vs. C) as well as p40 IL-12 mRNA expression (not shown) by DC upon antigen-specific activation of resting T cells, indicating that the CD40-L/CD40 interaction is indeed a major trigger for production of IL-12.

We next studied IL-12 production upon interaction of DC with peptide-specific, MHC class II–restricted T cell
Table 1. Murine Spleen Dendritic Cells Produce p70 IL-12 Protein and p40 IL-12 mRNA upon Interaction with Primary CD4+ T Cells and T Cell Hybridomas

| Row | DC | Stimulus 1 | Stimulus 2 | Inhibitory treatment | p70 IL-12 Expt. No. 1 | p70 IL-12 Expt. No. 2 | p40 mRNA Expt. No. 3 | p40 mRNA Expt. No. 4 | p40 mRNA Expt. No. 4 |
|-----|----|------------|------------|----------------------|------------------------|------------------------|-----------------------|------------------------|-----------------------|
| A   | DC | -          | -          | -                    | 0                      | 0                      |                       |                        |                       |
| B   | DC | CD4+ T cells | -          | -                    | 398                    | 597                    |                       |                        |                       |
| C   | DC | CD4+ T cells | -          | anti-CD40-L          | 124                    | -                      |                       |                        |                       |
| D   | -  | CD4+ T cells | -          | anti-CD40-L          | 0                      | -                      |                       |                        |                       |
| E   | -  | CD4+ T cells | -          | -                    | 0                      | 0                      |                       |                        |                       |
| F   | DC | -          | -          | -                    | 0                      | 0                      |                       |                        |                       |
| G   | DC | hybrid 1H3.1 | -          | -                    | 2890                   | 1800                   | 22                    |                       |                       |
| H   | DC | hybrid 1H3.1 | -          | anti-CD40-L          | 280                    | 239                    | 19                    |                       |                       |
| I   | DC | hybrid 1H3.1 | Eα 52-68   | -                    | 4601                   | 4342                   | 97                    |                       |                       |
| J   | DC | hybrid 1H3.1 | Eα 52-68   | anti-CD40-L          | 1077                   | 789                    | 29                    |                       |                       |
| K   | DC | hybrid 1H3.1 | PCC 88-104 | -                    | 2840                   | 1224                   | -                     |                       |                       |
| L   | DC | -          | Exα 52-68  | -                    | -                      | -                      | 0                     |                       |                       |
| M   | -  | hybrid 1H3.1 | -          | -                    | 0                      | 0                      | 0                     |                       |                       |
| N   | -  | hybrid 1H3.1 | Exα 52-68  | -                    | 0                      | 0                      | 0                     |                       |                       |

Dendritic cells (DC) were stimulated with primary CD4+ T cells (top, Rows A-E) or with T hybridoma cells (bottom, F-N). Representative experiments are shown. Concentrations of IL-12 p70 heterodimer were determined by ELISA in 72 h supernatants. Measurements after 36 h of stimulation were consistently negative. For PCR, DC were stimulated for 36 h. Values are expressed as attomol p40 RNA/mg total RNA. Due to the strong proliferation of hybridoma cells during the stimulation period the exact amount of DC RNA that was subjected to the quantitative PCR procedure was unknown. For this reason a meaningful control with DC alone could not be done. DC and periodated CD4+ cells were from C57BL/6 mice. Eα 52-68 and PCC 88-104 are I-Eα and pigeon cytochrome c peptides, respectively.

Figure 2. Expression of CD40 ligand on T cells. Periodated CD4+ T cells freshly isolated (A) or after 48 h of coculture with dendritic cells (B) and T cell hybridoma 1H3.1 (C and D) were analyzed by fluorescence flow cytometry using mAb anti-gp39. Large cells (i.e., activated T cell blasts) were selected by forward scatter/side scatter gating in B. CD40 ligand (arrowed curve in each panel) appears on primary T cells in response to activation by dendritic cells (A vs. B). The T-T hybrid shows low constitutive expression (C) and a marked upregulation following activation with 2 μM ionomycin for 12 h (D). Unlabeled curves represent controls with irrelevant hamster Ig.
Table 2. Murine Spleen Dendritic Cells Produce p70 IL-12 upon Ligation of MHC Class II and CD40 Molecules

