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Increased production of pro-inflammatory cytokines and enhanced T cell responses after activation of human dendritic cells with IL-1 and CD40 ligand

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Abstract

Background: Various microbial, inflammatory and immune signals regulate the activation of dendritic cells (DC), determining their ability to interact with naïve T cells and to produce cytokines that direct T cell development. In particular, CD40L and IL-1 cooperatively activate DC to secrete high levels of IL-12. The immuno-stimulatory capacity of such DC is otherwise not well-defined prompting further characterization of the effects of IL-1 and family members on DC activation in comparison with other pro-inflammatory stimuli.

Results: Human DC co-activated *in vitro* by CD40L and IL-1β expressed numerous cytokine genes including IL-12β, IL-23 p19, IL-1β, IL-1α, IL-1Ra, IL-10, IL-6, IL-18 and IFN-γ. These DC produced high levels of IL-12 protein and appeared capable of producing IFN-γ. Potent $CD4^+$ and $CD8^+$ T cell-stimulatory properties were acquired by DC under conditions that also induced IL-12. Notably, these DC induced rapid differentiation of fluMP-specific $CD8^+$ T cells. Molecules related to IL-1β, like IL-1α, co-induced IL-12 secretion whereas IL-18 did not. Conversely, the inhibitor IL-1Ra, produced endogenously by DC curtailed IL-12 production in response to CD40L.

Conclusions: IL-1 and IL-1Ra play a biologically-relevant role in the positive and negative regulation of DC activation. In conjunction with CD40L, IL-1 sends a powerful activation signal to DC that could be distinguished from other modes of activation. This signal enables the production of pro-inflammatory cytokines by DC, and enhances the differentiation of naïve T cells into effectors of type-1 cellular immune responses.

Background

Dendritic cells (DC) are highly effective antigen presenting cells (APCs) capable of stimulating the differentiation of naïve lymphocytes into effector cells (reviewed in [1]). Specific properties of the DC determine the quality of immune responses that they initiate. These properties are determined by complex and dynamic changes both in the DC and in its environment. Notably, DC undergo a so-called process of maturation that changes migratory properties, diminishes antigen uptake, enhances antigen

processing and presentation and induces the production of an array of cytokines (reviewed in [2]). The qualitative, quantitative and temporal regulation of cytokines produced by DC is thought to be a determining factor in the development of immune responses initiated by these APCs but molecules and mechanisms that regulate such process are not entirely understood.

Activated T cells provide strong stimuli that activate cytokine production in DC notably via interactions between CD40L and CD40 [3,4]. This interaction not only regulates the production of Th1 polarizing cytokines such as IL-12 by DC *in vitro* but is a central event *in vivo* that enables DC to prime effective MHC class I-restricted $CD8^+$ T cell responses [5–7]. IL-12 is a pivotal factor for the initiation of cellular immunity (reviewed in [8]). IL-12 promotes the polarization of Th1 T cell development, increases the differentiation and activation of cytolytic T cells and in concert with IL-18, induces IFN-γ production by T cells and NK cells. The mechanisms regulating IL-12 production are complex as IL-12 is composed of two chains IL-12α (IL-12p35) and IL-12β (IL-12p40) with independent transcriptional regulation. The two chains assemble as a disulfide-linked heterodimer which is glycosylated and secreted as a biologically active pro-inflammatory and immune mediator [9,10]. CD40L is known to induce the production of high levels of IL-12β in human monocyte-derived DC [11]. Yet, signalling through CD40 alone is not sufficient to induce the secretion of substantial amounts of IL-12αβ heterodimer. We and others have shown that a second signal, provided by a microbial product (lipopolysaccharide=LPS) or by immune mediators such as IFN-γ or IL-1β, is required to induce the secretion of high levels of IL-12 by DC in the presence of CD40L [12–15].

IL-1 is major pro-inflammatory cytokine with multiple activities in the regulation of immune, inflammatory, endocrine and neuronal systems (reviewed in [16]). The IL-1 family comprises several structurally-related ligands and the most extensively characterized include IL-1α, IL-1β, the inhibitory IL-1 receptor antagonist (IL-1Ra) and IL-18 (IL-1γ). These ligands bind to members of a family of receptors characterized by immunoglobulin folds in the extracellular portion and Toll/IL-1R (TIR) motif in the intracytoplasmic portion [17]. Whereas ligands of Toll-like receptors such as LPS and other microbial products have recently gained a lot of attention as regulators of immune responses in general (reviewed by [18]) and of DC activation in particular [19], ligands of IL-1R family have not been as extensively examined under this angle. On its own, IL-1 is known to be a weak maturing agent for DC [4]. However, we have recently shown that when combined with CD40L, IL-1β sends a powerful cooperative signal to monocyte-derived DC, inducing the secretion of high levels of IL-12 [14]. This was recently confirmed and extended to show that IL-1β and CD40L also induce the secretion of high levels of IL-12 in $CD34^+$ cell-derived DC [15] and A. Wesa, unpublished observation), and that this mode of activation also triggers the production of high levels of IL-6 and the production of IL-1. The effects of IL-1 can be explained at least at the transcriptional level, as CD40L up-regulates IL-12β mRNA and IL-1β complements this effect by up-regulating IL-12α mRNA [14]. Our own results suggest that CD40L co-activation with IL-1β yields high amounts of IL-12 but little IL-10 whereas LPS induces IL-12 but also more IL-10 [14]. As DC encounter various innate, microbial or immune stimuli that engage distinct intracellular signaling pathways, it seems likely that various combinations of these modes of activation might not produce equivalent APCs. The immuno-stimulatory properties of DC activated with IL-1 and C40L have not been well-defined and it is not clear that these cells which produce high amounts of IL-12 will be able to prime and activate naive T cells, in particular $CD8^+$ T cells. This prompted the present study to better characterize the effects of IL-1 and to better understand the molecular regulation of DC function. We found that CD40L+IL-1β stimulation effectively confers immuno-stimulatory activity and could be distinguished from other modes of pro-inflammatory activation of DC. We also found that the constitutive expression of IL-1Ra in DC constitutes a regulatory mechanism curtailing DC activation in the absence of specific inflammatory signals suggesting that the family of IL-1 molecules has biological relevance to DC activation.

