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# A new carboxamide compound exerts immunosuppressive activity by inhibiting dendritic cell maturation

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The immunosuppressive properties of a benzamide derivative, JM34, previously characterized as an anti-inflammatory compound are described. The immunosuppressive potential of JM34 was evidenced by inhibition of PBMC proliferation *in vitro* with an $IC_{50}$ of 20 μM. In contrast with classical immunosuppressive drugs, JM34 affected neither cytokine production nor IL-2R expression from activated T cell clones, and displayed only moderate inhibition of IL-2-induced or anti-CD3/anti-CD28-induced proliferation. We investigated its effects on dendritic cells (DC) *in vitro*. Addition of JM34 during DC maturation inhibited the expression of some maturation markers: specifically, MHC molecule up-regulation was totally inhibited and CD83 expression was significantly reduced, while up-regulation of CD86, CD80 or CD40 was less affected. Moreover, JM34-treated DC showed impaired IL-12 but not IL-10 secretion, and a markedly reduced ability to present antigens to naive T lymphocytes *in vitro*. We provide evidence that these JM34-induced alterations of DC were associated with a marked inhibition of NF-κB nuclear translocation. Finally, JM34 inhibited delayed type hypersensitivity dose dependently in mice. In conclusion, our data suggest that JM34 inhibited T lymphocyte activation mainly by targeting DC, and thus may represent a new class of therapeutic agents in the fields of transplantation and autoimmune diseases.

**Key words:** Dendritic cells · Antigen presentation · Immunosuppression · Lymphocytes · Transplantation

## Introduction

In keeping with the fundamental role of T cells in immune responses, much of the development of immunosuppressive drugs has focused on targeting T cells. Thus, all immunosuppressive drugs in clinical use today inhibit T cell activation either through a direct cytotoxic effect (cyclophosphamide), through inhibition of *de novo* purine or pyrimidine synthesis [azathioprine, Mycophenolate Mofetil (MMF)] or inhibition of lymphokine production [cyclosporin A (CsA)] or response (rapamycin) [1].

The two major fields of application of these drugs are graft rejection and autoimmune diseases. In transplantation, their use either alone or in combination has indeed significantly improved graft survival [2, 3]. However, since these drugs have broad immunosuppressive effects on all T cells populations, they impair memory response against virally infected cells and thus facilitate viral infection and development of lympho-

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**Abbreviations: iDC:** Immature DC · **mDC:** Mature DC · **CsA:** Cyclosporin A · **Dex:** Dexamethasone · **DTH:** Delayed type hypersensitivity · **KLH:** Keyhole limpet hemocyanin · **MFI:** Mean fluorescence intensity · **MMF:** Mycophenolate mofetil

 

proliferative disorders in long-term-treated patients [4, 5]. Therefore, an alternate immunosuppressive strategy is being considered, *i.e.* modulation of APC function [6]. Dendritic cells (DC), the major APC *in vivo*, represent a large family of different subsets and lineages of migratory APC [7]. During migration, DC undergo maturation which involves up-regulation of molecules such CD40, CD80/CD86, CD83, ICAM-1, MHC class II, decreased antigen-processing and increased antigen-presenting abilities [8, 9].

Different agents have been shown to alter DC maturation such as glucocorticoids [10, 11], aspirin [12], vitamin D [13, 14], or MMF [15]. We have previously described a family of benzamide derivatives that display anti-inflammatory activities *in vivo* [16] and inhibit TNF-α production from stimulated macrophages *in vitro* [17, 18]. In the present study, we provide evidence that the representative compound JM34 strongly inhibited DC maturation, but had little direct effect on T lymphocyte activation. This inhibition of DC maturation was associated with a significant inhibition of NF-κB translocation in these cells.

## Results

### JM34 inhibited PBMC proliferation

Fresh PBMC from healthy donors were stimulated with 0.3 μg/ml PHA (Fig. 1A) or irradiated allogeneic PBMC (MLR reaction) (Fig. 1B) for 48 h with various doses of JM34. As shown in Fig. 1A, JM34 inhibited mitogen-dependent PBMC proliferation in a dose-dependent manner. A plateau of inhibition of 80% (similar to the inhibition with 5 μM CsA) was obtained with JM34 at 100 μM. The 50% inhibitory concentration ($IC_{50}$) was about 20 μM, which was significantly lower than the $IC_{50}$ of 100 μM for anti-inflammatory properties [17]. Similarly, JM34 inhibited alloantigen-dependent T cell proliferation in a dose-dependent manner with a maximal inhibition of 84% at 100 μM (Fig. 1B). These anti-proliferative effects were not due to direct toxicity of JM34 since 95% of the cells were still viable after 48-h incubation with 100 μM JM34 (not shown).

### Effects of JM34 on T cell activation

We first examined whether cytokine production of $CD4^+$ and $CD8^+$ T cell clones activated by their specific targets was altered by JM34 addition. CsA at 0.5 μM, used as a positive control, induced a significant decrease in the percentages of IL-2-, IFN-γ- and TNF-α-producing cells (Table 1). In contrast, 100 μM JM34 reduced neither the percentage (Table 1) nor the mean fluorescence intensity (MFI) (not shown) of $CD4^+$ or $CD8^+$ T cells positive for IL-2, IL-4, IFN-γ or TNF-α.

Next, we analyzed whether expression of CD25 on T cell clones was affected by JM 34. $CD4^+$ and $CD8^+$ clones were stimulated by precoated anti-CD3 mAb with or without JM34 and CD25 expression was analyzed by flow cytometry. Results showed that CD3 cross-linking efficiently up-regulated CD25 expression both in terms of percentage of positive cells and MFI and that JM34 had no effect on this CD25 up-regulation (Table 2).

Since neither cytokine production nor IL-2 receptor expression was altered by JM34 treatment, we tested whether JM34 inhibited activation pathways triggered by IL-2R stimulation. We assessed the proliferative response of the CTL-L2 cell line to IL-2 in the presence of JM34. Rapamycin, which is known to block IL-2 transduction pathways [19] was used as positive control. As shown in Fig. 2, 50 nM rapamycin completely inhibited IL-2-dependent proliferation of CTL-L2 cells. In contrast, JM34 only showed a significant inhibition of CTL-L2 proliferation (around 30% inhibition) at the maximal dose of 100 μM, whereas at 50 μM, JM34 showed no significant inhibition (Fig. 2).