| Stimulus 1 | Stimulus 2 | BALB | BALB | BALB | C3H | C57 | C57* | C57* | BALB |
|------------|------------|------|------|------|-----|-----|------|------|------|
| -          | -          | 0    | 0    | 0    | 0   | 0   | 0    | 0    | 0    |
| anti-CD40  | -          | 3935 | 625  | 670  | 750 | 817 | 674  | 1188 | 94   |
| -          | anti-I-A   | 1695 | -    | 463  | 472 | 431 | 431  | -    | -    |
| -          | anti-I-A   | 5153 | -    | -    | -   | -   | -    | -    | -    |
| -          | anti-I-E   | -    | 184  | 431  | 417 | -   | -    | -    | -    |
| -          | anti-I-A + I-E | - | 200 | - | - | - | - | - | - |
| anti-CD40  | anti-I-E   | -    | 700  | -    | -   | -   | -    | -    | -    |
| Eq 52-68   | -          | 0    | 0    | 0    | 0   | 0   | 0    | 0    | 0    |
| Eq 52-68   | mAb Y-Ae   | 0    | 0    | 0    | 637 | 626 | 628  | 628  | -    |
| -          | mAb Y-Ae   | 0    | 0    | 0    | 0   | 0   | 0    | 0    | 0    |

Dendritic cells of different mouse strains were treated with the listed stimuli and p70 IL-12 was determined by ELISA in 72 h supernatants. Measurements after 36 h of stimulation were consistently negative. For quantitative PCR, IL-12 p40 (far right column), DC were stimulated for 36 h. Values are expressed as attomol p40 RNA/mg total RNA. (Upper part) Stimulation with anti-CD40 and anti-MHC class II mAbs; (lower part) stimulation with mAb Y-Ae, specific for the Eq 52-68/I-A complex.

*In these experiments control peptides (moth and pigeon cytochrome-c, myoglobin, and an undefined peptide; see Materials and Methods), all at 5–10 µM, were negative. Solvent control (DMSO) was also negative.

*A different anti-I-A mAb (MKD6) gave 535 pg/ml and a different anti-I-A mAb (M5-114) mAb yielded 483 pg/ml.

Dendritic Cells Produce IL-12 upon Ligation of CD40 and MHC Class II Molecules. We next used purified murine spleen DC and several mAbs to search for the molecular signals that trigger IL-12 production in DC. Binding of a stimulatory mAb to CD40 induced the release of large amounts of p70 IL-12 (Table 2, top two rows) substantiating the conclusion drawn from the anti-CD40-L blocking studies (see above) that ligation of CD40 triggers IL-12 production in DC. In 17 experiments the range for IL-12 production in this system was 263–4726 pg/ml of p70 heterodimer. Strain differences were not observed. Binding of anti-MHC class II mAbs also reproducibly induced IL-12 release although at ~50% lower levels as compared to triggering IL-12 production via the CD40 molecule (Table 2). Interestingly, simultaneous triggering of MHC class II as well as CD40 molecules had an additive effect.

In addition to the anti-MHC class II mAbs we also tested the peptide Eq 52-68 as a physiological ligand for MHC. This peptide binds to I-A^d and MHC class II molecules and can be detected by the Y-Ae mAb. This unique antibody binds to the complex of an I-E^o peptide and I-A^d (but not I-A^a), and, like a TCR recognizes both the Eq 52-68 peptide and polymorphic MHC class II residues (7). Upon binding of the mAb Y-Ae to the Eq 52-68 + I-A^d expression in response to either stimulus (C and D). Control hybridization of anti-class II-treated DC with sense RNA probe is shown in A. 150X (A and B); 250X (C and D).
Table 3. Interleukin-4 and Interleukin-10 Downregulate IL-12 p70 Production in Murine Spleen Dendritic Cells

| Stimulus/Cytokines | pg/ml IL-12 p70 |
|--------------------|---------------|
| No stimulus        | 0             |
| CD4+ T cells       | 0             |
| CD4+ T cells       | 0             |
| IL-4 (200 U/ml)    | 48            |
| CD4+ T cells       | 0             |
| IL-10 (2.5 U/ml)   | 0             |
| Anti-CD40          | 727           |
| IL-4 (50, 200 U/ml)| 43, 0         |
| Anti-CD40          | 0, 0          |
| IL-10 (0.1, 1 U/ml)| 0, 0          |

Dendritic cells from C57BL/6 mice were stimulated with primary CD4+ T cells or with mAb against CD40. IL-12 p70 heterodimer was determined by ELISA after 72 h of stimulation. IL-10 completely blocks IL-12 production. IL-4 leads to substantial inhibition and also blocks at higher concentrations.

