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CD40 Ligation Ablates the Tolerogenic Potential of Lymphoid Dendritic Cells

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The outcome of dendritic cell (DC) presentation of P815AB, a tolerogenic tumor/self peptide, depends on a balance between the respective immunogenic and tolerogenic properties of myeloid (CD8α−) and lymphoid (CD8α+) DC. We have previously shown that CD8α− DC can be primed by IL-12 to overcome inhibition by the CD8α+ subset and initiate immunogenic presentation in vivo when the two types of peptide-pulsed DC are cotransferred into recipient hosts. IFN-γ enhances the inhibitory activity of CD8α+ DC on Ag presentation by the other subset, blocking the ability of IL-12-treated CD8α− DC to overcome suppression. We report here that CD40 ligation on lymphoid DC ablated their inhibitory function on Ag presentation as well as IFN-γ potentiation of the effect. CD40 modulation of IFN-γ action on lymphoid DC involved a reduction in IFN-γR expression and tryptophan-degrading ability. This effect was accompanied in vitro by an impaired capacity of the CD40-modulated and IFN-γ-treated DC to initiate T cell apoptosis. In vivo, not only did CD40 triggering on lymphoid DC abrogate their tolerogenic activity, but it also induced the potential for immunogenic presentation of P815AB. Importantly, a pattern similar to P815AB as well as CD40 modulation of lymphoid DC function were observed on testing reactivity to NRP, a synthetic peptide mimotope recognized by diabetogenic CD8α+ T cells in nonobese diabetic mice. The Journal of Immunology, 2001, 166: 277–283.

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urine dendritic cells (DC) are freshly isolated from the spleen fall into two subsets. In addition to a population resembling CD34−-derived myeloid DC, a second subset expresses both CD8α and DEC-205 and is considered to be of lymphoid origin (1, 2). Lymphoid DC are thought to be important in deletional tolerance, as they appear to be resident, sedentary cells present in secondary lymphoid organs (3, 4). These cells have the ability to phagocytose other cells, including myeloid DC, and cross-present Ag derived from phagocytosis, inducing deletional tolerance (5). CD8α+ DC also produce high amounts of IL-12 when pulsed with Ag in vitro. When injected into recipient hosts, they initiate T cell priming, resulting in a Th1-type response (6). These two apparently conflicting properties of lymphoid DC, namely their ability to mediate tolerogenic effects and their Th1-promoting activity, could be reconciled by the idea that the induction of deletional tolerance is accompanied by very early production of Th1 (IL-2, IL-3, and IFN-γ), but not Th2 (IL-4 or IL-10), cytokines (7), which is consistent with the high level of IL-12 production by stimulated lymphoid DC (6, 8). Interesting in this regard is the finding that lymphoid DC may themselves produce significant amounts of IFN-γ in response to IL-12 (9).

Using an in vivo model of tumor/self peptide (P815AB) presentation for induction of class I-restricted skin test reactivity (10, 11), we have previously shown that CD8α− DC negatively regulate the induction of T cell reactivity by peptide-loaded myeloid DC. By virtue of their respective actions on CD8α− (12, 13) and CD8α+ (14) DC, IL-12 and IFN-γ have functionally opposing effects on peptide presentation by the CD8α− DC subset, and IFN-γ-activated lymphoid DC mediate tolerogenic effects that prevail over the immunoadjuvant activity of IL-12 on myeloid DC. Interestingly, the negative regulatory effect triggered by IFN-γ in lymphoid DC appears to be restricted to P815AB, as it is not observed with evolutionarily distant Ags and involves interference with tryptophan metabolism in vivo. We have suggested that 1) DC might discriminate self from nonself in an inflammatory context dominated by IFN-γ and that 2) IFN-γ acting on lymphoid DC might contribute to the maintenance of T cell tolerance to self Ags via tryptophan degradation affecting T cell responses (14).

As we expand on this hypothesis, we demonstrate here that triggering of CD40 on CD8α− DC will abrogate their susceptibility to the tolerogenic activity of IFN-γ in the induction of T cell reactivity to P815AB. The effect involves down-regulation of IFN-γR expression and leads to reduced ability of CD8α− DC to metabolize tryptophan and mediate T cell apoptosis in vitro. In addition, CD40 activation on CD8α− DC makes these cells capable of immunogenic presentation of P815AB. Finally, a pattern similar to that of P815AB and a similar effect of CD40 ligation are observed with a synthetic peptide that acts as a mimotope for autoimmune diabetes model. Collectively, these findings may help to explain recent evidence in different experimental systems that CD40-CD40 ligand interactions are crucial in determining whether naive peripheral T cells are primed or tolerated upon DC presentation of tumor/ self peptides (15).