To further evaluate the direct effect of JM34 on T cell activation, we tested it on freshly purified T cells

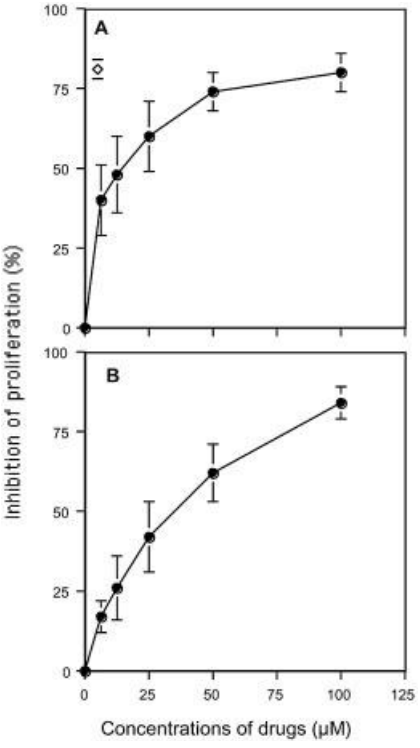


**Fig. 1.** Effect of JM34 on T cell proliferation. PBMC were stimulated for 48 h with either 0.3 μg/ml of PHA (A) or with irradiated allogeneic stimulated PBMC ($1\times10^5$/well) (B) in the presence of increasing concentrations of JM34 (filled circles) or 5 μM CsA (open diamond). Mean ± SEM of three independent experiments is presented.

 

**Table 1.** Effect of JM34 on cytokines production by activated $CD4^+$ and $CD8^+$ T cells clones [a]

| | | IL-2 | IL-4 | TNF-α | IFN-γ |
|---|---|---|---|---|---|
| CD8 clones | | | | | |
| A4-5 | unstim. | 0.4 | 0.1 | 0 | ND |
| | Stim. | 43.1 | 5.4 | 66.2 | ND |
| | Stim. + JM34 100 μM | 43.2 | 5.4 | 68.1 | ND |
| A2-10 | Unstim. | 0.9 | 0.2 | ND | ND |
| | Stim. | 51.1 | 45.9 | ND | ND |
| | Stim. + JM34 100 μM | 50.9 | 43.2 | ND | ND |
| 10-C10 | Unstim. | 0.5 | ND | 0.7 | 0.1 |
| | Stim. | 55.9 | ND | 78.5 | 73.2 |
| | Stim.+ JM34 100 μM | 56.3 | ND | 72.2 | 66.0 |
| | Stim.+ CsA 0.5 μM | 14.0 | ND | 4.9 | 1.3 |
| 40-D2 | unstim. | 4.2 | ND | ND | ND |
| | Stim. | 52.8 | ND | ND | ND |
| | Stim.+ JM34 100 μM | 50.8 | ND | ND | ND |
| | Stim.+ CsA 0.5 μM | 11.4 | ND | ND | ND |
| CD4 clones | | | | | |
| 201 | Unstim. | 0.2 | ND | 1.7 | 0.5 |
| | Stim. | 0.5 | ND | 35.9 | 26.1 |
| | Stim.+ JM34 100 μM | 0.5 | ND | 32.1 | 20.3 |
| 401 | Unstim. | 0.2 | ND | 1.1 | 0.1 |
| | Stim. | 0.7 | ND | 17.0 | 4.7 |
| | Stim.+ JM34 100 μM | 0.4 | ND | 14.3 | 3.7 |

[a] Cytokines production was assessed by intracellular staining after stimulation by T2 cells alone (Unstim.) or pulsed with the relevant EBV or Melan-A epitopes for CD8 clones or allogeneic stimulation by B-EBV cell line for CD4 clones (Stim). Results are expressed as the percentage of positive cells. Data are from one representative experiment of three performed. ND: not done.

stimulated with microbeads precoated with anti-CD3 mAb alone or in combination with anti-CD28 mAb. Purified T cells proliferated poorly in response to anti-CD3 alone, but significantly when CD28 co-stimulation was provided (2,514±159 cpm with anti-CD3 mAb alone *vs.* 39,547±2,798 cpm for anti-CD3 + anti-CD28 mAb) (Fig. 3). This confirmed that our T cell preparations were devoid of monocytes/macrophages since very few APC are sufficient to provide co-stimulation to T cells and would have triggered proliferation in response to anti-CD3 alone. JM34 at 100 μM inhibited 47% of the proliferative response to anti-CD3 + anti-CD28 mAb, but this inhibition was no longer significant at 50 μM (Fig. 3). These data were thus in agreement with the CTL-L2 experiments and suggested that only at 100 μM did JM34 display a significant yet partial inhibition of T cell activation.

**Table 2.** Effect of JM34 on CD25 expression by activated $CD4^+$ and $CD8^+$ T cells clones[a)]

| | | CD25 | |
|---|---|---|---|
| | | % positive cells | MFI |
| CD8 clones | | | |
| 10C10 | Alone | 0 | 7.9 |
| | + anti-CD3 | 96.7 | 70.9 |
| | + anti-CD3+JM34 50 μM | 95.1 | 75.8 |
| | + anti-CD3+JM34 100 μM | 97.7 | 83.3 |
| 24G7 | Alone | 1.12 | 5.3 |
| | + anti-CD3 | 85.2 | 65.1 |
| | + anti-CD3+JM34 50 μM | 83.8 | 66.0 |
| | + anti-CD3+JM34 100 μM | 83.5 | 60.1 |
| CD4 clones | | | |
| M576 | Alone | 0 | 3.06 |
| | + anti-CD3 | 97.2 | 135.0 |
| | + anti-CD3+JM34 50 μM | 97.6 | 155.8 |
| | + anti-CD3+JM34 100 μM | 97.8 | 149.6 |

[a)] CD25 expression was assessed after stimulation with anti-CD3 mAb (5 μg/ml) for 18 h. Results are expressed as percentage of positive cells or MFI. Data are from one representative experiment of three performed.

