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The address for Mirko Vukcevic was incorrectly assigned in the e-press version.

In addition, in both the online and print versions, the addresses for Susan Treves were incorrectly assigned.

The correct version is shown below.

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Summary

Increases in intracellular Ca^{2+} concentration accompany many physiological events, including maturation of dendritic cells, professional antigen-presenting cells characterized by their ability to migrate to secondary lymphoid organs where they initiate primary immune responses. The mechanism and molecules involved in the early steps of Ca^{2+} release in dendritic cells have not yet been defined. Here we show that the concomitant activation of ryanodine receptor-induced Ca^{2+} release together with the activation of Toll-like receptors by suboptimal concentrations of microbial stimuli provide synergistic signals, resulting in dendritic cell maturation and stimulation of T cell functions. Furthermore, our results show that the initial intracellular signaling cascade activated by ryanodine receptors is different from that induced by activation of Toll-like receptors. We propose that under physiological conditions, especially when low suboptimal amounts of Toll-like receptor ligands are present, ryanodine receptor-mediated events cooperate in bringing about dendritic cell maturation.

Key words: Dendritic cell, Maturation, Ryanodine receptor, Signaling

Introduction

Ryanodine receptors (RyR) are intracellular Ca^{2+} channels mainly found in excitable tissues, mediating Ca^{2+} release from intracellular stores (Sutko and Airey, 1996; Franzini-Armstrong and Protasi, 1997). The functional Ca^{2+} release channel is composed of four ryanodine receptor monomers (each of which has a molecular mass of approximately 560 kDa), which assemble into a large macromolecular structure with a molecular mass of more than 2×10^6 Da (Bers, 2004; Serysheva et al., 2005; Samso et al., 2005). Three isoforms of the RyR have been identified at the molecular level: they share an overall amino acid identity of approximately 60% and experimental evidence suggests that they are structurally similar, with a large hydrophilic NH_2-terminal domain and a hydrophobic C-terminal domain containing several transmembrane domains as well as the channel pore (Bers, 2004; Serysheva et al., 2005; Samso et al., 2005). Type 1 RyR (RyR1), encoded by a gene located on human chromosome 19, is mainly expressed in skeletal muscle where it mediates Ca^{2+} release from the sarcoplasmic reticulum, following depolarization of the plasmalemma (Phillips et al., 1996; Takeshima et al., 1989; Zorzato et al., 1990). Type 2 RyR, encoded by a gene located on chromosome 1, is mainly expressed in the heart and in certain areas of the cerebellum and is activated through a Ca^{2+}-induced Ca^{2+}-release mechanism (Otsu et al., 1990; McPherson and Campbell, 1993). Type 3 RyR, encoded by a gene located on chromosome 15, is expressed in several tissues, including the central nervous system; its expression in some tissues appears to be developmentally regulated (Sorrentino et al., 1993; Tarroni et al., 1997). In recent years, more detailed investigations have revealed that this isoform-specific tissue distribution of RyR may in fact be more complex. Sei et al. (Sei et al., 1999) and Hosoi et al. (Hosoi et al., 2001) showed that circulating leukocytes, as well as leukocyte-derived cell lines, express different RyR transcripts. We have shown that Epstein Barr Virus (EBV)-immortalized B-lymphocytes express the transcript, the protein and the functional RyR1 Ca^{2+}-release channel (Girard et al., 2001), and O'Connell et al. (O'Connell et al., 2002) have demonstrated that the RyR1 is expressed in immature mouse dendritic cells (DCs). Immature DCs (iDCs) act as sentinels in peripheral tissues, continuously sampling the antigenic environment. Upon Toll-like receptor engagement by microbial products or tissue debris, DCs undergo maturation and become the most potent antigen-presenting cells. At this point, Toll-like receptor-activated DCs upregulate costimulatory and antigen-presenting molecules and migrate to secondary lymphoid organs for the interaction with naive T-cells and the priming of immune responses in vivo (Banchereau and Steinman, 1998). For several years it has been known that increases in the intracellular calcium concentration ($[Ca^{2+}]_i$) participate in the regulation and maturation of DCs (Koski et al., 1999; Czerniecki et al., 1997), although the mechanism and molecules involved in the early steps of the Ca^{2+}-release event have not been clearly defined.