Materials and Methods

Mice, cytokines, and reagents

DBA/2J (H-2d) mice were obtained from Charles River Laboratories (Calco, Milan, Italy). Male mice were used at the age of 2–4 mo. The source and characteristics of murine rIL-12 (12), hamster anti-murine CD40 (HM40-3) mAb used in combination with goat anti-hamster IgG
Results

IFN-γ and CD8+ DC induce T lymphocyte tolerance

Our previous studies have shown that IL-12 in vitro confers priming ability on splenic DC pulsed with an otherwise tolerogenic tumor/self peptide (P815AB) (10, 12, 13). In fact, the adjuvant activity of IL-12 on CD8+ DC counteracts a tolerogenic effect of CD8+ DC that can be detected even when these cells represent as little as 3% of the DC population (11). In contrast, IFN-γ acts on CD8+ DC to abolish the adjuvant activity of IL-12 on myeloid DC (14). Using IL-12-activated CD8+ DC in a model system for assessment of T cell tolerance (11), we investigated whether IFN-γ acting on lymphoid DC would initiate long-lasting effects on host responsiveness to P815AB. Peptide-loaded CD8+ DC, either alone or in combination with 3% CD8+ DC, were injected into recipient mice to be assayed for skin test reactivity to the eliciting peptide. Each DC fraction was used either as such or after treatment with IL-12 (for CD8+ DC) or IFN-γ (for CD8+ DC; Fig. 1A). As expected, IFN-γ completely abolished the adjuvant activity of IL-12 on myeloid DC when the animals were assayed for skin test reactivity to P815AB at 15 days after cell transfer. To study the impact of a previous exposure to IFN-γ-treated CD8+ DC, groups of mice were further immunized on day 15 with IL-12-activated and peptide-pulsed CD8+ DC. The animals were then assayed for skin test reactivity to P815AB after 2 wk. Fig. 1B shows that exposure of
mice to IFN-γ-treated CD8+ DC resulted in a tolerant state that could not be reversed by the use of IL-12-activated CD8+ DC. As unresponsiveness persisted when the second cell transfer was delayed up to 40, 60, or 90 days after the tolerogenic priming (data not shown), these findings suggested the occurrence of deletional tolerance initiated by the action of IFN-γ on lymphoid DC. This condition appeared to be different from the anergic state induced by host transfer with unfractionated DC pulsed with P815AB, as the latter represents a reversible phenomenon that is no longer observable at 40–60 days after tolerogenic priming (11).

**CD40 ligation blocks the effect of IFN-γ on CD8+ DC**

In several experimental models it has been shown that the induction of tolerance vs immunity is determined by resting vs activated APC (15), which can be effectively modulated via CD40-CD40 ligand interactions (21). Therefore, we became interested in ascertaining whether CD40 ligation on CD8+ DC might affect their negative regulatory function in the priming to P815AB as well as their susceptibility to the effect of IFN-γ. Recipient mice were injected with a combination of peptide-pulsed CD8+ and 3% CD8+ DC. Each DC fraction was used either as such or after cytokine treatment in vitro. Groups of CD8+ DC were exposed to anti-CD40 mAb plus goat anti-hamster Ab as described in Materials and Methods before IFN-γ treatment (200 U/ml for 6 h). Fig. 2 shows that CD40 activation on CD8+ DC blocked their inhibitory function on peptide presentation by CD8+ DC. In addition, this maneuver reversed the effect of IFN-γ on CD8+ DC. These data demonstrated that CD40 ligation on CD8+ DC could prevent T cell anergy induced by a tolerogenic peptide alone or by a combination of the peptide with IFN-γ.

**Down-regulation of IFN-γR expression by anti-CD40 mAb**

Final maturation of DC is known to be associated with impaired responsiveness to IFN-γ and to be correlated with reduced expression of IFN-γR (22). In addition, CD40 activation is one of the critical signals that allow the full maturation of DC (21, 23). Therefore, we examined any possible regulation of IFN-γR expression by CD40 activation in lymphoid vs myeloid DC. We first assayed the qualitative expression of mRNA specific for the α-chain of murine IFN-γR. Using RT-PCR, we obtained evidence that CD40 ligation on CD8+ DC completely ablated the expression of CD119-specific messages (Fig. 3). We next examined the surface expression of the IFN-γR α-chain by flow cytometry using biotinylated rat IgG to murine CD119 (14). The results showed that triggering of CD40 on lymphoid DC resulted in a marked decrease (at 24 h) or even disappearance (at 48 h) of the α-chain of the IFN-γR. This effect was in contrast with that of CD40 ligation on CD8+ DC, where no significant changes in CD119 expression were observed at 48 h (Fig. 3). Although down-regulation of IFN-γR α-chain expression may be a major mechanism by which CD40 ligation blocks the effect of IFN-γ on CD8+ DC, these data did not exclude the possibility of a differential effect of CD40 ligation on the survival of lymphoid vs myeloid DC.