Therefore, the major inhibition of PBMC proliferation observed with JM34 at doses of 50 μM and lower (Fig. 1) could not be explained by a direct effect of JM34 on T cells.

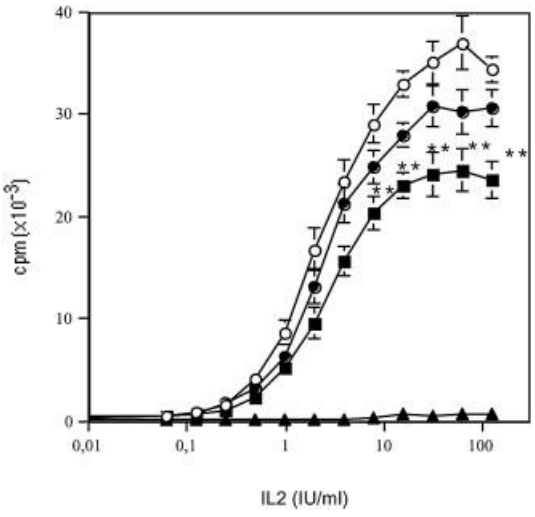


**Fig. 2.** Effect of JM34 on IL-2-dependent CTL-L2 proliferation. CTL-L2 cells were cultured for 18 h with a range of IL-2 concentrations either alone (open circles) or with 50 μM JM34 (closed circles) or 100 μM JM34 (closed squares) or with 50 nM rapamycin (closed triangles). Results are expressed as mean ± SEM of three experiments. $^{**}p<0.01$ compared to CTL-L2 alone.

 

## JM34 inhibited maturation and function of DC

We next hypothesized that JM34 may target APC rather than T lymphocytes, and analyzed its effects *in vitro* on the expression of DC maturation markers. Immature DC (iDC), differentiated from peripheral monocytes were matured with either 1 μg/ml LPS (Fig. 4A) or with 500 ng/ml recombinant Flag-CD40L + 1 μg/ml anti-Flag mAb (Fig. 4B). DC maturation was shown by the induction of the maturation marker CD83 and up-regulation of CD40, MHC class I and II, CD80 and CD86 molecules. A summary of four experiments using LPS-matured DC (mDC) is shown in Table 3. Incubation with JM34 during the maturation phase dose-dependently inhibited the induction of CD83, as assessed by the percentage of positive cells (27±3% for JM34 at 100 μM *vs.* 63±7% for untreated mDC; $p<0.01$) and slightly decreased the up-regulation of CD86 assessed by MFI, although not significantly (41±17 for JM34 at 100 μM *vs.* 70±6 for untreated mDC). By contrast, JM34 had little effect on the expression of CD80 and CD40 (Table 3). In the case of HLA-ABC molecules, maturation-induced up-regulation was totally abrogated with 20 μM JM34 (260±56 for untreated mDC *vs.* 139±29

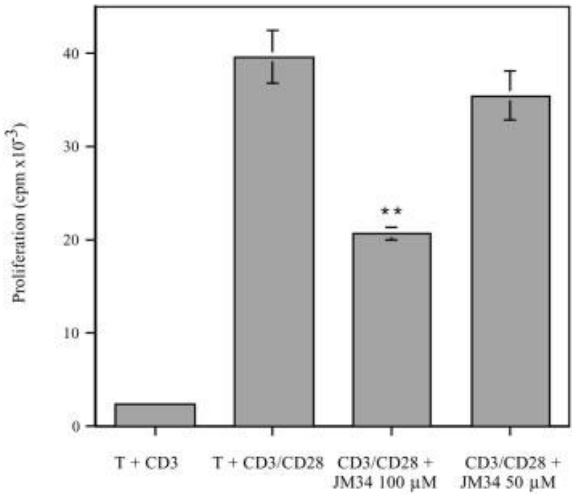


**Fig. 3.** Effect of JM34 on the proliferation of purified T lymphocytes stimulated with anti-CD3 + anti-CD28 mAb. T cells were incubated for 72 h with microbeads coated either with anti-CD3 alone (to control for the absence of APC in the preparation) or with a combination of anti-CD3/CD28 mAb in the presence or not of JM34. Mean ± SEM of three independent experiments is presented. $^{**}p<0.01$ for 100 μM JM34 *vs.* anti-CD3/CD28 alone.