As part of an investigation aimed at identifying the role of RyR1 in cells of the immune system, we showed that in freshly isolated human B-lymphocytes, activation of the RyR1 leads

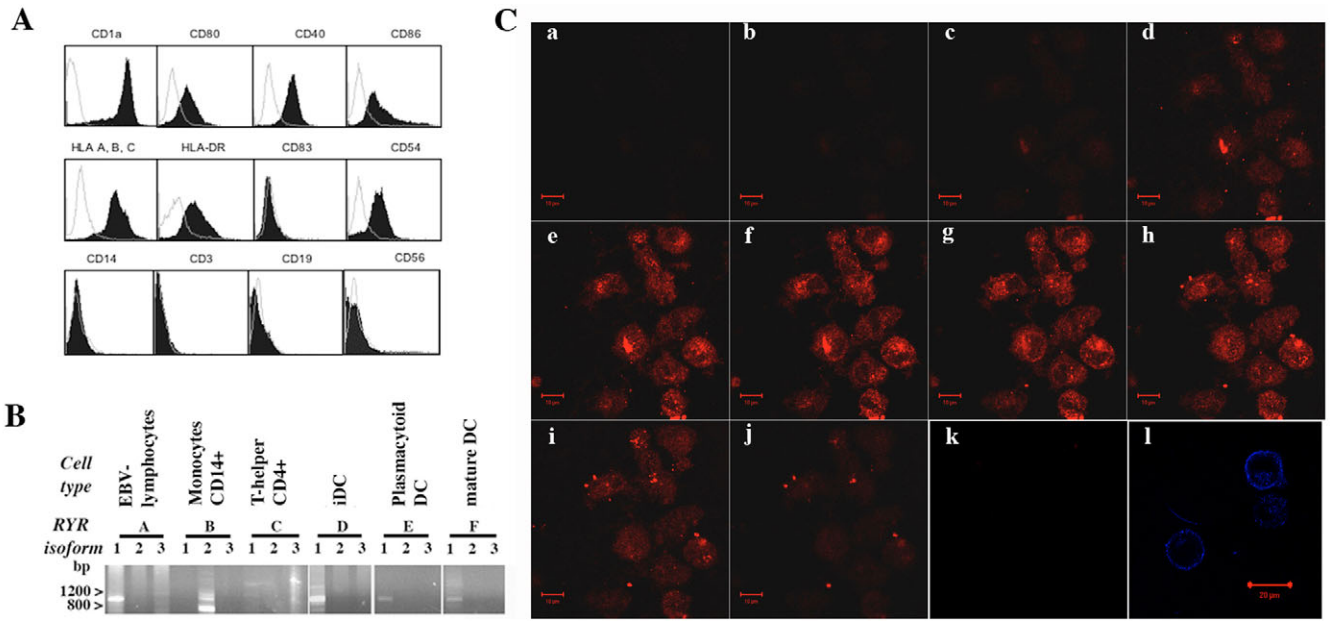

Fig. 1. Expression of RyR in human DCs. Peripheral blood monocytes were induced to differentiate into iDCs by 5-day culture in IL4 and GM-CSF. (A) Cells were then washed and incubated in the presence of fluorochrome-labeled monoclonal antibodies (mAbs) recognizing the indicated surface markers. Specific fluorescence (full histograms) was evaluated by taking advantage of a FACSCalibur flow cytometer equipped with Cell Quest software (Becton Dickinson) using, as negative controls, isotype-matched irrelevant reagents (empty histograms). One representative experiment out of five is shown. (B) Total RNA was extracted from immature (iDCs), LPS-matured (mature DCs) and from other cell types, and the expression of genes encoding the different RYR isoforms was evaluated by RT-PCR. RNA from EBV-transformed lymphocytes was used as control for RYR1 isoform gene expression. (C) Immunofluorescence analysis of iDCs. Cells were stained with goat anti-RyR polyclonal Ab followed by Alexa Fluor-555-conjugated anti-goat Ab (a-j), Alexa Fluor-555-conjugated anti-goat Ab alone (k) or with allophycocyanine-conjugated anti-CD1a (l). Immunofluorescence analysis on acetone:methanol-fixed iDCs reveals strong perinuclear RyR fluorescence that extends into the endoplasmic reticulum network. Panels a-j show 1 μm optical slices from the bottom upwards; bar, 10 μm. Images were acquired with a 100× Plan Neofluar oil immersion objective (NA 1.3) mounted on a Zeiss Axiovert 100 confocal microscope. Panel l: paraformaldehyde fixed DCs display typical plasma membrane fluorescence for the CD1a marker (bar, 20 μm).

to the rapid release of the proinflammatory cytokine IL1B. Furthermore, cells from patients with the malignant hyperthermia susceptible phenotype, a pharmacogenetic hypermetabolic disease caused by *RYR1* mutations (Treves et al., 2005), released more proinflammatory cytokines than cells from controls, indicating that one of the downstream effects of human RyR1 activation is coupled to cytokine release (Girard et al., 2001). In the present study we investigated the effects of pharmacological activation of RyR1 in human DCs. Our results show that treatment of iDCs with RyR1 agonists is accompanied by an increase in the intracellular calcium concentration. Furthermore, treatment of iDCs with a suboptimal concentration of bacterial lipopolysaccharide (LPS) in the presence of RyR1 agonists induces activation of cytokine transcription and upregulation of surface markers that are typically associated with cell maturation as well as with an increased capacity to stimulate allospecific T-cells. These effects are specifically associated with RyR1 activation as they could be blocked by pretreatment with the RyR1 antagonist dantrolene (Zhao et al., 2001) and could not be induced by addition of ATP, an agonist releasing calcium through IP_3 mobilization (Ralevic and Burnstock, 1998; Schnurr et al., 2004). These results provide for the first time evidence for the involvement of RyR1 in DC maturation and indicate a functional cooperation between RyR1-mediated and Toll-like receptor-mediated intracellular signaling.