**Antagonistic effect of CD40 activation on IDO induction by IFN-γ**

There is now enough evidence to support a protective role of IFN-γ in experimental models of T cell-mediated autoimmune. In many of these models, IFN-γ may have an essential role in stimulating APC to produce NO (24–26). NO is an inducer of apoptosis in a variety of cell types, including T cell clones and activated CD4+ T cells (27). However, it is also evident from these models that induction of NO does not account for all the negative regulatory effect of IFN-γ (27). IFN-γ-dependent production of IDO by macrophages is known to result in inhibition of T cell proliferation through tryptophan degradation (28). Recent evidence indicates that IDO production by human DC may suppress T cell proliferation (19). We have already shown that the effect triggered by IFN-γ in CD8+ DC involves interference with tryptophan metabolism in vivo upon transfer of P815AB-pulsed DC (14). Therefore, we sought to determine whether CD40 ligation on CD8+ DC might interfere with IDO activity as induced by IFN-γ treatment. The functional activity of IDO produced by activated CD8+ DC was measured in terms of the ability to metabolize tryptophan to kynurenine. Although not entirely quantitative, this assay provides a reliable means of measuring functional IDO activity, reflecting a multifaceted combination of IDO expression, tryptophan transport into the cells, and intracellular conditions that post-translationally affect enzyme activity (28). CD8+ DC were treated overnight with IFN-γ or were exposed sequentially to anti-CD40 mAb and IFN-γ. Cells were then washed and resuspended in HBSS containing 100 μM tryptophan and incubated at 37°C for 4 h. Supernatants were harvested, and kynurenine was measured by HPLC. Fig. 4 shows that virtually no kynurenine was detected in supernatants of DC unexposed to IFN-γ. However, DC activation with the cytokine alone resulted in high levels of tryptophan breakdown product. This effect of IFN-γ was completely blocked by pre-exposure of CD8+ DC to anti-CD40 mAb. These data demonstrated that at least one functional property of IFN-γ, namely, the induction of IDO activity, is abolished by CD40 activation that results in down-modulation of IFN-γR expression.

**Antagonistic effect of CD40 activation on induction of apoptosis by lymphoid DC**

We next examined whether expression of IDO might contribute to the negative regulatory and tolerogenic properties of IFN-γ via induction of T cell apoptosis in our model system with P815AB. We used a P815AB-specific CD4+ T cell clone (20) for measurement of apoptosis upon coculture of the latter cells with unfractionated DC exposed to IFN-γ, either alone or in combination with anti-CD40 mAb. Fig. 5 shows that the addition of IFN-γ-treated DC to clone F76 cells cultured in the presence of P815AB caused a significant proportion (~40%) of these cells to undergo apoptosis. The effect was inhibited either by pre-exposure of the DC to
anti-CD40 mAb or by the addition of the competitive inhibitor of IDO, 1-MT, to the coculture of DC and CD4\(^+\) T cells. Fig. 5 also shows that no effect was induced by IFN-\(\gamma\) when the purified CD8\(^+\) fraction was used in the place of unfractionated DC. These data suggest that IFN-\(\gamma\) may act through expression of IDO in CD8\(^+\) DC to regulate T cell apoptosis and could explain our previous observation that inhibition of IDO will abrogate the negative regulatory role of CD8\(^+\) DC in vivo (14).

**CD40 ligation renders lymphoid DC capable of presenting P815AB in an immunogenic fashion**

We next examined whether triggering of CD40 on lymphoid DC might not only abolish their tolerogenic activity but also render the cells capable of effective presentation of P815AB in the absence of the CD8\(^-\) component. Recipient mice were transferred with \(3 \times 10^5\) CD8\(^-\) or CD8\(^+\) DC; the latter cells were used either as such or after treatment with anti-CD40 mAb. Mice were assayed for skin test reactivity to P815AB at 2 wk after DC transfer. Fig. 6 shows that in contrast to untreated cells, the CD40-modulated lymphoid DC were fully capable of presenting P815AB in an immunogenic fashion. Although the number of CD40-modulated CD8\(^-\) DC injected per mouse was considerably higher than that occurring in a DBA/2 spleen, these data indicated that CD40 activation might induce the potential for immunogenic presentation of P815AB in lymphoid DC. Furthermore, these data tend to rule out the possibility that selective death of CD8\(^-\) DC is a major mechanism through which CD40 ligation exerts its effect on presentation of P815AB in vivo and breakdown of tryptophan into kynurenine in vitro.