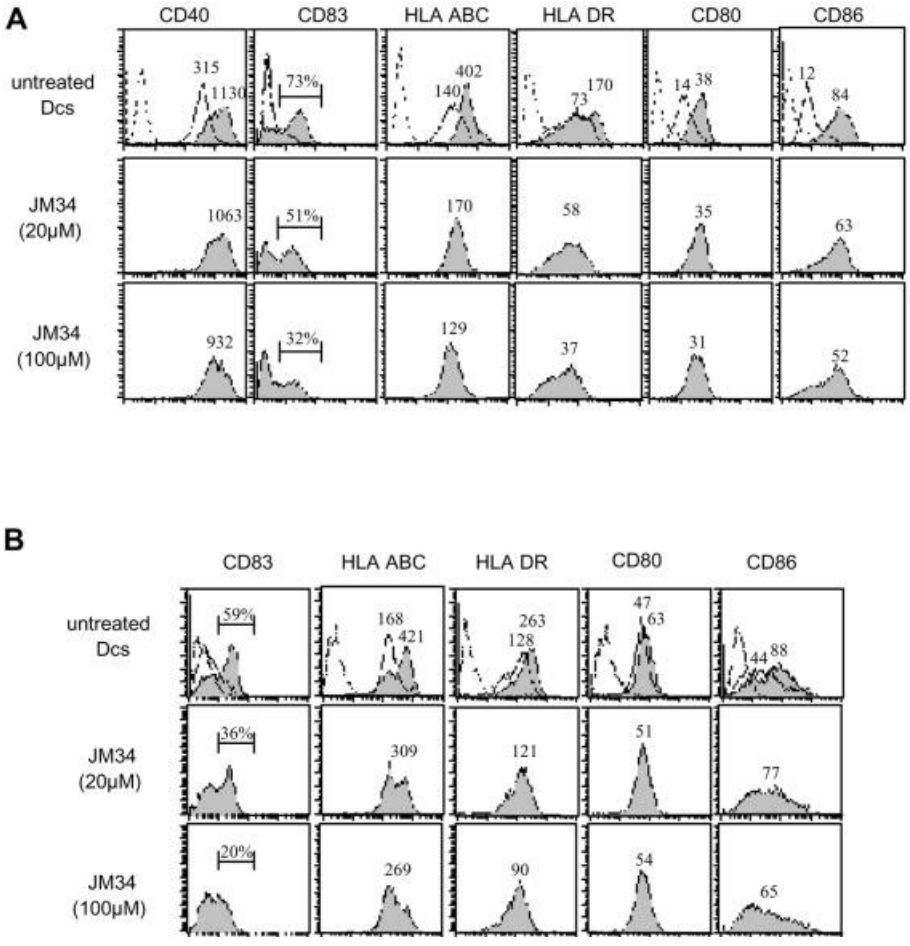


**Fig. 4.** Effect of JM34 on DC maturation. DC were generated as described in Material and methods. Maturation was induced by incubation of cells with 1 μg/ml LPS (A) or with 500 ng/ml Flagged-rCD40L + 1 μg/ml anti-Flag mAb (B) for 48 h and expression of surface markers was analyzed by flow cytometry. Dotted histograms represent staining with a control isotype, open histograms staining of iDC and dark histograms staining of mDC. Percentages of positive cells or MFI are indicated. Data are from one representative experiment out of four in each panel.

 

**Table 3.** Phenotypic analysis of DC matured in the presence of JM34[a)]

| | iDC | | mDC | | mDC + JM34 (20 μM) | | mDC + JM34 (100 μM) | |
|---|---|---|---|---|---|---|---|---|
| | % positive cells | MFI | % positive cells | MFI | % positive cells | MFI | % positive cells | MFI |
| CD83 | 3±0.5 | - | 63±7 | - | 43±6* | - | 27±3*** | - |
| CD80 | - | 16±4 | - | 48±5 | - | 46±11 | - | 35±6 |
| CD86 | - | 10±2 | - | 70±6 | - | 43±16 | - | 41±17 |
| CD40 | - | 383±67 | - | 1105±25 | - | 1009±85 | - | 889±47 |
| HLA I | - | 140±11 | - | 260± 56 | - | 139±29* | - | 118±25* |
| HLA DR | - | 89±12 | - | 139±10 | - | 69±8** | - | 38±7*** |

a) DC were generated as described in Material and methods. Maturation was induced by incubation of cells with LPS (1 μg/ml) for 48 h with or without JM34. Percentage of positive cells or MFI are expressed as mean ± SEM of four different experiments.
*$p<0.05$; **$p<0.01$; ***$p<0.001$ when compared to mDC.

for treated mDC, $p<0.05$ *vs.* 140±11 for iDC) and concerning HLA class II, the levels of expression of treated mDC were even lower than that of iDC (139±10 for untreated mDC *vs.* 69±8 for treated mDC, $p<0.01$ *vs.* 89±12 for iDC) (Table 3). Similar results were obtained using CD40L as maturation agent (Fig. 4B) or a combination of TNF-α/poly IC (data not shown).

We next studied the effect of JM34 on IL-12p70 and IL-10 secretion measured by ELISA on 48 h supernatants from DC matured with either LPS or TNF-α/poly IC. As shown in Fig. 5A, mDC produced significant levels of IL-12 (151±47 pg/ml) but very little secretion by iDC. Treatment of DC with JM34 during the maturation phase markedly decreased IL-12 secretion (79±21 pg/ml at 20 μM and 50±12 pg/ml at 100 μM, $p<0.05$). Maturation of DC also induced a significant increase in IL-10 secretion (256±58 pg/ml *vs.* 44±8 pg/ml for iDC) but in contrast with IL-12, treatment with JM34 did not significantly alter IL-10 secretion (239±45 pg/ml at 20 μM and 208±48 pg/ml at 100 μM) (Fig. 5B).

Finally, we tested the capacity of iDC pulsed with antigen and subsequently matured with LPS in the presence of JM34 to sensitize autologous naive T lymphocytes. None of the donors had been previously exposed to this antigen, and a previous study has shown that keyhole limpet hemocyanin (KLH) alone does not induced DC maturation [20]. As shown in Fig. 6A, KLH-pulsed mDC induced a significantly higher lymphocyte proliferation than KLH-pulsed iDC at all stimulator/responder (S/R) ratios tested. In contrast, KLH-pulsed DC matured in the presence of JM34 displayed a dramatic decrease in their T cell-activating properties such that they did not differ from KLH-pulsed iDC at S/R ratio of 1:40 and 1:80. Maximal inhibition was reached as soon as 20 μM JM34. Similar results were obtained in allogeneic stimulations (Fig. 6B). Finally, it should be pointed out that mDC were washed extensively before being mixed with T lymphocytes and thus only trace amounts of JM34, released by DC, could have been present during the mixed cultures.