Results

Regulation of cytokine gene expression in DC

Cytokine gene expression in human monocyte-derived DC was analyzed in response to various pro-inflammatory stimuli. Immature DC were produced by culture of adherent blood monocytes with GM-CSF and IL-4 and these cells were matured by treatment with CD40L combined with either IL-1β or IFN-γ or for comparison by treatment with LPS+IFN-γ effective inducers of IL-12 in DC [12]. The expression of IL-12α, IL-12β, IL-10, IL-1α, IL-1β, IL-1Ra, IL-6, IL-18, IFN-γ and the housekeeping L32 and GAPDH genes were assessed using a non-isotopic multiple probe RPA. Levels of mRNAs were calculated in relation to housekeeping gene expression. Results from 2 to 7 independent experiments are presented in Figure 1. Treatment with CD40L+IL-1β induced the expression of multiple genes as IL-12β, IL-10, IL-1α, IL-1β, IL-6, IL-18 and IFN-γ were more highly expressed than in DC untreated or treated with IL-1β alone. The conjunction of CD40L with either IL-1β or IFN-γ induced statistically higher levels of IL-12β than CD40L alone (8.00 ± 4.0 with CD40L+IL-1β or 5.68 ± 1.6 with CD40L+IFN-γ; *versus* $1.53 \pm .0.5$ for CD40L alone; $p < 0.05$). However, these two co-stimulato-

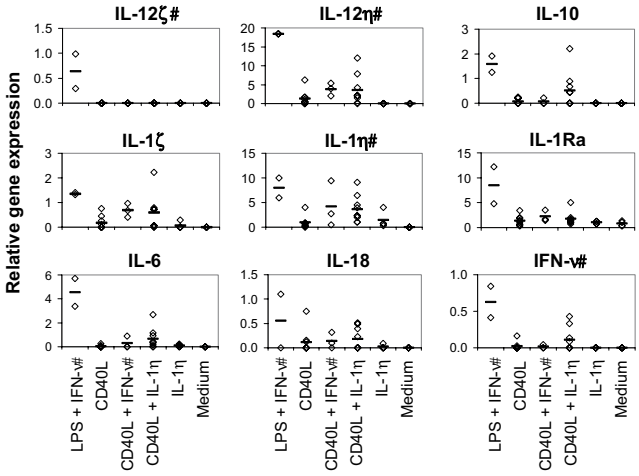

Figure 1
Comparison of different activation signals on gene expression in DC. Normalized gene expression levels from 2–7 independent experiments with averages indicated by a horizontal bar. DC were cultured with LPS+IFN-γ (n = 2); CD40L+IFN-γ (n = 3); CD40L+IL-1β (n = 7); CD40L (n = 7); IL-1β (n = 4); medium n = 4 for 8 hours and total RNA was extracted and hybridized to biotin-labeled RNA probes. After RNase treatment, digested probes were separated on a 5% acrylamide/8 M urea gel, blotted to a positively charged membrane for detection with CDP-Star substrate solution, and exposed to film or directly to the Kodak Image Station. Controls include yeast RNA incubated with probes followed or not by RNase treatment. Relative gene expression levels were calculated by dividing the net intensity of a gene (calculated with Kodak 1D software) with the net intensity of L32 in the same lane.

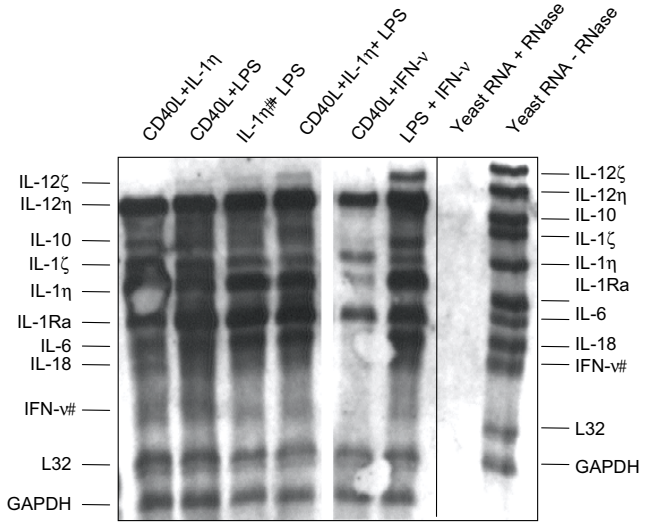

Figure 2
Effect of combined signals on cytokine gene expression in DC. DC (1×10^6) were treated with CD40L+IL-1β, CD40L+LPS, LPS+IL-1β, LPS+CD40L+IL-1β, CD40L+IFN-γ or LPS+IFN-γ for 8 hours. Following stimulation, RNA was isolated and subjected to RPA analysis (as described in Figure 1). Controls are shown of yeast RNA incubated with probes followed by RNase treatment (background) or mock treatment (undigested probes).

ry signals had distinct effects. Treatment with CD40L+IFN-γ induced IL-12β, IL-1α, IL-1β, and low levels of IL-18 but no IL-6, IL-10 or IFN-γ. Statistical analysis confirmed that DC treated with CD40L+IFN-γ produced significantly less IL-6, IL-10 and IL-1β than DC treated with CD40L+IL-1β ($p < 0.05$, n = 3). This difference was confirmed over time in kinetics studies examining gene expression at 4, 8 and 16 hours post-stimulation (data not shown). The overall strongest induction of gene expression was obtained by treatment with LPS+IFN-γ (Figure 1) which was uniquely able to induce high levels of IL-12α mRNA by RPA. Furthermore, IL-1Ra mRNA was upregulated in response to LPS+IFN-γ whereas it remained relatively constant with all other stimuli.

As DC respond to complex signals including autocrine signals such as IL-1β and IFN-γ, it seemed imperative to analyze how these complex combinations regulate gene expression in comparison with a strong pro-inflammatory activator such as LPS. Combinations of CD40L, IFN-γ, LPS and IL-1β were therefore compared (Figure 2). Each of the combination tested induced the production of IL-12β and the overall transcription profiles examined appeared to be qualitatively similar. However, the presence of LPS clearly correlated with higher levels of IL-12α and IL-10 mRNAs. Thus, DC treated with CD40L+IL-1β induced gene expression patterns that were qualitatively similar to those induced by LPS+CD40L, LPS+IL-1β or LPS+CD40L+IL-1β but qualitatively distinct from those induced by CD40L+IFN-γ. This transcriptional analysis implies that activation of DC by diverse pro-inflammatory agents may have distinct functional outcome.