Results

Fig. 1A shows that peripheral blood monocytes cultured for 5 days in the presence of GM-CSF and IL4 differentiate into iDCs (Sallusto and Lanzavecchia, 1994). These cells display a typical immature phenotype in as much as they express the surface markers CD1a, CD80, CD40, CD86 and CD54 and are negative for CD83 (maturation marker) and CD14 (monocyte marker). Furthermore, no positivity for CD3 (T-lymphocytes), CD19 (B-lymphocyte marker) and CD56 (NK cell marker) could be detected. Immature CD1a-positive DCs can be matured by treatment with Toll-like receptor agonists, including bacterial LPS (Sallusto et al., 1998). Fig. 1B shows that, irrespective of their degree of maturation, DCs express the gene encoding the skeletal muscle RyR1 but not the other isoforms. This transcript is not expressed by other peripheral blood leukocyte populations such as monocytes (which express the RyR2 isoform transcript) and T-lymphocytes (Fig. 1B), but is expressed by plasmacytoid DCs, which are natural circulating DCs isolated from peripheral blood as opposed to cells obtained upon in vitro culture. Immunofluorescence analysis shows that the intracellular distribution of the RyR1 in in vitro-derived CD1a-positive DCs is concentrated in a reticulum extending from the perinuclear area towards the plasma membrane. Confocal analysis of 1 μm optical slices shows no surface fluorescence, confirming that the RyR1 is expressed in intracellular membrane compartments, most likely the endoplasmic reticulum (Fig. 1C).

Single-cell intracellular Ca^{2+} measurements on fura-2-loaded iDCs show that addition of 10 mM caffeine leads to a rapid and transient increase in the $[Ca^{2+}]_i$ (Fig. 2A). Data presented in Fig. 2B confirm that the RyR1 can also be activated pharmacologically with specific agonists such as caffeine and 4-chloro-m-cresol (Zucchi and Ronca-Testoni, 1997) as well as by KCl. The addition of 100 μM ATP, which induces Ca^{2+} release through $InsP_3$ receptor activation in many cell types (Ralevic and Burnstock, 1998; Schnurr et al., 2004), was also accompanied by an increase in $[Ca^{2+}]_i$ in iDCs (Fig. 2C). Interestingly, the peak amplitude induced by ATP was significantly larger than that observed after RyR1 activation (compare Fig. 2B and Fig. 2D; $P<0.00001$). These results unequivocally demonstrate that increases in intracellular $[Ca^{2+}]$ in human iDCs can be stimulated both by $InsP_3$ mobilizing agonists as well as by RyR1 activators. We next investigated whether the Ca^{2+} released through RyR1 activation plays a specific function in DC maturation.

Immature DCs generated by culturing peripheral blood monocytes for 5 days in the presence of IL4 and GM-CSF were induced to mature by 18 hours of incubation with increasing concentrations of LPS (from 1 ng/ml to 1 μg/ml) in the presence of 10 mM caffeine. The expression of genes associated with DC maturation and their capacity for stimulating T-cell responses was then evaluated. Fig. 3 shows the results obtained in a typical experiment representative of data obtained with four different donors. Low (sub-optimal) concentrations (1 ng/ml) of LPS only slightly activated the transcription of the genes under investigation, whereas the addition of an optimal LPS concentration (1 μg/ml) strongly stimulated the transcription of genes encoding the maturation marker CD83 and IFNα, IL12B and IL23A, cytokines that are associated with a high capacity of stimulating T-cell responses. Importantly, costimulation of DCs for 18 hours with 10 mM caffeine plus 1 ng/ml LPS stimulated transcription of all the genes under investigation to extents similar to those obtained using a 1000-fold higher concentration of LPS alone. The involvement of RyR1 activation is supported by the fact that pretreatment of cells with the RyR1 antagonist dantrolene followed by the addition of caffeine and LPS inhibited the synergistic effects of caffeine and LPS on gene expression (Fig. 3A). Quantitatively and qualitatively similar results were obtained when iDCs were treated with 4-chloro-m-cresol and LPS, but in some cases the presence of the latter RyR1 agonist was accompanied by apoptosis, resulting in more variable results (data not shown). Fig. 3B shows that in human DCs Ca^{2+} release mediated by the activation of the $InsP_3$-signaling pathway induced by 100 μM ATP in the presence of 1 ng/ml LPS could only partially activate DCs, as detected by the expression of the *IL23A* gene, albeit at a fourfold lower extent than that observed upon RyR1 activation. By contrast, it was not potent enough to induce transcription of the genes encoding CD83, IL12B and IFNα. The addition of 10 mM caffeine alone (i.e. in the absence of LPS) caused an increase in *IL23A* gene expression, but did not affect transcription of the other genes under investigation (Fig. 3B, right panel).