**Inhibitory activity of CD8\(^+\) DC and opposing effect of CD40 ligation on presentation of a peptide mimotope for autoimmune diabetes**

To investigate whether the distinct patterns of activity observed with different DC subsets and the effect of CD40 ligation could be observed with self peptides other than P815AB, we used a peptide mimotope recognized by diabetogenic CD8\(^+\) T cells in NOD mice. NRP is a synthetic nonapeptide that elicits the proliferation, cytokine secretion, differentiation, and cytotoxicity of a diabetogenic DC.
H-2Kd-restricted CD8+ T cell specificity that uses a TCRβ rearrangement frequently expressed by CD8+ T cells propagated from the earliest insulitic lesions of NOD mice (17, 18). In an experimental model analogous to that of P815AB, NRP-pulsed DC were transferred into recipient hosts either as such or after cell fractionation and treatment with anti-CD40 mAb. After 2 wk the animals were assayed for skin test reactivity (Fig. 7). Analogous to the pattern of P815AB, unfractionated DC pulsed with NRP failed to induce a delayed-type hypersensitivity response. In contrast, a strong response was induced by the CD8- cell fraction alone, an effect that was negated by the addition of 3% CD8+ DC. Remarkably, treatment of the CD8+ fraction with anti-CD40 mAb restored the ability of CD8- DC to present NRP in an immunogenic fashion.

Discussion

In this paper we present additional in vivo and in vitro evidence that IFN-γ may limit the T cell response to a tumor/self peptide by inducing IDO expression in lymphoid DC (14). Furthermore, we demonstrate that CD40 activation in these cells 1) ablates their basal inhibitory activity on peptide presentation by CD8+ DC, 2) down-regulates IFN-γR expression and makes lymphoid DC unresponsive to IFN-γ, and 3) induces the potential for immunogenic presentation of P815AB in this otherwise tolerogenic DC subset.

Lymphoid DC are considered to be of primary importance in deletional tolerance (2–4). These cells have the ability to phagocytose other cells and may be able to cross-present Ag obtained from phagocytosis, inducing deletional tolerance (5, 29, 30). The hypothesis that CD8- DC are stimulatory whereas CD8+ DC may restrict the immune response and play a role in tolerance induction is further supported by the observation that DC within the T cell areas (the majority of which expresses CD8αβ) present high levels of self peptides (31). However, two recent studies have revealed that both CD8- and CD8+ DC can sensitize naïve T cells and direct the development of distinct Th subpopulations (32), thus calling attention to a possible major role of DC in regulating Th1 vs Th2 development. In a study by Maldonado-López et al. (6), splenic CD8+ DC transferred intrafootpad were found to prime an immune response to keyhole limpet hemocyanin that was dominated by Th1 cytokines. In contrast, administration of CD8+ DC induced a Th2 response. In another study using DC subsets pulsed with an OVA peptide (33), CD8+ DC were found to induce high concentrations of the Th1 cytokines IFN-γ and IL-2, but little or no Th2 cytokines. In contrast, myeloid DC induced large amounts of the Th2 cytokines IL-4 and IL-10, in addition to IFN-γ and IL-2.

The reason for the different regulations of IFN-γ and IL-12 in these studies is not clear, but could be related to differences in the purification procedures, the maturation state of the transferred DC, the form of Ag, and/or the frequency of Ag-specific T lymphocytes in the recipients (32).

The apparent contradiction between the tolerogenic properties of lymphoid DC and their ability to produce large amounts of IL-12 (6, 8) could be explained in several ways. In autoimmunity, it has been proposed that proinflammatory cytokines may be required at an early time to induce self-reactive responses by priming inflammatory Th1 responses, however, the late expression of the same cytokines could drive the terminal differentiation and death of T cells, including those engaged in autoreactive responses (34). Smith et al. (7) recently reported that tolerance induction may indeed require an early production of Th1 cytokines, accounting for the ability of lymphoid DC to produce IL-12. DC themselves may produce significant amounts of IFN-γ in response to IL-12, with the cytokine being released mainly by CD8+ DC (9). It has been recently shown that paralysis of IL-12 production by CD8+ DC may prevent infection-induced immunopathology in response to microbial products (35). In our model system with a tumor/self peptide, we have proposed the occurrence of a negative feedback loop in which IFN-γ down-regulates the presentation of self Ags via a direct action on lymphoid DC (14). The hypothesis of such a loop is not in conflict with the widely accepted idea that IFN-γ activates different types of myeloid APC to produce IL-12, according to a positive feedback system that is probably required for optimal production of IL-12 in the early response to foreign entities, such as conserved molecules on bacteria and other evolutionarily distant organisms (36).