Regulation of IL-12 and IL-23 secretion in DC

We previously reported that IL-12 αβ heterodimer was being secreted after stimulation with IL-1β+CD40L [14]. To verify these findings, supernatants of DC were immunoprecipitated with a mAb against IL-12β and separated by non-reducing SDS-PAGE. Detection with a mAb specific for IL-12α/β heterodimer (Figure 3-A) confirmed that CD40L+IL-1β, LPS+IFN-γ, and CD40L+IFN-γ induced the secretion of the 70 kD heterodimer IL-12αβ at levels correlated to those detected by ELISA (data not shown). These data confirmed the synergy of CD40L+IL-1β+IFN-γ on the secretion of IL-12α/β. The secretion of the 70 kD IL-12 was further confirmed by blotting the immuno-precipitants with a polyclonal IL-12 Ab (Figure 3-B). This polyclonal Ab also revealed the secretion of free IL-12β at 40

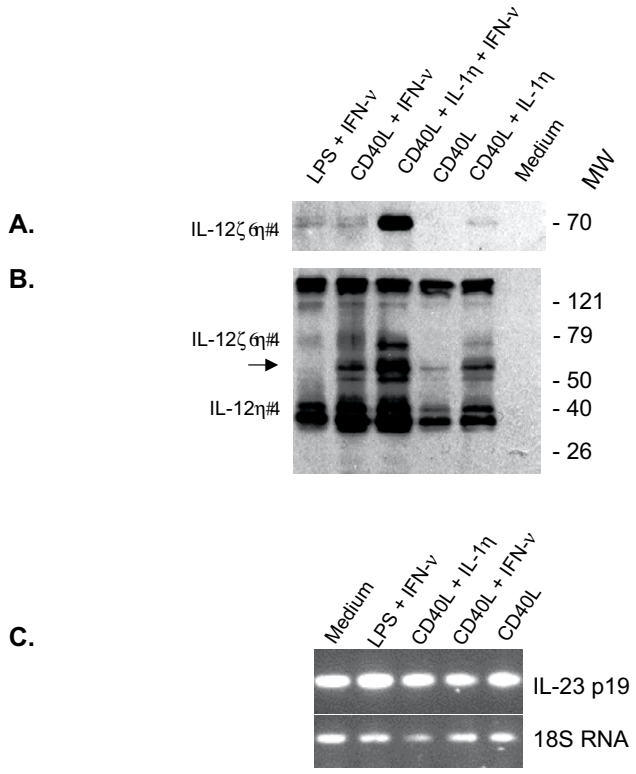

Figure 3

CD40L+IL-1β-treated DC secrete IL-12 and IL-23 heterodimersA-B. Immature DC were stimulated for 24 hours with LPS+IFN-γ, CD40L+IFN-γ, CD40L+IL-1β+IFN-γ, CD40L or CD40L+IL-1β. Supernatants and control AIM-V medium were concentrated by centrifugation, then subject to immunoprecipitation with anti-IL-12p40/p70 monoclonal antibody (clone C8.6, PharMingen) and A/G agarose beads. Immunoprecipitants were separated by 10% SDS PAGE under non-reducing conditions. **A**. Detection of IL-12p70 using IL-12p70-specific rat mAb 20C2 (PharMingen). **B.** Detection using anti-IL-12 polyclonal goat Ab (R&D Systems). **C**. RT-PCR analysis for p19 and 18s RNA. DC were stimulated with indicated stimuli for 8 h. Total RNA was extracted, reverse-transcribed and used as template for amplification during 35 cycles. Results show ethidium bromide detection of PCR products on agarose gel (represents one of 3 separate experiments).

kD in all conditions tested and the presence of a 60 kD complex in some conditions (arrow). It is very likely that this 60 kD complex formed between IL-12β (40 kD) and the recently described p19 subunit chain form the IL-23 heterodimer [20]. RT-PCR confirmed that p19mRNA was found in DC stimulated with CD40L and IL-1 (Figure 3-C) and the detection of IL-23 in CD40L-activated DC is consistent with prior reports [20]. Interestingly, immunoprecipitation studies suggest that the IL-23 60 kD complex is produced at a higher level upon treatment with CD40L+IL-1β+IFN-γ.

Production of IFN-γ by DC

RPA studies indicated that IFN-γ mRNA was induced after stimulation of DC with CD40L+IL-1β, CD40L+LPS or LPS+IFN-γ (Figures 1 and 2). Production of IFN-γ has been reported in murine DC [21] but not to our knowledge in human DC. To verify these findings, we analyzed the production of IFN-γ and IL-12β in DC by flow cytometry. Results of 4 separate experiments showed the presence of intracellular IFN-γ as demonstrated by staining levels above isotype-matched controls in cells of large size, expressing HLA-DR or CD1a but lacking CD3 or CD56 (Figure 4 and not shown). Under these conditions, DC cultured in medium or with LPS or LPS+IL-1 did not express significant levels of IFN-γ (data not shown), thus arguing in favor of a specific response. Co-staining for IFN-γ and IL-12p40 further showed that a subset of human monocyte-derived DC could produce both cytokines. Statistical analysis showed that DC stimulated with CD40L or CD40L+IL-1β (respectively containing 3.4 ± 2.5 % and 3.9 ± 2.8 % cells expressing IFN-γ) produced significantly more IFN-γ than control DC (0.08 ± 0.1 %), $p < 0.05$. This is consistent with mRNA data shown in Figure 1.

Regulation of cytokine production by molecules related to IL-1

IL-1β, like other ligands of TLR/IL1R (LPS or peptidoglycan) [19] is able to co-induce the secretion of IL-12 prompting us to ask if signaling through the IL-1R superfamily could represent a general mechanism for the induction of IL-12 in DC. Like IL-1β, IL-1α acted in combination with CD40L to increase the secretion of IL-12 in a dose-dependent manner (Figure 5-A). Comparable dose-response curves were obtained with IL-1α and IL-1β which at doses of 10 ng/ml co-induced the secretion of 14.9 ± 3.8 and 13.0 ± 4.3 pg IL-12/1000 cells, respectively, while CD40L alone induced 0.3 ± 0.4 pg IL-12/1000 cells (n = 2). On the other hand, IL-18 showed no effect, even at concentrations up to 500 ng/ml (Figure 5-A) as IL-18+CD40L was not different from CD40L alone (1.12 ± 0.9 and 0.4 ± 0.3 pg/1000 cells respectively, n = 2). While IL-1α and IL-1β share the same signal-transmitting receptor complex of IL-1RI and IL-1R3, IL-18 engages a distinct receptor complex IL-1R5/IL-1R7 [17,22]. The expression of IL-18R was undetectable on DC by flow cytometry, although it was found on the surface of activated T cells (data not shown). The lack of responsiveness by DC to IL-18 appears to be due to the lack of IL-18R expression on monocyte-derived DC.