Finally, increasing concentrations of caffeine (from 1-10 mM + 1 ng/ml LPS) resulted in a proportional increase of *IL23A* gene transcription, reflecting the calcium-dependent nature of this activation event (Fig. 3C); this result was further confirmed by the observation that addition of 1 μM thapsigargin [an inhibitor of sarcoplasmic and endoplasmic reticulum Ca^{2+}-ATPases (SERCA)] in the presence of 1 ng/ml LPS resulted in the activation of *IL23A* gene transcription (Fig. 3C).

We then examined in more detail the functional consequences of the maturation signals generated by simultaneous RyR1 and Toll-like receptor activation. The capacity of iDCs treated with LPS plus caffeine to stimulate allospecific T-cell proliferation was assayed by measuring [^{3}H]thymidine incorporation. As shown in Fig. 3D, iDCs are relatively poor allostimulatory cells (white bars) and the low dose of LPS used to activate DCs in this assay (1 ng/ml) was also not so efficient, causing a 1.6-fold increase in T-cell proliferation (grey bars). Incubation of iDCs with 100 μM ATP (which induces a large $[Ca^{2+}]_i$ transient) plus 1 ng/ml LPS did not significantly improve [^{3}H]thymidine incorporation by T cells (horizontally lined bars), as compared with LPS treatment alone. However, treatment of iDCs with 10 mM caffeine plus 1 ng/ml LPS provided synergistic signals resulting in a twofold increase in [^{3}H]thymidine incorporation in T cells (hatched bars). This effect was specific as it could be blocked by inhibiting RyR1-mediated Ca^{2+} signaling by pretreating iDCs

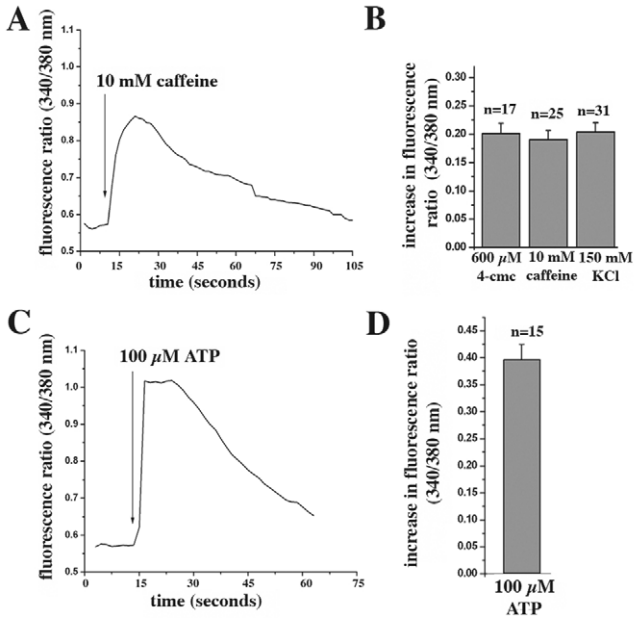

Fig. 2. Single-cell intracellular calcium imaging on iDCs. (A,C) Single-cell intracellular calcium measurements in fura-2-loaded iDCs obtained after 5 days of culture. The traces show changes in fura-2 fluorescence ratio (340/380 nm) in a single iDC after addition of caffeine or ATP. Experiments were performed in Krebs-Ringer containing 1 mM Ca^{2+}. (B,D) Average peak increases in fluorescence ratio induced by the addition of the indicated agonists to fura-2-loaded iDCs. Results represent the mean Δ increase in fluorescence (calculated by subtracting the peak fluorescent ratio from the resting fluorescent ratio) obtained after addition of 600 μM 4-chloro-m-cresol, 10 mM caffeine or 150 mM KCl (B) or 100 μM ATP (D). No difference was found in the peak Ca^{2+} in response to RyR activation (P=0.820), but the amount of Ca^{2+} released by 100 μM ATP was significantly higher ($P<0.00001$). Results represent the mean (± s.e.m.) Δ increase in fluorescence in DCs isolated from four different donors.