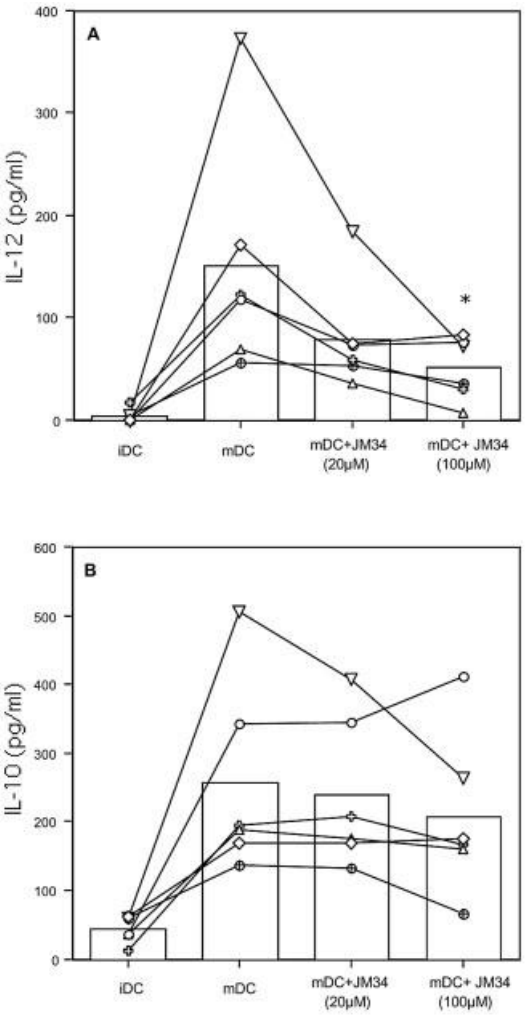


**Fig. 5.** Effect on JM34 on DC cytokine production. IL-12 (A) and IL-10 (B) production was assessed by ELISA in supernatants of DC matured with 1 μg/ml LPS or TNF-α/Poly IC for 48 h with or without JM34. Bars represent the mean of the six independent experiments depicted. *$p<0.05$ for mDC + 100 μM JM34 *vs.* mDC alone.

 

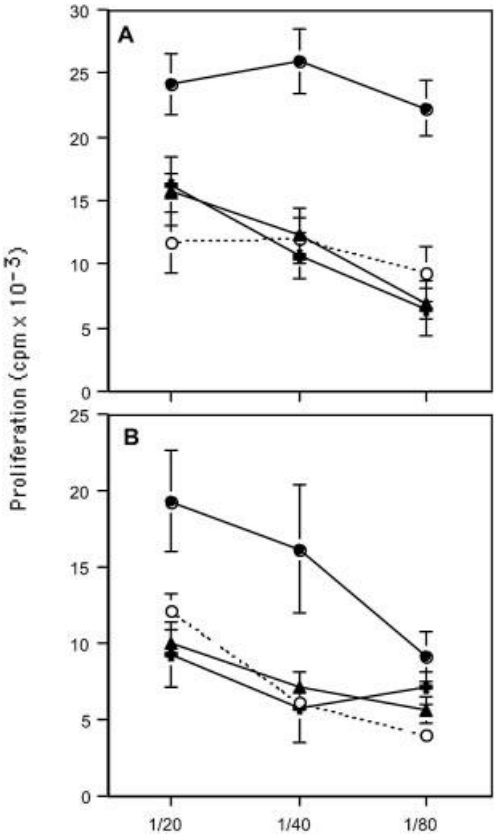


**Fig. 6.** (A) Effect of JM34 on autologous lymphocytes proliferation against KLH. Immature DC (open circles) were pulsed for 12 h with KLH, then matured by LPS for 48 h without JM34 (closed circles), with 20 μM JM34 (crosses) or 100 μM JM34 (closed triangles). mDC were co-cultured with autologous T cells, and proliferation was measured at day 7. (B) Effect of JM34 on allogeneic proliferation. After maturation with or without JM34, DC were mixed with $10^5$ T allogenic lymphocytes, and proliferation was measured at day 4. Symbols are the same as in (A). In each panel, data represent mean ± SEM of three different experiments.

### JM34 inhibited NF-κB translocation in DC

Since NF-κB activation has been shown to be essential for DC maturation [21], we next investigated the effect of JM34 on maturation-induced NF-κB nuclear translocation in DC by immunofluorescence staining and confocal analysis. As shown on Fig. 7, NF-κB p65 was exclusively localized in the cytoplasm in immature DC with very little to none detected in the nuclei. In contrast, maturation for 1 h with TNF-α/poly IC (Fig. 7) or with LPS (not shown) induced massive nuclear translocation of NF-κB p65, which was partially or totally inhibited by addition of JM34 (Fig. 7).

To evaluate the effect of JM34 on this nuclear translocation, we compared percentages of cells negative for nuclear NF-κB in the different conditions. This represented a stringent assessment of JM34 activity since only total inhibition of NF-κB p65 translocation was taken into account. Following TNF-α/poly IC maturation, only 13 out of 217 DC (6%) were negative for nuclear NF-κB, and this ratio increased to 68/246 (27.6%) after treatment with 200 μM JM34 ($p<0.0001$, Fisher's exact test). Similarly, 3/115 (2.6%) were negative after LPS maturation *vs.* 28/153 (18.3%) after addition of JM34 ($p<0.0001$, Fisher's exact test). These experiments, which were reproduced twice, strongly suggested that JM34 inhibited maturation-induced NF-κB p65 translocation in DC.

### JM34 inhibited DTH *in vivo*

We compared the effect of JM34, Dex and CsA in a DTH reaction in mice. Animals were sensitized with $5\times10^6$ SRBC by i.v. and challenged 4 days later by injection of SRBC into the hind footpad. Drugs were administered orally from the day of priming until the day of challenge. Fig. 8 shows the inhibition of DTH footpad swelling in mice treated either with JM34 (50, 100, 150 mg/kg) or Dex (0.5 mg/kg) or CsA (50 mg/kg). The drug JM34 significantly inhibited the DTH response in a dose-dependent manner, and, at the highest dose, inhibition (53.5%) was comparable to that of Dex (49.4%) but lower than that of cyclosporin (73.9%). These results demonstrated that the drug JM34 exerted significant immunosuppressive effects *in vivo*.

## Discussion

An ideal immunosuppressive drug in transplantation would be a compound that prevents immune response against graft alloantigens without impairing the recall of pre-existing memory immune responses. Drugs that prevent full activation of APC during primary sensitization may be better candidates than conventional immunosuppressive agents that broadly inhibit T lymphocytes activation. Indeed, many reports suggest that maintaining DC in an immature or semi-mature stage can be effective in preventing allograft rejection and favor graft tolerance [22, 23]. Various compounds such as MMF, vitamin D or glucocorticoids have been shown to inhibit DC maturation, but all these drugs also strongly inhibit T cell activation [24–26].