Both unstimulated DC and activated DC constitutively expressed IL-1Ra mRNA. This IL-1 family member negatively regulates IL-1 by competitively binding to IL-1RI without inducing the signaling cascade (reviewed in [23]). This suggested that IL-1Ra could perturb the effects of IL-

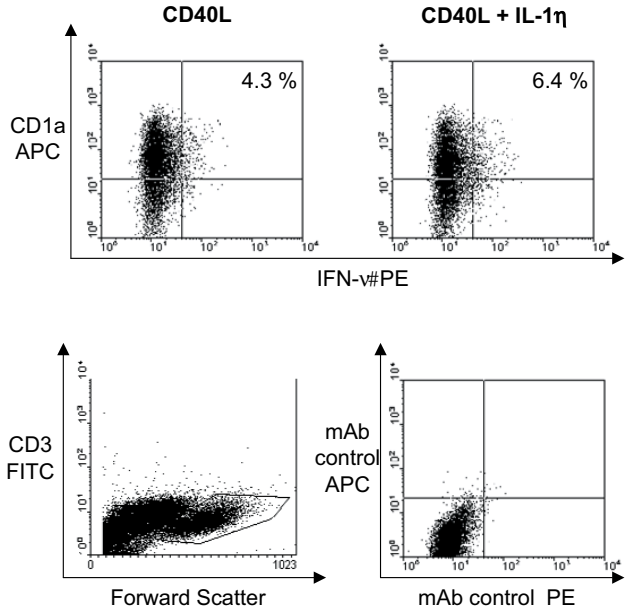

Figure 4
Production of IFN-γ by human monocyte-derived DC DC were treated with CD40L (top left panel) or CD40L+IL-1β (top right panel) for 24 hours, monensin being present during the last 5 hours of incubation. After staining for surface markers and intracellular IFN-γ, DC were analyzed by flow cytometry. Large $CD3^-$ cells were analyzed (as shown in gate represented in bottom left dot-plot) to exclude T cells, and results show staining above isotype control (bottom right dot-plot), being expressed as percentages of $CD1a^+$ cells expressing IFN-γ. Result representative of 4 experiments.

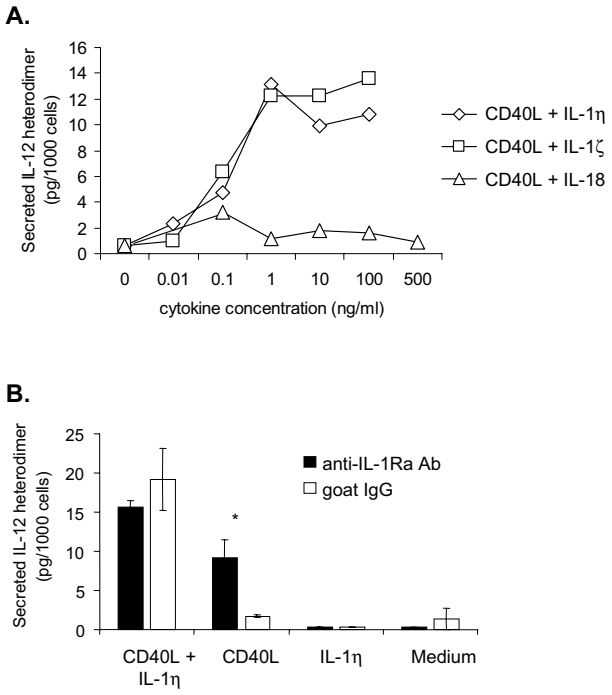

Figure 5
Effects of IL-1 family membersA, IL-12 secretion is modulated by IL-1α or IL-1β, but not by IL-18. Human monocyte-derived DC were treated with CD40L (1 μg/ml) for 24 hours in the presence of increased concentrations of IL-1α, IL-1β or IL-18 (0 to 100 or 500 ng/ml). Secreted IL-12 heterodimer was measured in supernatants by ELISA. **B**, Blocking IL-1Ra increases CD40L-induced secretion of IL-12 heterodimer. DC were activated as indicated in the presence of either anti-IL-1Ra neutralizing Ab or normal goat IgG for 24 hours. Supernatants were harvested and analyzed for IL-12 heterodimer by ELISA. The average secretion in duplicate wells was calculated as pg/1000 cells. Results are representative of three experiments. * indicates statistical significance of anti-IL-1Ra Ab treatment.

1 signaling in DC and could impact on the regulation of IL-12 production. Figure 5-B shows a representative experiment with the specific inhibition of IL-1Ra by neutralizing antibodies. Anti-IL-1Ra Ab significantly increased the secretion of IL-12 induced by CD40L relative to DC treated with control IgG and CD40L (4.65 ± 3.7 and 0.85 ± 0.7 respectively; $p < 0.02$, n = 3). The neutralizing anti-IL-1Ra antibody did not affect the production of IL-12 when DC were activated with medium or IL-1β alone which is consistent with the fact that these conditions do not up-regulate IL-12β on their own. The neutralizing antibody also did not affect the levels of IL-12 produced in response to CD40L+IL-1β. In this case, the exogenous IL-1 provided maximal induction of IL-12 and was probably largely in excess of the endogenous IL-1Ra, therefore inhibition of IL-1Ra did not provide additional induction of IL-12. Altogether, these results provide evidence that both IL-1Ra mRNA and IL-1 mRNA are translated in CD40L-treated DC, and act in a biologically relevant manner. Further, this demonstrates that constitutive IL-1Ra expression regulates the response of DC to endogenous IL-1.