There is now substantial evidence to indicate a protective role for proinflammatory cytokines in experimental models of T cell-mediated autoimmunity (34, 37). In many of these models, IFN-γ has been shown to act via induction of NO by APC (24–26), although NO may not account for all the observed regulatory effects of IFN-γ (27). Although IFN-γ-dependent production of IDO by macrophages is known to result in inhibition of T cell proliferation through tryptophan degradation (28), recent evidence suggests that...
IDO production by human DC may also suppress T cell proliferation (19). In our experimental model of P815AB-specific CD4+ cells, we found that the blockade of IDO activity by a competitive inhibitor would negate the induction of apoptosis in vitro by IFN-γ-treated DC. This suggests that IDO induction is a major mechanism by which IFN-γ acts on DC to mediate apoptosis of T cells. Our current data may represent the first experimental evidence for the involvement of tryptophan degradation in the regulation of T cell apoptosis. A recent study indicates that a metabolite of the tryptophan/kynurenine pathway, 3-hydroxyanthranilic acid, can act as an endogenous inducer of apoptosis in monocyte/macrophage cell lines (38).

Triggering of DC in vivo through CD40 is a powerful activation stimulus, causing these cells to express the full array of Ag-presenting/costimulatory molecules (21, 39) and regulating their migration in vivo (40). Moreover, injection of CD40-modulated DC restores Ag-specific CTL responses in CD4+ T cell-depleted mice (41). These data indicate that the function of CD4+ T cells is mediated through CD40-dependent activation of APC. Recent evidence suggests that the CD40-CD40 ligand pair can act as a switch in vivo, determining whether naive peripheral CTL are primed or tolerized (42) and accounting for the ability of CD40 ligation to convert tumor-specific CD4+ T cell tolerance into T cell priming (43). In line with these observations, we demonstrate here that CD40 activation on lymphoid DC overcomes T cell unresponsiveness to an otherwise tolerogenic tumor/self peptide even when the potentiating effect of IFN-γ on lymphoid DC would result in the induction of peripheral tolerance. Under these conditions, triggering of CD40 is sufficient to prevent tolerization of T cells as assessed by CD4+ T cell-dependent induction of skin test reactivity mediated by CD8+ T cells (10, 11). These findings may be important for improved understanding of the requirements for Th cell function in avoiding tolerance induction, particularly in regard to the idea that Ag-specific T cell tolerance is known to limit the efficacy of therapeutic cancer vaccines.

Spontaneous autoimmune diabetes in NOD mice is the result of a CD4+ and CD8+ T cell-dependent autoimmune process directed against the pancreatic β-cells. In an attempt to extend our current results with P815AB to different peptides, we have resorted to a recently described peptide mimotope recognized by CD8+ T cells from NOD mice (17, 18). Besides representing a peptide ligand for CD8+ T cells in autoimmune diabetes, NRP will induce the deletion of specific CD8+ T cells under selected conditions of immunization (18). We found that the pattern of immune response induced by transfer of NRP-pulsed DC was similar to that of P815AB when different DC subsets were injected either singly or in combination. Most importantly, CD40 ligation on lymphoid DC negated their ability to modulate the immunogenic presentation of NRP by myeloid DC.

In conclusion, the data reported here reinforce our previous suggestion that murine DC are able to discriminate self from nonself in an inflammatory context dominated by IFN-γ, calling attention to the possibility that tryptophan degradation may be one mechanism by which IFN-γ acting on CD8+ DC contributes to the maintenance of peripheral T cell tolerance. As it is known that CD40/CD40 ligand interactions are crucial in conditioning immunogenic vs tolerogenic presentation of tumor/self peptides to T cells by DC (15, 21, 42–44), the finding that CD40 activation on CD8+ DC abrogates their tolerogenic potential in vivo may have important implications for autoimmunity and immunotherapy of cancer. It is likely that a variety of different effects contributes to the ability of CD40 activation to prevent IFN-γ-driven tolerance mediated by lymphoid DC, including down-regulation of IFN-γR expression. However, the finding of antagonistic effects of IFN-γ treatment and CD40 activation on lymphoid DC suggests that IFN-γ modulation of these cells may be a useful means of limiting autoimmune disease sustained by activated T cells and further supports the clinical use of CD40-stimulating agents as components of anti-cancer vaccines.

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References