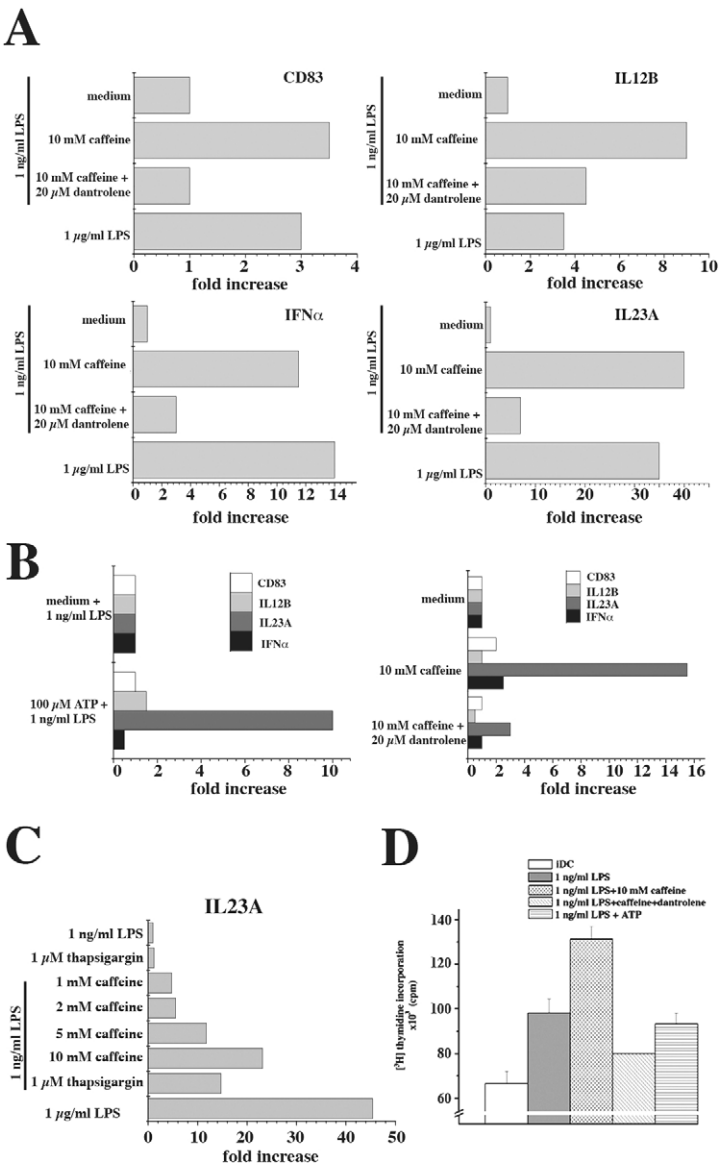

Fig. 3. RyR activation induces transcription of genes involved in DC maturation and potentiates allospecific T-cell stimulation. (A) In vitro-derived iDCs were cultured for 18 hours in the presence of the indicated concentrations of LPS and with 10 mM caffeine and 20 μM dantrolene, as indicated. Total RNA was extracted and *CD83*, *IFNα*, *IL12B* and *IL23A* gene expression was evaluated by quantitative real-time PCR. Gene expression results are expressed as fold-increase as compared with values obtained in iDCs treated with medium + 1 ng/ml LPS. One representative experiment out of four is shown. (B) Experiment as in A except that in vitro-derived iDCs were cultured for 18 hours in the presence of 1 ng/ml LPS + 100 μM ATP (left panel) or in the presence of 10 mM caffeine and 20 μM dantrolene, as indicated (right panel). Gene expression results are expressed as fold-increase as compared with values obtained in iDCs treated with medium + 1 ng/ml LPS (left panel; ATP experiments) or as fold-increase as compared with values obtained in iDCs treated with medium alone (right panel). One representative experiment out of four is shown. (C) Experiment as in A except that in vitro-derived iDCs were cultured for 18 hours in the presence of 1 μM thapsigargin, or the indicated concentration of caffeine or 1 μM thapsigargin + 1 ng/ml LPS. Total RNA was extracted and *IL23A* gene expression was evaluated by quantitative real-time PCR. One representative experiment out of four is shown. (D) Immature DCs were harvested on day 5 of differentiation and stimulated with 1 ng/ml LPS (grey box), with 10 mM caffeine + 1 ng/ml LPS (hatched box), with 20 μM dantrolene + 10 mM caffeine + 1 ng/ml LPS (diagonal lines) or with 100 μM ATP + 1 ng/ml LPS (horizontal lines), or left untreated (empty box). DCs were then washed and added to allogenic PBMC at a ratio of 10:1 for 5 days. [^{3}H] thymidine was then added and cells were cultured for another day. The bars indicate the mean value of c.p.m. (± s.e.m.) from triplicate samples from one donor. The experiment was repeated with similar results at least four times with different donors.

with 20 μM dantrolene, prior to the addition of 10 mM caffeine and 1 ng/ml LPS (diagonally lined bars). Taken together, these results suggest that the signals generated in human DCs through Toll-like receptors (by the addition of LPS) and by RyR1 activation are different, and that their combined activation results in synergistic effects.