DC stimulated by CD40L+IL-1β effectively prime and stimulate the differentiation of $CD4^+$ and $CD8^+$ T cells

DC treated with CD40L+IL-1β produced high levels of pro-inflammatory cytokines such as IL-12 and IL-23 that are known to augment T cell activation but also produce inhibitory molecules such as IL-10 or IL-1Ra with opposite effects. To assess the net outcome of this stimulation, T cell-stimulating properties of these DCs were examined using priming of naïve cord blood $CD8^+$ T cells to the HLA-A2-restricted fluMP peptide. The detection of fluMP-specific cells in culture was done by flow cytometry using Flu-MP-HLA-A2 tetramers. DC activated with CD40L+IL-1β appeared to prime more effectively than DC activated with CD40L or than immature DC (Table 1 and Figure 6-A). Absolute numbers of tetramer-positive cells induced by CD40L+IL-1β-primed DC are significantly higher than

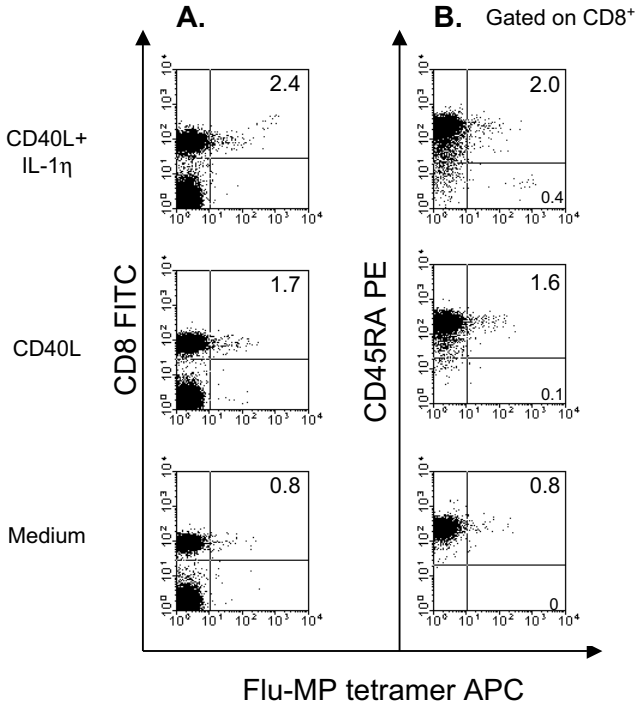

Figure 6
CD40L+IL-1β stimulated DC are highly effective APCs for CD8+ T cell priming. HLA-A2+ cord blood-derived immature DC were treated with CD40L+IL-1β, CD40L or medium for 16 hours, pulsed with Flu-MP peptide, washed and used to prime naïve autologous T cells. On day 7, T cell cultures were analyzed for the presence of antigen-specific CD8+ T cells using Flu-MP-HLA-A2 tetramers. Representative dot plots are shown of CD8 vs. Flu-MP tetramer (**A**), and CD45RA vs. Flu-MP tetramer (gated on CD8+ cells) (**B**). All were gated on live, PI- cells; quadrants were set based on background staining with control mAbs. Percent positivity is indicated in corresponding quadrants.

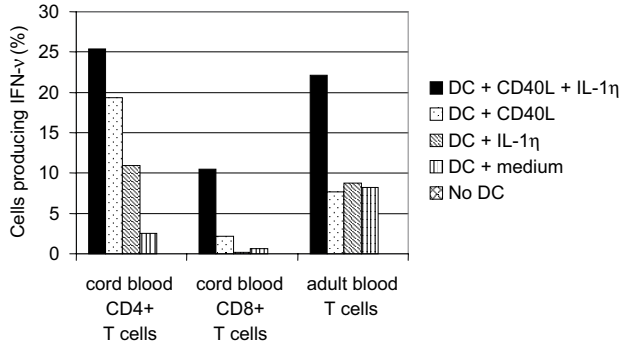

Figure 7
CD40L+IL-1β stimulated DC induce high levels of IFN-γ in MLR. DC were stimulated as indicated, then washed prior to culture with purified, allogeneic T cells. T cell priming was assessed by measuring IFN-γ producing cells with flow cytometry after a brief restimulation on anti-CD3/anti-CD28 coated plates. Data represent results of three independent experiments.

in cultures primed by CD40L-activated DC ($p < 0.05$, t test). These enhancing effects of CD40L + IL-1 over CD40L were blocked by polyclonal Abs to IL-12 recognizing IL-12β-associated proteins suggesting that the enhanced production of IL-12 and/or IL-23 is responsible for this activity (Table 1, experiment 4). Strikingly, peptide presentation by DC matured with CD40L+IL-1β induced the rapid differentiation of antigen-specific cells as seen by the presence of Flu-MP-HLA-A2^{high} CD45RA- CD8+ T cells (Figure 6-B). This was confirmed at day 14 post priming (data not shown). Induction of allogeneic responses was examined by measuring the production of IFN-γ in MLR. Naïve cord blood CD4+ or CD8+ T cells or adult blood T cells produced more IFN-γ when primed with DC treated with CD40L+IL-1β than with DC treated with CD40L alone or IL-1β alone (Figure 7). Thus, these DC are highly effective and functional APCs that act in a manner consistent with the production of high levels of pro-inflammatory cytokines such as IL-12, IL-23, IL-18 and IFN-γ that are known to activate CD4+ and CD8+ T cells and cellular immunity in general.

Discussion

We herein show that various pro-inflammatory molecules can generate distinct activation profiles in DC and that members of the IL-1 family of proteins play a biologically relevant role in the regulation of the functional differentiation of DC.