IL-4 and IL-10 Downregulate IL-12 Production by Dendritic Cells. Downregulation of IL-12 production by DC might be biologically relevant with regard to Th1 skewing of the immune response (1) and tolerance (16). We therefore tested the effect of IL-4 and IL-10 which both can suppress IL-12 production by mononuclear phagocytes (17, 18). IL-10 as well as IL-4 were also able to suppress T cell-induced as well as anti-CD40 mAb-triggered IL-12 production in DC (Table 3). In a pilot experiment DC were stimulated for 24 h with anti-CD40 mAb in the presence of IL-4 or IL-10 followed by thorough washing and continued culture for another 56 h in the absence of IL-4 or IL-10. Induction of IL-12 was much lower (272 and 90 pg/ml, respectively) as compared with control cultures that had been without cytokines and/or without anti-CD40 during the first 24 h (885 and >1000 pg/ml, respectively). This suggests that the inhibitory effects of IL-4 and IL-10 are not fully irreversible. Downregulation of IL-12 production in DC as well as other antigen presenting cells (17, 18) explains why both IL-10 and IL-4 suppress Th1 development (19). Downregulation of IL-12 production by IL-10 may also contribute to development of hapten-specific tolerance (20) as the induction of contact hypersensitivity critically depends on DC-derived IL-12 (16). It appears also that IL-4 drives Th2 development so potently as it does not only directly act on precursor T cells (19) but in addition also downregulates IL-12 production in antigen-presenting cells including DC.

Possible Biological Relevance of T Cell-induced IL-12 Production by Dendritic Cells. It is evident that antigen-presenting cells including DC release IL-12 upon antigen-specific interaction with T cells, and can thus skew the immune response to Th1 including IFNγ production from resulting Th1 cells (2, 5). We have shown here that ligation by T cells of MHC class II as well as CD40 molecules independently triggers high levels of IL-12 production in DC. Relative to macrophages and B cells, DC can make larger amounts of IL-12. The induction of IL-12 production via CD40 molecule readily explains our finding that helper T cells, upon activation and expression of CD40-L, can induce IL-12 production even in the absence of cognate antigen recognition by T cells. By making so much IL-12 upon contact with T cells, either via MHC class II or CD40, the DC would skew these T cells towards a Th1 phenotype (5) and thus promote strong cell mediated immunity.

This work was supported by a grant of the Austrian Science Foundation (P9967Med) to N. Romani and G. Schuler. We thank Susanne Ebner and Daniela Reider for expert assistance with FACS® analyses and Hella Stössel for in situ hybridizations. The continued support of Dr. P. Fritsch, Chair of Dermatology, University of Innsbruck, is greatly appreciated.
References

1. Trinchieri, G. 1995. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Annu. Rev. Immunol.* 13:251–276.

2. Macatonia, S.E., N.A. Hosken, M. Litton, P. Vieira, C.-S. Hsieh, J.A. Culpepper, M. Wysocka, G. Trinchieri, K.M. Murphy, and A. O’Garra. 1995. Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T cells. *J. Immunol.* 154:5071–5079.

3. Scheicher, C., M. Mehlig, H.-P. Dienes, and K. Reske. 1995. Uptake of microparticle-adsorbed protein antigen by bone marrow-derived dendritic cells results in up-regulation of interleukin-1α and interleukin-12 p40/p35 and triggers prolonged, efficient antigen presentation. *Eur. J. Immunol.* 25:1566–1572.

4. Kang, K.F., M. Kubin, K.D. Cooper, S.R. Lessin, G. Trinchieri, and A.H. Rook. 1996. IL-12 synthesis by human Langerhans cells. *J. Immunol.* 156:1402–1407.