Although RyR1 can be pharmacologically activated by a variety of agonists, how its activation occurs in iDCs in vivo remains puzzling. We were intrigued by the finding that the addition of KCl causes a rise in the $[Ca^{2+}]_i$ in iDCs and reasoned that this may have physiological relevance. In fact, cells dying in the vicinity of iDCs in a restricted microenvironment such as an inflamed tissue could release their intracellular K^+ into the extracellular milieu, thereby providing the necessary costimulating signal(s) to iDCs residing in neighboring areas. In order to verify our hypothesis, we set up a series of experiments; first we tested whether the effects of caffeine on DC maturation could be monitored by flow cytometry, by following the surface expression of CD83, a good phenotypic indicator of DC maturation (Zhou and Tedder, 1996; Lachmann et al., 2002). Fig. 4A shows that as early as 4 hours after stimulation 1 μg/ml LPS induced significant surface expression of CD83; this incubation time was chosen for all subsequent experiments and the results obtained by adding different stimuli were compared with those obtained by treating cells with 1 μg/ml LPS, which was set at 100% induction of CD83 expression. Fig. 4A shows that addition of 10 mM caffeine alone, 100 μM ATP plus 1 ng/ml LPS, or pretreatment of cells with 20 μM dantrolene followed by the addition of 10 mM caffeine plus 1 ng/ml LPS did not result in significant induction of CD83 expression. Pretreatment of DCs with 20 μM dantrolene did not affect the induction of CD83 surface expression stimulated by 1 μg/ml LPS. However, the addition of 10 mM caffeine plus 1 ng/ml LPS caused a significant induction of CD83 surface expression (P<0.001; Student's t-test).

We followed the same protocol to verify whether the hypothesis that KCl or the content of necrotic cells could