Multiple activation signals appear to be required to induce the production of high levels of cytokines by DC. The production of IL-12 which is a pivotal event in the development of type-1 cellular immunity is effectively induced *in vitro* by treating DC with CD40L+IL-1β and even more so by CD40L+IL-1β+IFN-γ, in agreement with recent findings by Luft et al. [15]. Such synergistic concept is consistent with *in vivo* studies showing the dependence on both microbial signals and endogenous CD40L to induce effective immunity [13]. Exposure to multiple pro-inflammatory mediators is likely to occur under biological circumstances as these mediators can be transcriptionally induced in a contemporary manner and are regulated by autocrine loops, commonly described in inflammation [24]. In combination with CD40L, IL-1 induces the production of IL-12, IL-1, IFN-γ and IL-18 in DC. IL-18, acting in combination with IL-12 could induce large amounts of IFN-γ by lymphocytes, further sensitizing DC to produce extremely high levels of IL-12. The role played by DC-derived IFN-γ remains unclear. Neutralization studies suggest that IFN-γ is not responsible for IL-12 production in

Table 1: Effects of DC activation on priming of CD8+ T cell responses

Exp. [a]	DC used in priming [b]		Percent of CD8+ cells binding Flu-MP-HLA-A2 tetramer	Absolute number of Flu-MP specific cells	Fold expansion Flu-MP specific T cells
1	CD40L + IL-1β		2.39	5020	Not determined
	CD40L		1.68	2900	"
	Immature		0.84	1190	"
2	CD40L + IL-1β		0.28	1400	7.0
	CD40L		0.24	528	2.6
	Immature		0.25	656	3.3
3	CD40L + IL-1β		0.26	594	2.9
	CD40L		0.14	309	1.5
	Immature		0.00	0	0
4	CD40L + IL-1β	IgG	1.39	2870	19.1
	CD40L	"	0.64	945	6.3
	Immature	"	0.61	1130	7.5
	CD40L + IL-1β	Anti-IL-12	0.57	1270	8.5
	CD40L	"	0.78	1200	8.0
	Immature	"	0.41	700	4.7

(a) Neonatal umbilical cord blood was used for exp. 1–3, adult mobilized peripheral blood was used in exp. 4. (b) Monocyte-derived DC cultured in GM-CSF+IL-4 were used as immature DC (unstimulated), or after activation with CD40L (1 ug/ml) or CD40L + IL-1β (1 ug/ml, 10 ng/ml respectively) for 24 hours. In experiment 4, polyclonal neutralizing Abs to IL-12 or normal goat IgG (1 μg/ml), were added at time of priming.

response to IL-1β and CD40L [14]. As IFN-γ is produced approximately 24 hours after stimulation it seems that its role would be to contribute to T cell activation rather than to DC activation itself. DC-derived IFN-γ has been reported in the mouse [21], albeit under distinct activating conditions. Based on these studies it remains to be tested if IFN-γ production by human DC might also be indirectly due to the presence of IL-12 and IL-18.

Here, we report distinct pro-inflammatory stimuli that all induce high levels of IL-12 but appear to provide otherwise distinct responses. In contrast to IL-1, co-stimulation with IFN-γ induced relatively little IL-10, IL-6, IL-18 and IFN-γ. LPS induced higher IL-10, higher IL-1Ra and strikingly less IL-23. The difference in the ability to produce IL-10 was confirmed at the protein level (data not shown and [14]). While IL-12 is important for naïve CD4+ T cell priming, IL-23 is reportedly important for memory CD4+ cells [20]. Differences between IL-12 and IL-23 production suggest that DC may display distinct cytokine profiles that correspond to distinct phases of an immune response. Another interpretation is that DC may interpret distinct pathogen signals, for instance IL-1 or LPS, to mount different types of cytokine reponses [19,25,26]. This would be desirable to optimize host defense against particular aggressions or to be effective in particular tissues. The biological consequences of these differences will have to be determined in future studies.

An important aspect of the autocrine regulation of cytokines in DC is the production of negative regulators such as IL1Ra. Genetic invalidation of IL-1Ra not only upregulates inflammation but also affects the immune system, causing spontaneous development of auto-immune arthritis in certain strains of mice and rendering other strains more susceptible to immunogens causing this disease [27]. The precise mechanisms underlying the role of IL-1Ra in immune responses are complex, but implicate antigen presentation. APCs of mice lacking IL-1Ra induce higher proliferation and higher activation of wild-type CD4+ T cells than control APCs, resulting in the up-regulation of CD40L, OX40 and IL-2Rα, and thus have the opposite effects of APCs lacking IL-1 [28]. Our own data provide a potential mechanistic explanation clearly showing for the first time that IL-1Ra can have a direct role on APC function by regulating the secretion of IL-12. Neutralization experiments in DC showed that IL-1Ra inhibited the secretion of IL-12 in response to CD40L. This is

most likely through negative regulation of endogenous IL-1 responses as well as CD40L-induced IL-1 in DC. While the RPA technique did not reveal IL-1 transcripts in unstimulated immature DC, it has been reported that IL-1β mRNA are detected by the more sensitive RT-PCR analysis in freshly-isolated Langerhans cells, generally considered a paradigm for unstimulated immature DC [29]. Furthermore, direct evidence of IL-1β protein secretion has been shown in monocyte-derived DC [15]. This demonstrates that autocrine cytokine regulation via IL-1 family members is a biologically relevant mechanism in DC. As IL-1Ra appears to be a negative regulator of DC activation, it is notable that the LPS+IFN-γ stimulation induces the highest levels of IL-1Ra mRNA in DC. As high levels of IL-1α, IL-1β are also induced, IL-1Ra may provide a negative balance to prevent the establishment of an IL-1-mediated autocrine stimulation cascade and to curtail the effects of LPS. Thus, IL-1Ra may be one significant built-in mechanism in the APC to regulate its immuno-stimulatory properties and to prevent the development of uncontrolled immunity. The biological relevance of these conclusions seems further supported by genetic analyses studies in humans. Polymorphisms in the IL-1Ra gene such as the IL1RN*2 allele cause quantitative differences in both IL-1Ra and IL-1β production and associate with the severity of inflammatory and auto-immune diseases such as ulcerative colitis and Crohn's disease, lupus erythematosus and possibly with coronary artery disease. Conversely, these polymorphisms negatively associate with various infections such as vaginal colonization with mycoplasmas, cytomegalovirus, Epstein-Barr virus, human immunodeficiency virus and with the occurrence of ovarian cancer (recently reviewed in [30]).