In the present study, we demonstrated the immunosuppressive potential of JM34 *in vitro* by showing a dose-dependent inhibition of PBMC proliferation after PHA or allogeneic stimulation. However, since both assays require the presence of APC [27], we could not discriminate between an inhibition of APC-stimulating abilities or a direct inhibition of T cell activation. Therefore, to evaluate the effect of JM34 on T cell activation *per se*, we chose a short-term assay on pre-activated T lymphocytes that requires minimal APC

 

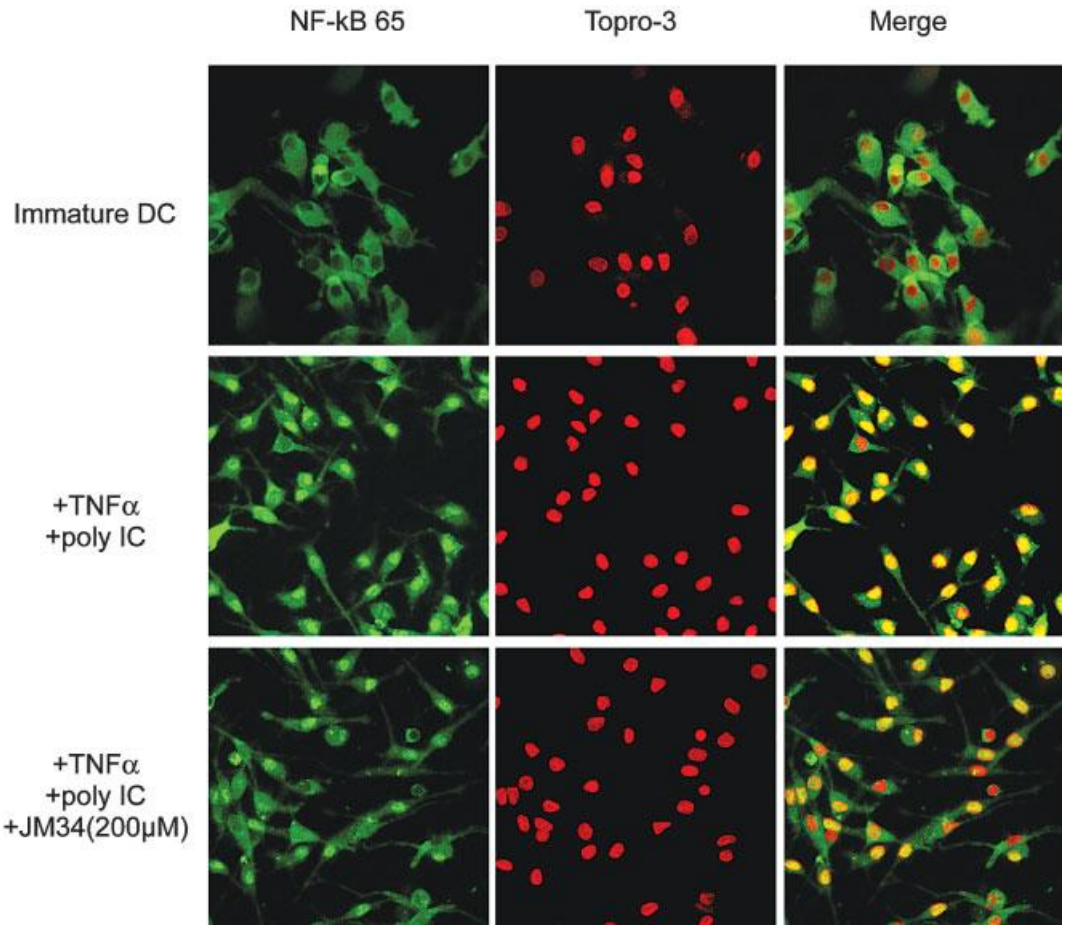


**Fig. 7.** Effect of JM34 on NF-κB nuclear translocation. Permealized DC, stained with an FITC-conjugated anti-NF-κB p65 mAb and counterstained with Topro-3 (DNA marker), were analyzed by confocal microscopy. Left and center columns show z projection (average intensity) of stacked green and red fluorescence and right column represents merged images.

activation, i.e., cytokine production of pre-activated T cell clones following specific restimulation. Our results indicated that JM34 did not inhibit IL-2, IL-4, IFN-γ or TNF-α production at doses at which it inhibited 80% of mitogen and allogeneic-induced proliferation. We next examined the effect of our compound on APC-independent T cell activation, and demonstrated that JM34 had no effect on IL-2R up-regulation by T cell clones stimulated with anti-CD3 mAb. Finally, we tested the effect of JM34 on the proliferation of the IL-2-dependent CTL-L2 cell line and on freshly purified T cells stimulated with anti-CD3/anti-CD28 mAb. In both assays, we observed a significant inhibition with the highest dose of JM34 (100 μM) that disappeared with lower doses. It was thus unlikely that the strong inhibition of PBMC proliferation that we already observed with 20 μM JM34 could have resulted exclusively from a direct inhibition of T cell activation. This compound thus differed from classical immunosuppressive drugs such as CsA, FK506 or rapamycin, which exert their immunosuppressive effect mainly by inhibiting T lymphocyte cytokine production or response [1] (although recent evidence suggest that they also alter DC function [28]).

Since our previous studies had shown that JM34 inhibited macrophage activation in a mouse model [17, 18], we suspected that the inhibition of PBMC proliferation could have resulted from an inhibition of APC activation. We therefore investigated the effects of JM34 on the maturation of human monocyte-derived dendritic cells induced either by LPS, TNFα/poly I:C or by rCD40L. Our results showed that JM34 differentially affected the expression of DC maturation markers: thus, we observed a major inhibition of the expression of MHC molecules, especially HLA class II, and a significant inhibition of CD83 but no significant effect on CD80, CD86 or CD40 expression. This altered phenotype was associated with dramatically impaired antigen-presenting capacity in functional assays: T cell proliferation against DC matured in the presence of JM34 was markedly decreased as compared to untreated DC, and, in fact, similar to that obtained with iDC in KLH-autologous assay and in MLR (Fig. 6). It is noteworthy that DC seemed to be permanently blocked in their semi-

 

