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

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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 1×10^6 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.

IL-12, a 70-kD heterodimeric cytokine composed of covalently linked p35 and p40 chains has emerged as a central cytokine in the immune response (1). IL-12 stimulates NK cells, mediates Th1 development, and fosters CTL development. It can be produced by monocytes and macrophages in response to intracellular pathogens, bacteria (e.g., staphylococci) and bacterial products. Recent reports indicate that dendritic cells (DC) also release bioactive IL-12. One report described that anti–IL-12 blocks the capacity of murine DC to skew the response of naive transgenic T cells to the Th1 phenotype (2), and another shows induction of IL-12 p40/p35 mRNA in bone-marrow derived murine DC upon uptake of microparticle-absorbed protein antigen (3). Human epidermal Langerhans cells are also a source of IL-12 (4). We have recently used several criteria for demonstration of IL-12 p40 and p35 mRNA as well as IL-12 p40 and bioactive p70 proteins, to show that murine and human DC release IL-12 upon conventional stimuli such as staphylococcus aureus (5). We also found that DC produced bioactive IL-12 upon interaction with T cells without standard stimuli such as bacterial products. Here, we describe the regulation of IL-12 in DC. We show that ligation of CD40 and MHC class II molecules independently trigger IL-12 production by DC in large amounts, and that IL-12 release by DC is downregulated by IL-4 as well as by IL-10.

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 MR1, IgG; PharMingen, San Diego, CA), rat anti–mouse CD40 (clone 3/23, IgG2a; Serotec, Oxford, UK), rat anti–mouse MHC class II/I-$A^{b,d}$ (clone B21-2, IgG2b; TIB229 from the American Type Culture Collection [ATCC], Rockville, MD), mouse anti-I-$E^{k,d}$ (clone 14-4-4S, IgG2a, HB32 from ATCC), and mouse anti-I-A^k (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-Ae (7) and T cell hybridoma 1H3.1 (8), both specific for the I-Eα peptide 52-68 bound to I-A^b 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^k); moth cytochrome c (m-cyt-c 88-103, I-$E^{k/b}$); undefined (I-A^b). All peptides were used at 5–10 μM final concentration. Myoglobin peptide-specific T cell hybridomas 11.3.7 ($A_\beta{}^dE_\alpha$-restricted) and 13.26.8 (I-E^d-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+ 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 plus complement. Periodation was performed as described (10). Regulation of IL-12 production by DC was studied using recombinant murine IL-4 (specific activity 1×10^7 U/mg) and IL-10 (specific activity 5×10^5 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 (1×10^6 DC /1 ml/24 well) ± stimulation with the mAbs or cytokines mentioned above. Likewise, 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 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, 5C3 (anti–IL-12 p40) as detection antibody (11). Detection limit was about 20 pg/ml of IL-12. IL-12 was also measured indirectly via determination of INFγ produced by Th1 type T cell blasts as described (5).

Detection of IL-12 mRNA by In Situ Hybridization. This was done essentially as described (5) using a non-radioactive labeling system (Boehringer Mannheim, Mannheim, Germany). For detection of bound digoxigenin-labeled probes we used FITC-conjugated sheep anti-digoxigenin antibodies (Boehringer Mannheim).

Detection of IL-12 mRNA by quantitative PCR and liquid hybridization. For the quantitative PCR the PCR Mimic Construction Kit™ (No. 1700-1; Invitrogen, Leek, The Netherlands) was used to produce a neutral DNA fragment spanning 548 bp, flanked by the IL-12 primers used for the PCR. For quantitation, IL-12 p40 PCR was performed under standard conditions using the same amount of template cDNA with serial dilutions of mimic DNA (10-0,001 attomol/μl in tenfold dilutions or in some cases twofold dilutions spanning the concentration range of interest). The template DNA was quantitated by comparison of the brightness of ethidium bromide stained bands of amplified p40 versus minic DNA. Wincam™ software was employed for evaluation. The primers for murine IL-12 p40 were designed according to published sequences (12) and spanned one intron (bp 173–481): 3′ primer: AACCTCACCTGTGACACGCC, 5′ primer: CAAGTCCATGTTTCTTTGCACC.