Our results therefore identify IL-1 as an effective co-activator of DC in conjunction with CD40L. This is consistent with the fact that IL-1 has been recognized as an adjuvant for almost 2 decades [31] and may refine our understanding of the effects of IL-1 in the context of DC-initiated immunity. Thus, IL-1 which is produced locally and systemically in response to inflammation or infection may play an important role as a T cell-independent factor complementing the effects of T cell-derived CD40L. Upon encountering these two molecules, DC would become more effective APCs, producing IL-12, IL-23, IL-18 and IFN-γ and stimulating the differentiation of $CD4^+$ and $CD8^+$ T cells. Besides implications in inflammatory, infectious or auto-immune diseases, our data are also consistent with the identification of IL-1 as an important adjuvant of CTL immunization *in vivo* in conjunction with IL-12, IL-18 or IFN-γ [32]. Like IL-1, IL-12 is not critically required for the generation of CTL, yet the addition of exogenous IL-12 or the inhibition of IL-12 respectively improve or inhibit the development of CTL [33,34]. Our results confirm that IL-12 is not essential for priming against fluMP since neutralizing Abs do not block the development of specific T cells. However, we show that enhanced production of IL-12 or IL-23 by CD40L+IL-1-activated DC mediates the enhancing effects on the priming of $CD8^+$ T cell responses.

Conclusions

Altogether, we predict that CD40L+IL-1β-treated DC will function as effective vaccines for the induction of cellular immunity *in vivo*. As we see that various degrees of pro-inflammatory functional differentiation can be acquired by DC, it seems that the rational use of adjuvants based on molecular regulation of cytokines in DC may yield useful vaccines for immunotherapy.

Methods

Source of cells

Human blood samples were obtained with approval from the Institutional Review Board of Wayne State University. G-CSF mobilized peripheral blood was obtained from breast cancer patients at the Karmanos Cancer Institute. Cord blood samples were obtained from Hutzel Hospital, Detroit MI. Mononuclear cells (MNC) were isolated by centrifugation over Ficoll (Amersham Pharmacia Biotech, Piscataway, NJ) ($d < 1.077$ g/ml) and were cryopreserved in liquid nitrogen using a 10% DMSO freezing solution.

Culture of DC

Monocytes were obtained by incubating MNC on tissue culture plates (2×10^6 cells per ml per well in 24 well plates) in RPMI medium with 10% fetal bovine serum (FBS) (R10) [35] in a humidified atmosphere at 37°C, 5% CO_2 for 2 hours, followed by washing to remove non-adherent cells. These adherent cells were cultured in R10 medium with GM-CSF (25 ng/ml, Immunex, Seattle, WA), and IL-4 (100 U/ml, kind gift from Dr. H Yssel, DNAX Research Institute, Palo Alto, CA) to induce DC differentiation.

DC activation

Following 6 days of culture, cells were harvested and washed twice in cytokine-free medium, prior to incubation with stimuli such as: human recombinant CD40L trimer (1 μg/ml; kind gift of Immunex), IL-1β (10 ng/ml, unless stated otherwise; Biological Resource Branch Repository, Rockville, MD), LPS (100 ng/ml; Sigma, St. Louis MO), IFN-γ (100 ng/ml; R&D Systems), IL-1α (various concentrations; R&D Systems), IL-18 (various concentrations; R&D Systems), TNF-α (50 ng/m; RDI, Flanders, NJ). To measure intracellular cytokine production, monensin (2 μM; Sigma) was added to the DC culture for the last 5 hours. To measure cytokine secretion by ELISA, supernatants were collected after 24 hours and stored frozen at -20°C until tested. Endotoxin levels in working concentrations of cytokines, media and CD40L were determined to

be less than 0.01 ng/ml by Limulus amoebocyte lysate gel assay (Sigma).

Immuno-staining and flow cytometric analysis

Intracellular staining was done as previously described [14], Briefly, after fixation in 4% formaldehyde, cells were washed twice in PBS then permeabilized in saponin buffer (0.1% saponin (Sigma), 0.2% BSA, 0.02% sodium azide, in PBS). Non-specific Fc binding was blocked by incubation for 10 min. on ice with excess human gamma-globulin in saponin buffer (1 mg/ml; Gamimune, Miles, Eckhert, IL), then cells were incubated for 30 minutes on ice with mAbs diluted in saponin buffer followed by 2 washes in saponin buffer. Cells were resuspended in wash buffer without saponin prior to analysis. FITC or APC-conjugated anti-IL-12 (BD-PharMingen), which recognizes the IL-12 β-chain either alone or in IL-12 α/β heterodimer, was used for detection of intracellular IL-12β in DC, while PE-conjugated anti-IFN-γ (clone 4S.B3, Pharmingen) were used for measuring intracellular IFN-γ by DC or T cells. Irrelevant control mAbs were used as negative controls of non-specific staining. Cells were analyzed on a FACSCalibur instrument (Becton Dickinson) and data were analyzed using WinMDI (Version 2.8) software.

ELISA

IFN-γ was measured in supernatants using the OptEIA ELISA set for IFN-γ according to manufacturer's instructions (BD-PharMingen). The lower limit of detection was 4 pg/ml.

Non-isotopic RNase protection assay (RPA)

Following activation with various stimuli for 8 hours (unless otherwise indicated), total RNA was isolated from DC using RNA Isolator (Sigma-Genosys, The Woodlands, TX). Biotin labeled RNA probes were transcribed from PharMingen's Multi-Probe template set using Maxiscript transcription kit (Ambion Inc., Austin, TX) withT7 RNA polymerase and biotin-14-CTP (Gibco-BRL, Rockville, MD) used at a 40:60 ratio with unlabeled CTP. RPAs were performed using Ambion's RPA III kit according to manufacturer's instructions. Briefly, sample RNA was hybridized to 8 ng biotin-labeled probes overnight at 56°C, then digested with RNase A/T. Digested probes were analyzed on a 8 M urea/5% polyacrylamide gel (19:1acrylamide:bis-acrylamide, (BioRad, Hercules, CA)) in TBE. Separated probes were transferred to a positively charged nylon membrane by a semi-dry electroblotter (Owl Separation Systems, Portsmouth, NH), UV cross-linked (Stratagene, La Jolla, CA), then subjected to chemiluminescent detection using Ambion's BrightStar Biodetect kit with CDP-Star substrate. Membranes were exposed either to Kodak Biomax ML film, or directly to the Kodak Image station 440 CF for a one-hour capture. Images analysis was performed using the Kodak Image station 440 CF and 1D Image Analysis software. Results are expressed as the ratio of net signal intensity for a particular mRNA to the net signal intensity of the L32 housekeeping gene.