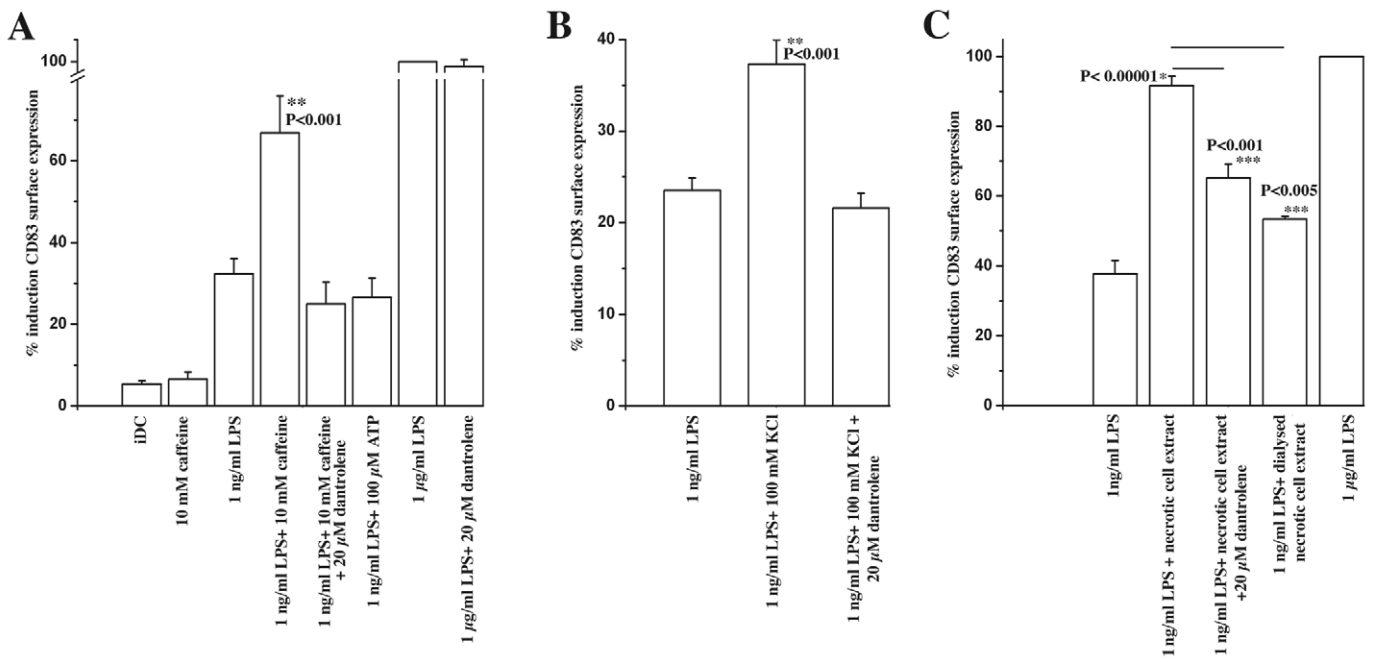

Fig. 4. Incubation of iDCs with KCl or soluble extracts from necrotic cells promotes surface expression of CD83. iDCs were treated for 4 hours as indicated and the percent positive CD83 cells was determined by flow cytometry. (A) Stimulation with 10 mM caffeine alone or 100 μM ATP + 1 ng/ml LPS did not significantly increase CD83 surface expression; incubation with 10 mM caffeine + 1 ng/ml LPS significantly increased CD83 surface expression (P<0.001). This effect was blocked by pretreatment with 20 μM dantrolene. (B) When KCl was added, cells were incubated with Krebs-Ringer saline (in which the NaCl had been substituted for KCl) for 30 minutes at 37°C; they were then centrifuged, and fresh medium containing 1 ng/ml LPS was added and cells were incubated at 37°C for 4 hours. (C) For experiments in which iDCs were incubated with the supernatant from necrotic HEK293 cells, extracts were prepared as described in the Materials and methods section and either used directly (+ necrotic cell extract ± 20 μM dantrolene) or dialysed overnight against 1×PBS (dialysed necrotic cell extract). Results are expressed as mean (± s.e.m.) percentage induction of CD83 surface expression of at least three experiments performed on iDCs purified from blood of different donors; values obtained by treating iDCs with 1 μg/ml LPS was considered 100%. *P and **P, significant difference in the treated population compared with iDCs treated with 1 ng/ml. ***P, significant difference from iDCs treated with 1 ng/ml LPS + necrotic cell extracts.

mimic the synergistic effects of caffeine on iDC maturation. Immature DCs were incubated with Krebs-Ringer in which NaCl was substituted for KCl (in order to maintain the osmolarity), plus 1 ng/ml LPS for 30 minutes at 37°C; the medium was then replaced by differentiation medium containing 1 ng/ml LPS and the cells were incubated for 4 hours. The KCl-washout step was necessary as iDCs were sensitive to prolonged exposure to KCl. As shown in Fig. 4B, the addition of KCl and sub-optimal LPS (1 ng/ml) to iDCs promoted surface expression of CD83, which could be inhibited by pretreatment with 20 μM dantrolene. Fig. 4C shows that the addition of filtered soluble extracts from necrotic HEK293 cells in the presence of 1 ng/ml LPS caused a significant number of cells to express surface CD83; this stimulation of CD83 surface expression was significantly decreased by preincubation with 20 μM dantrolene (P<0.0001), or by dialysis of the filtered extracts (P<0.005). In the former cases, however, some induction of CD83 expression was still present, indicating that other factors promoting iDC maturation independently of RyR activation are also released from dying cells.

In order to dissect the intracellular pathways involved in Toll-like receptor and RyR activation, we examined (1) the effects of LPS and caffeine on the translocation of p65 (RelA, a component of the NF-kB complex) to the nucleus and (2) the sensitivity of DC maturation to cyclosporine A, which would suggest the involvement of the Ca^{2+}/calmodulin phosphatase, calcineurin. Fig. 5A (top panels) shows that in untreated iDCs and in cells treated for 5 minutes with 10 mM caffeine and/or 1 ng/ml LPS, p65 was mainly distributed in the cytoplasm; however, incubation with 1 μg/ml LPS for 60 minutes caused its nuclear translocation in a large number of cells (Fig. 5A, bottom panel, arrowheads). Caffeine stimulation of iDCs evokes a low-amplitude calcium signal (Fig. 2), and this low-amplitude calcium signal alone or in combination with 1 ng/ml LPS is not sufficient to activate the nuclear translocation of NF-kB. These results support the hypothesis that some intracellular signals generated by LPS-mediated activation of Toll-like receptors in DCs are led by NF-kB-dependent events, whereas caffeine-activated pathways are NF-kB independent.