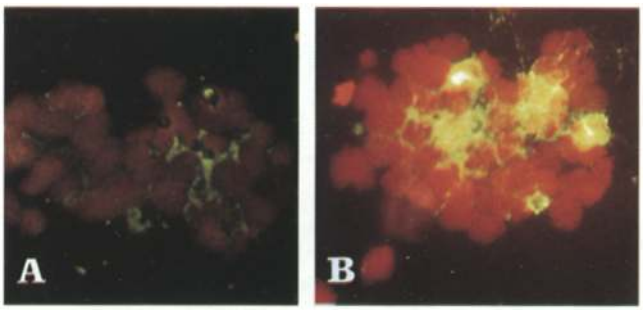

Figure 1. Non-radioactive in situ hybridization demonstrates that murine spleen DC express IL-12 p40 mRNA upon contact with CD4+ T cells. A population of >95% DC was cocultured with allogeneic periodated CD4+ T cells at a ratio of 1:100 for 36 h. Clusters of T cells and DC were analyzed. IL-12 p40 mRNA expression is evident in dendritic appearing cells (*A*) within the cluster. On parallel slides from the same experiment DC within the aggregates are identified by means of anti–MHC class II staining (*B*). IL-12-negative and MHC class II–negative T cells were made visible by counterstaining nuclei in red with propidium iodide. DC by themselves express very little IL-12 mRNA (see Fig. 3 *B*). 150×.

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 10^6 DC) of bioactive p70 heterodimer were released, yet only after 72 h. This was detected by a p70-specific ELISA (Table 1, 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		p40 mRNA
					Expt. No. 1	*Expt. No. 2*	
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	
					Expt. No. 3	*Expt. No. 4*	*Expt. No. 4*
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	–	Eα 52-68	–	–	0	–
M	–	hybrid 1H3.1	–	–	0	0	0
N	–	hybrid 1H3.1	Eα 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.

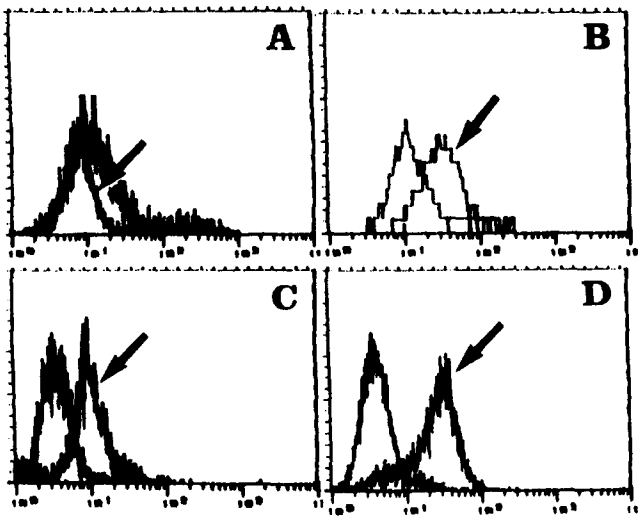

Figure 2. Expression of CD40 ligand on T cells. Periodated CD4+ T cells freshly isolated (*A*) or after 48 h of coculture with dendritic cells (*B*) and T cell hybridoma 1H3.1 (*C* and *D*) were analyzed by fluorescence flow cytometry using mAb anti-gp39. Large cells (i.e., activated T cell blasts) were 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.

hybridomas as a model for activated T cells. We noted that even an antigen-nonspecific interaction (i.e., no peptide or control peptide added) with T cell hybridomas induced high levels of IL-12 (Table 1, rows *F* vs. *G* and *K*). Production of IL-12 was, however, still higher in the presence of the appropriate peptide enabling antigen-specific activation of the T cell hybridomas (Table 1, rows *G* vs. *I*). Using anti–CD40-L mAb, we found that the T cell hybridomas increased their constitutive CD40-L expression further, as expected, upon activation (Fig. 2, *C* and *D*). Anti-CD40-L prevented most of the IL-12 production, that occurred upon mixing DC with T-T hybrid cells. However, blocking was incomplete notably in the case of the antigen-specific interaction (Table 1, rows *B* vs. *C* and *I* vs. *J*). The same observations were made with two myoglobin-specific T cell hybridomas (not shown).

Table 2. *Murine Spleen Dendritic Cells Produce p70 IL-12 upon Ligation of MHC Class II and CD40 Molecules*

		p70 IL-12							mRNA
Stimulus 1	Stimulus 2	BALB	BALB	BALB	C3H	C57	C57*	C57*	BALB
				pg/ml					
–	–	0	0	0	0	0	0	0	26
anti-CD40	–	3935	625	670	750	817	674	1188	94
–	anti–I-A	1695	–	463‡	472	431§	–	–	40
anti-CD40	anti–I-A	5153	–	–	–	–	–	–	80
–	anti–I-E	–	184	431	417	–	–	–	–
–	anti–I-A + I-E	–	200	–	–	–	–	–	51
anti-CD40	anti–I-E	–	700	–	–	–	–	–	–
Eα 52-68	–			0	0	0	0	0	
Eα 52-68	mAb Y-Ae			0	0	637	626	628	
–	mAb Y-Ae			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 for 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 Eα 52-68/I-A^b 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^d mAb (MKD6) gave 535 pg/ml and § a different anti–I-A^b (M5-114) mAb yielded 483 pg/ml.