Immunoprecipitation and western analysis

DC supernatants were concentrated by centrifugation through YM-10 concentrators (Millipore Corp., Bedford, Massachusetts) then immunoprecipitated with anti-IL-12β (C8.6, PharMingen) and A/G agarose bead conjugates (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Precipitants were separated by 10% SDS-PAGE under non-reducing conditions, transferred to PVDF membrane, and blotted with anti-IL-12p70-specific rat monoclonal Ab 20C2 (PharMingen) or anti-IL-12 goat polyclonal Ab (R&D Systems). HRP-conjugated goat-anti-rat or donkey-anti-goat polyclonal secondary Abs were visualized using the ECL detection system (Amersham Biosciences, Piscataway, NJ). Blots were exposed to Biomax ML film as above.

Blocking with neutralizing antibodies

To examine the effects of IL-1ra on DC activation, goat anti-human neutralizing IgG specific for IL-1Ra (R&D Systems) and normal goat IgG (R&D Systems) were added at the start of DC stimulation with CD40L. To examine the effects of IL-12 in priming experiment 4, polyclonal neutralizing Abs to IL-12 ((R&D Systems) or normal goat IgG (1 μg/ml), were added in the T cell/DC culture at initiation of priming.

Naïve CD8+ T cell priming

DC, prepared from HLA-A2$^+$ cord blood (CB) MNC, were activated overnight as described above, then pulsed with 10 μg/ml influenza matrix peptide Flu-MP (58–66 GILGFVTL; Genemed Synthesis, San Francisco, CA). Autologous purified T cells were obtained by negative selection, using mAbs specific for CD14 (3C10), CD40 (G28.5), CD32 (IV-3), CD11b (OKM1), glycophorin A (10F7MN) (ATCC), and HLA-DP/DQ (SPVL-3) (kind gift of Dr. H Yssel), followed by incubation with sheep anti-mouse magnetic beads (Dynal, Lake Success, NY). Cells were further depleted with mAbs to HLA-DR and CD16 (Caltag) and a second round of selection with beads. The resulting cell preparations contained > 95% CD3$^+$ T cells. Peptide pulsed-DC were irradiated (400-cGy ^{137}Cs source), washed twice, then incubated with purified T cells in R10 medium supplemented with 10 ng/ml IL-7 (R&D Systems) at 37°C, 5% CO_2. Cultures were replenished with R10 and IL-7 on day 3–4 by demi-depletion, and were examined for the presence of antigen specific cells by flow cytometry on day 7. Briefly, T cells were counted then incubated in the dark at 37°C for 15 min. with APC-labeled Flu-MP-HLA-A2 tetramer constructs (NIAID Tetramer Facility and the NIH AIDS Research and Reference Reagent Program, Atlanta, GA). After washing,

cells were incubated with anti-CD8-FITC (OKT8, ATCC, conjugated in our lab) and anti-CD45RA-PE (Caltag) mAbs, then washed. Cells were resuspended with 5 μg/ml propidium iodide (Sigma) for exclusion of dead cells and analyzed by flow cytometry. The limit of detection of $CD8^+$ Flu-MP-HLA-A2 $tetramer^+$ T cells was 0.02–0.04% as determined by dilutions of a flu-MP specific $HLA\text{-}A2^+$ T cell line in $HLA\text{-}A2^-$ cord blood MNC. Negative control consisted of $HLA\text{-}A2^-$ cord blood MNC in which < 0.01% (n = 3) $CD8^+$ Flu-MP-HLA-A2 $tetramer^+$ cells were detected. Percent of $CD8^+$ cells binding Flu-MP-HLA-A2 tetramer were calculated by setting quadrants based on staining with isotype control Abs (staining <0.01%), and calculated as (% Flu-MP-HLA-A2 $tetramer^+$ $CD8^+$ cells) / (% total $CD8^+$ cells). Absolute number of $tetramer^+$ cells was calculated as (% $CD8^+$ Flu-MP-HLA-A2 $tetramer^+$ cells) × (total number of cells in culture after priming). Fold expansion specific T cells was calculated as (absolute number of $tetramer^+$ cells after priming) / (absolute number of $tetramer^+$ cells in initiating cultures).

Mixed lymphocyte reaction (MLR)

The procedure has been described elsewhere [14]. Briefly, preparations >90% pure T cells were obtained from MNC by removal of cells recognized by mAbs to CD14 (3C10), CD40 (G28.5), CD32 (IV-3), CD11b (OKM1), glycophorin A (10F7MN), (ATCC, Manassas, VA) HLA-DP/DQ (SPVL-3, kind gift of Dr. H Yssel) and CD16 (KD1, kind gift of Dr. Ferlazzo, Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy), HLA-DR and CD15 (Caltag) using indirect panning on goat-anti-mouse IgG (Sigma)-coated plates, followed by a second round of negative selection with sheep anti-mouse magnetic beads (Dynal, Lake Success, NY). Populations of $CD4^+$ or $CD8^+$ T cells were obtained by removing the opposite subset in the second round of purification. Allogeneic DC were irradiated and cultured with pure T cells at the concentration of 20% DC:T cell ratio in R10 medium for 8 days. On day 8, supernatant fluids were collected to measure cytokine content by ELISA and cells were harvested to be re-stimulated for 5 hours on plates coated with mAbs to CD3 and CD28 (2 μg/ml each) (PharMingen, San Diego, CA). Monensin (2 μM; Sigma) was added to enable the detection of intracellular IFN-γ by flow cytometry.

RT-PCR

Total RNA prepared from DC was reverse transcribed after incubation with Superscript II reverse transcriptase (Gibco) and random hexanucleotide primers for one hour at 37°C. The resulting cDNA was amplified by PCR using AmpliTaq DNA polymerase (Applied Biosystems (PE Corp), Foster City, CA) and primers for p19 (forward: 5' AGC AGC TCA AGG ATG GCA CTC AG 3'; reverse: 5' CCC CAA ATT TCC CTT CCC ATC TA 3') and for 18S ribosomal RNA (Ambion) for 35 cycles (1 min 95°C, 2 min 56°C, 1 min 70°C). PCR products were separated on a 2% agarose gel, stained with ethidium bromide.

Statistical analysis

Paired t-Test analysis was used to assess statistical differences where indicated.

Authors' contributions

Author AW carried out the cellular and molecular experiments, participated in the design of the study and performed the statistical analysis. Author AG conceived of the study, and participated in its design and coordination. All authors read and approved the final manuscript.

Abbreviations

CD40L = recombinant human CD40 ligand, DC = dendritic cells, TLR = Toll-like receptor, RPA = RNase protection assay

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