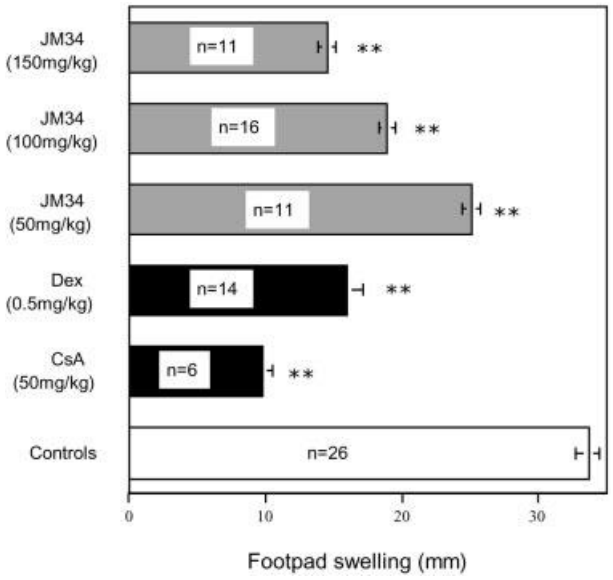


**Fig. 8.** Effect of JM34 on DTH response. Each bar represents the mean difference ± SEM in swelling between the two hind footpads, one injected with saline, the other with SRBC, measured 24 h after challenge. Drugs were administered daily *per os* from the time of priming until SRBC challenge. The number of mice (*n*) in each group is indicated. $^{**}p<0.01$ compared to the control group.

mature stage since they did not recover their antigen-presenting abilities during the 7-day mixed culture with T lymphocytes.

In addition, we report that JM34 significantly decreased IL-12 production by mDC (Fig. 5A). The major role of the active form of IL-12 (p70 IL-12) is to promote the differentiation of naive $CD4^+$ T cells into Th1 cells, a cell type which is critically involved in acute allograft rejection [29]. A recent report from Viera et al. [30] describes a new immunosuppressive compound, glatiramer acetate, which acted by favoring Th2 development through both inhibition of p70-IL12 secretion and enhanced IL-10 production. We therefore tested whether JM34 enhanced IL-10 production by DC, and this was not the case (Fig. 5B). However, the possibility remains that JM34-treated DC induced the differentiation of IL-10-producing CD4 regulatory T cells, and this will require further investigation. In the present report, we provide evidence that JM34 showed a significant inhibition of DTH in the mouse (Fig. 8), a response that is mainly due to the proliferation of Th1 lymphocytes [31]. It will therefore be interesting to test whether JM34 induces the development of regulatory T cells in this model.

Optimal maturation of DC typically requires three distinct stimuli: (1) products of pathogens recognized by Toll-like receptors (TLR) [32], (2) proinflammatory cytokines [33], and (3) T cell dependent signals [34]. Our present data demonstrated that JM34 inhibited maturation induced by each of the three stimuli, namely LPS, TNF/poly IC and CD40L. This suggests that JM34 inhibited a pathway that is common to all three stimuli. TLR, IL-1R, TNF and CD40 receptors lead to activation of downstream signaling cascades such as the NF-κB pathway or the MAP kinase pathways [35]. Thus, it was reported that the ERK1/2 and PI3K pathways were involved in DC survival, whereas the p38 MAPK and the NF-κB pathways were critical for DC maturation [21, 36, 37]. We have previously shown that JM34 inhibited the PKC-dependent activation of ERK2 in macrophages [18], but this is unlikely to account for the present inhibition of DC maturation. Here, we provide evidence that JM34 significantly inhibited maturation-induced NF-κB translocation in DC (Fig. 7), which could account for its major impact on DC maturation and its minor effect on T cell activation. Further investigations on the IκB phosphorylation pathway will be required to determine the precise target of JM34.

In conclusion, we provided evidence that the carboxamide JM34 inhibited T cell primary proliferative responses mainly by targeting DC with little effect on the secondary proliferative response of pre-activated T lymphocytes. This family of compounds thus displays an original pattern of immunosuppression and may allow a new therapeutic approach to autoimmune diseases and allograft rejection.

## Materials and methods

### Drugs

JM34 [*N*-(4,6-dimethylpyridin-2-yl)-furane-2-carboxamide] was used as maleate salt. CsA (Tocris, Illkirch, France) was dissolved in absolute ethanol for *in vitro* experiments or in olive oil for *in vivo* experiments. Rapamycin (Rapamune®) was diluted in PBS (1 μg/ml) and dexamethasone sodium salt (Dex) (Soludecadron®) in sterile water.

### Cell isolation and culture

PBMC were isolated from healthy donors by Ficoll-Hypaque gradient centrifugation and were resuspended in RPMI medium (Sigma, St Quentin Fallavier, France) with 1% L-glutamine (Sigma) and 10% heat-inactivated FCS (Sigma) referred to as complete medium. Purified T cells were obtained from fresh blood using RosetteSep isolation kit according to the manufacturer's instructions (StemCell Technologies, Meylan, France). The purity of the T lymphocyte preparation was checked by flow cytometry with a FITC-conjugated anti-CD3 mAb (BD Biosciences, Le Pont de Claix, France) and was always above 95%.

$CD8^+$ and $CD4^+$ T cell clones were generated previously and recognized specific EBV epitopes [38] or Melan-A/MART1 epitopes [39] in the HLA-A 0201 context or allogeneic B-EBV cell line (for $CD4^+$ clones) [40].

 

Immature DC were generated from PBMC of HLA-A 0201$^+$ healthy donors obtained from the Etablissement Français du Sang of Nantes after informed consent. Briefly, PBMC were cultured in hydrophobic bags in 200 ml X-Vivo 15 medium (BioWhittaker, Walkersville, MD) with 500 IU/ml GM-CSF (Leucomax, Novartis, Rueil-Malmaison, France) and 15 ng/ml IL-4 (AbCys SA, Paris, France) for 7 days. Fresh cytokines were added on day 4 of culture. On day 7, iDC were purified with an elutriator system (Beckman Coulter, Villepinte, France) and seeded in 24-well plates at $1\times10^6$ DC/ml in X-Vivo 15 medium with GM-CSF (500 IU/ml) and IL-4 (15 ng/ml). DC maturation was induced by addition of either LPS (1 μg/ml), a combination of TNF-α (20 ng/ml)/poly IC (50 μg/ml) or Flagged rCD40L (500 ng/ml) (Alexis, Paris, France) + anti-Flag mAb (1 μg/ml) (Sigma) for 48 h. JM34 was added 2 h before the maturation-inducing agents.