5. Heufler, C., F. Koch, U. Stanzl, G. Topar, M. Wysocka, G. Trinchieri, A. Enk, R.M. Steinman, N. Romani, and G. Schuler. 1996. Interleukin-12 is produced by dendritic cells and mediates Th1 development as well as Interferon-γ production by Th1 cells. *Eur. J. Immunol.* 26:659–668.

6. Crowley, M.T., K. Inaba, M. Wittem-Pack, and R.M. Steinman. 1989. The cell surface of mouse dendritic cells: FACs analyses of dendritic cells from different tissues including thymus. *Cell. Immunol.* 118:108–125.

7. Murphy, D.B., S. Rath, E. Pizzo, A.Y. Rudensky, A. George, J.K. Larson, and C.A. Janeway, Jr. 1992. Monoclonal antibody detection of a major self peptide: MHC class II complex. *J. Immunol.* 148:3483–3491.

8. Rudensky, A.Y., S. Rath, P. Preston-Hurlburt, D.B. Murphy, and C.A. Janeway, Jr. 1991. On the complexity of self. *Nature (Lond.*) 353:660–663.

9. Danska, J.S., A.M. Livingstone, V. Paragas, T. Ishihara, and C.G. Fathman. 1990. The presumptive CD83 regions of both T cell receptor α and β chains determine T cell specificity for myoglobin peptides. *J. Exp. Med.* 172:27–33.

10. Schuler, G., and R.M. Steinman. 1985. Murine epidermal Langerhans cells mature into potent immunostimulatory dendritic cells in vitro. *J. Exp. Med.* 161:526–546.

11. Wilkinson, V.L., R.R. Warrier, T.P. Truitt, P. Nunes, M.K. Gately, and D.H. Presky. 1996. Characterization of anti-mouse IL-12 monoclonal antibodies and measurement of mouse IL-12 by ELISA. *J. Immunol. Methods.* 189:15–24.

12. Schoenhaut, D.S., A.O. Chua, A.G. Wolitzky, P.M. Quinn, C.M. Dwyer, W. Comosas, P.C. Familleti, M.K. Gately, and U. Gubler. 1992. Cloning and expression of murine IL-12. *J. Immunol.* 148:3433–3440.

13. Banchereau, J., F. Bazan, D. Blanchard, F. Brière, J.P. Galizzi, C. Van Kooten, Y.J. Liu, F. Rousset, and S. Saeland. 1994. The CD40 antigen and its ligand. *Annu. Rev. Immunol.* 12:881–922.

14. Caux, C., C. Massacrier, B. Vanbervliet, B. Dubois, C. Van Kooten, I. Durand, and J. Banchereau. 1994. Activation of human dendritic cells through CD40 cross-linking. *J. Exp. Med.* 180:1263–1272.

15. Shu, U., M. Kiniwa, C.Y. Wu, C. Maliszewski, N. Vezzio, J. Hakimi, M. Gately, and G. Delespesse. 1995. Activated T cells induce interleukin-12 production by monocytes via CD40-CD40 ligand interaction. *Eur. J. Immunol.* 25:1125–1128.

16. Müller, G., J. Saloga, T. Germann, G. Schuler, J. Knop, and A.H. Enk. 1995. IL-12 as mediator and adjuvant for the induction of contact sensitivity in vivo. *J. Immunol.* 155:4661–4668.

17. D’Andrea, A., M. Aste-Amezaga, N.M. Valiante, X. Ma, M. Kubin, and G. Trinchieri. 1993. Interleukin 10 (IL-10) inhibits human lymphocyte interferon gamma-production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. *J. Exp. Med.* 178:1041–1048.

18. D’Andrea, A., X. Ma, M. Aste-Amezaga, C. Paganini, and G. Trinchieri. 1995. Stimulatory and inhibitory effects of interleukin (IL)-4 and IL-13 on the production of cytokines by human peripheral blood mononuclear cells: priming for IL-12 and tumor necrosis factor α production. *J. Exp. Med.* 181:537–546.

19. Paul, W.E., and R.A. Seder. 1994. Lymphocyte responses and cytokines. *Cell.* 76:241–251.

20. Enk, A.H., J. Saloga, D. Becker, M. Mohamadzadeh, and J. Knop. 1994. Induction of hapten-specific tolerance by interleukin 10 in vivo. *J. Exp. Med.* 179:1397–1402.