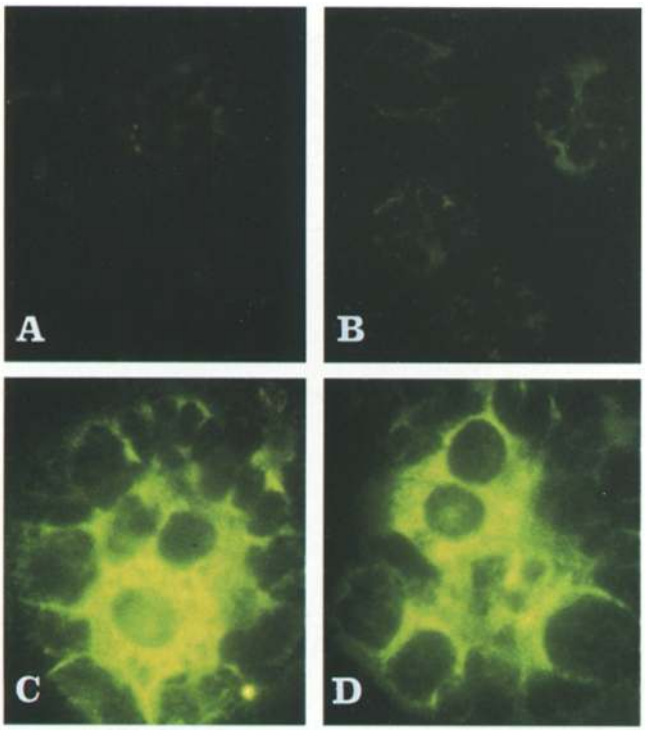

Figure 3. Non-radioactive in situ hybridization demonstrates that murine spleen DC express IL-12 p40 mRNA upon ligation of CD40 and MHC class II molecules. A population of >95% DC was incubated for 36 h in the absence of stimuli (*B*) or in the presence of anti-CD40 mAbs (*C*) or anti–MHC class II mAbs (*D*). Compare the very weak signal in unstimulated DC (*A*) with the strongly upregulated IL-12 p40 mRNA 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. 150× (*A* and *B*); 250× (*C* and *D*).

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 Eα 52-68 as a physiological ligand for MHC. This peptide binds to I-$A^{b \text{ and } d}$ MHC class II molecules and can be detected by the Y-Ae mAb. This unique antibody binds to the complex of an I-Eα peptide and I-A^b (but not I-A^d), and, like a TCR recognizes both the Eα 52-68 peptide and polymorphic MHC class II residues (7). Upon binding of the mAb Y-Ae to the Eα 52-68 + I-A^b

Table 3. *Interleukin-4 and Interleukin-10 Downregulate IL-12 p70 Production in Murine Spleen Dendritic Cells*

Stimulus/Cytokines	p70 IL-12		
	pg/ml		
No stimulus	–	0	0
CD4+ T cells	398	–	–
CD4+ T cells IL-4 (200 U/ml)	48	–	–
CD4+ T cells IL-10 (2.5 U/ml)	0	–	–
Anti-CD40	–	727	321
Anti-CD40 IL-4 (50, 200 U/ml)	–	43, 0	0, 0
Anti-CD40 IL-10 (0.1, 1 U/ml)	–	0, 0	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.

complex, DC released substantial amounts of IL-12 (Table 2). I-A^b-binding (an undefined peptide) and non-binding (PCC 88-104, m-cyt-c 88-103, myoglobin 132-147) control peptides did not induce IL-12 production irrespective of the presence of mAb Y-Ae. Conversely, peptide Eα 52-68, with or without mAb Y-Ae, was inactive on DC of binding (BALB/c - H-2^d) and non-binding (C3H - H-2^k) MHC class II haplotypes (Table 2). These findings supported the conclusion drawn from the experiments using DC and T cell hybridomas (including the T cell hybridoma 1H3.1 that recognizes the very same Eα 52–68 + I-A^b complex [8], see above and Table 1) that ligation of MHC class II molecules directly signals IL-12 production in DC, independent of the indirect effect of CD40-L upregulation on T cells via TCR-mediated activation (13).

Quantitative RT-PCR and non-radioactive in situ hybridization demonstrated that the induction of IL-12 production via CD40 and MHC class II molecules is transcriptionally regulated (Tables 1 and 2, Fig. 3). In situ analyses showed furthermore that IL-12 mRNA is induced in virtually all DC and is not restricted to subsets.

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 the 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.

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