### Flow cytometry

Cytokine production was assessed by intracellular staining. CD8$^+$ clones were incubated with the peptide transporter-deficient T2 cells loaded for 1 h with 10 μM of the relevant peptide. Alloreactive CD4$^+$ clones (anti-DP 0401 and anti-DP 0201) were incubated with their relevant B-EBV lines. Incubations were performed for 6 h in the presence of 10 μg/ml brefeldin A (Sigma). Cells were then fixed in 4% w/v paraformaldehyde, washed in 0.1% BSA/PBS, permeabilized in 0.1% saponin and stained with FITC-conjugated anti-IL-2, anti-IL-4, or anti-TNF-α mAb (BD Biosciences). CD25 surface expression on clones was induced by anti-CD3 mAb stimulation. CD8$^+$ or CD4$^+$ clones were cultured for 18 h in plates precoated with 5 μg/ml anti-CD3 mAb (Orthoclone OKT3®) and then stained with FITC-conjugated anti-CD25 mAb (BD Biosciences).

For phenotypic analysis, DC were stained with a panel of mAb including anti-CD80 (BD Biosciences), anti-CD83, anti-HLA ABC and anti-CD40 (Immunotech, Villepinte, France), anti-CD86 and anti-HLA-DR (Caltag, Burlingame, CA) or with appropriate isotype controls. Stained cells were resuspended in PBS/0.1% BSA containing Topro-3-iodide, and $10^4$ events were analyzed with a FACSCalibur (BD Biosciences). Dead cells were excluded on the basis of Topro-3-iodide staining.

### Proliferation assays

PBMC ($1\times10^5$/well in U-bottom 96-well plates; Falcon) were incubated with 0.3 μg/ml PHA (Sigma) or irradiated allogeneic PBMC ($1\times10^5$/well) (MLR). Proliferation was assessed in sextuplicate after 48 h of culture. CTL-L2, maintained in complete medium supplemented with rIL-2 (50 IU/ml) and 2-ME, were washed before the assay and incubated ($5\times10^3$/well) with a range of IL-2 concentrations (0–125 IU/ml) with or without JM 34 or rapamycin (50 nM). Proliferation was assessed in triplicate after 18 h of culture.

Freshly isolated T cells were stimulated for 72 h with biotinylated anti-CD3 and/or anti-CD28 mAb precoated on streptavidin microbeads M280 (Dynal Biotech SA, Compiegne, France). Proliferation was assessed in triplicate after 72 h of culture.

To evaluate antigen presentation by DC, iDC were pulsed with 25 μg/ml KLH (Calbiochem, Meudon, France) for 12 h and subsequently matured for 48 h. Matured DC were co-cultured with $10^5$ autologous T cells at different DC/T ratios in complete medium. Proliferation was determined in triplicate after 7 days of culture. For allogeneic stimulation, mDC were cultured with allogenic T cells in the same conditions as above except that proliferation was assessed after 4 days. In all experiments, cells were pulsed with [$^3$H]thymidine (1 μCi/well) during the last 16 h of culture and their thymidine uptake was measured with a Beta counter (Wallac, PerkinElmer Life Sciences, Zaventem, Belgium).

### Detection of IL-12 and IL-10

Levels of p70 IL-12 and IL-10 in supernatants of DC cultures were quantified using ELISA kits (BD Biosciences) according to the manufacturer's instructions.

### Immunofluorescence staining and confocal microscopy analysis

DC were seeded in 24-well plates at $5\times10^5$ cells/well and allowed to adhere to polylysine-coated glass slides for 18–36 h at 37°C. Cells were then fixed for 10 min in fresh 4% paraformaldehyde in PBS, washed and permeabilized with PBS/0.1% BSA and 0.1% saponin for 2 min at room temperature and finally blocked for 10 min at room temperature with PBS/3% BSA. Staining was performed with an anti-NFκB p65 mAb (Santa Cruz Biotechnology Inc, Santa Cruz, CA) (5 μg/ml in PBS/BSA/saponin) at room temperature for 1 h followed by FITC-conjugated goat anti-mouse IgG (Sigma). To identify nuclei, FITC-labeled samples were counterstained with 1 μM Topro-3 iodide (Molecular Probes, Montluçon, France) for 30 min in PBS. Confocal analysis was performed with a TCS NT microscope (Leica Instruments, Heidelberg, Germany). Percentages of translocation in the different experimental conditions was assessed as follows: DC nuclei were localized and counted using red fluorescence and then translocation was assessed using green fluorescence.

### Delayed-type hypersensitivity

DTH experiments were performed as previously described [41]. Groups of five female BALB/C mice (Janvier, Laval, France), 8–9 weeks of age, were injected with $5\times10^6$ SRBC (BioMerieux, Marcy-l'Etoile, France) into the tail vein. Four days after immunization, mice were tested for DTH with a subinflammatory challenging dose ($3\times10^8$–$4\times10^8$ SRBC) injected into the right hind footpad. The left food pad was used as control and injected with PBS alone. The size of both hind footpad was measured with an Oditest (Kroeplin, Germany) 24 h after challenge. DTH reaction was determined by the difference in size between the SRBC- and PBS-injected footpads. Mice were treated daily *per os* for 5 days with different doses of JM34, Dex or CsA, from the time of priming until the SRBC challenge. The DTH inhibition index was calculated as the ratio of DTH reaction in the treated animal versus the DTH reaction in control mice.

 

### Statistical analysis

Results are expressed as mean ± SEM. All results were compared using ANOVA analysis followed by Dunnett post-tests. Post-tests were only performed when the ANOVA test showed a significant difference ($p<0.05$) between groups. In translocation experiments, differences between percentages of translocation in treated *vs.* untreated cells were assessed by Fisher's exact test.

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