We next investigated whether the caffeine low-amplitude calcium signal is sufficient to activate an alternative signaling pathway, namely Ca^{2+}/calmodulin-dependent phosphatase calcineurin. We probed the effect of caffeine on maturation of iDCs by using the calcineurin pathway inhibitors cyclosporine A (Barford, 1996) and deltamethrin (Enan and Matsumara, 1992). Fig. 5B shows that the synergistic effects of 1 ng/ml LPS and 10 mM caffeine (light-grey bars) on maturation of iDCs could be completely abrogated by pretreatment of iDCs with 2 μM cyclosporine A and 10 μM deltamethrin. These

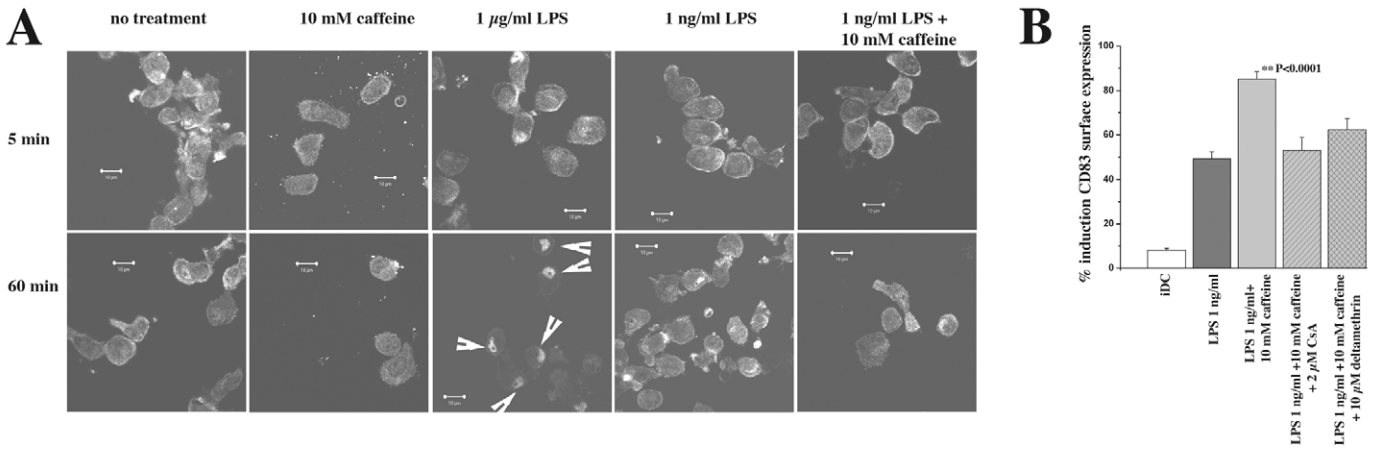

Fig. 5. LPS but not caffeine induces nuclear translocation of NF-kB, whereas caffeine-induced maturation is sensitive to cyclosporine A (CsA). iDCs were left untreated or stimulated for 5 or 60 minutes at 37°C, as indicated. Cells were allowed to stick to poly-L-lysine-treated coverslips and permeabilized with acetone:methanol. Cells were incubated with rabbit anti-p65 polyclonal antibodies (Ab), followed by Alexa Fluor-488-labeled secondary Ab and visualized with a confocal microscope as indicated in the Materials and Methods section. Arrowheads indicate nuclear fluorescence. Images were acquired with a 100× Plan Neofluar oil immersion objective (NA 1.3) mounted on a Zeiss Axiovert 100 confocal microscope. Horizontal bar, 10 μm. Arrowheads point to nuclear translocation of p65. (B) Surface expression of CD83 in unstimulated iDCs (empty bars) or iDCs stimulated with 1 ng/ml LPS (dark-grey bars) ± 10 mM caffeine (light-grey bars) or pretreated with 2 μM CsA for 30 minutes and then with 10 mM caffeine + 1 ng/ml LPS (slashed grey bars), or pretreated with 10 μM deltamethrin for 30 minutes and then with 10 mM caffeine + 1 ng/ml LPS (hatched grey bars). Results are expressed as percent (mean ± s.e.m.; n=3) induction of CD83 surface expression: 100% was the value obtained by stimulating cells with 1 μg/ml LPS for 4 hours. **P, significant difference in the treated population compared with iDCs treated with 1 ng/ml.

results are consistent with the idea that in addition to NF-kB the Ca^{2+}-calcineurin-sensitive signaling pathway is also involved in the maturation of human DCs.

Discussion

The present study shows that activation of RyR1 in iDCs generates, through a calcineurin-sensitive pathway, a costimulatory signal that enhances the sensitivity of iDCs to bacterial stimuli, leading to their maturation. These effects are unveiled when subthreshold concentrations of LPS are used. DCs express both IP_3R and RyR1 intracellular Ca^{2+} channels (Hosoi et al., 2001; Stolk et al., 2006; Goth et al., 2006) and the involvement of Ca^{2+}-dependent signaling events in their activation has been clearly established. In fact, (1) treatment of human iDCs with the Ca^{2+} ionophore A23187 induces upregulation of major histocompatibility complex (MHC) and costimulatory molecules and CD83 expression (Czerniecki et al., 1997) and activates the transcription of *IL23A* (this study), and (2) promotes T-cell activation (Faries et al., 2001). Moreover, (3) treatment of mature human DCs with ionomycin triggers release of pro-IL-1β (Gardella et al., 2001).

As to the types of intracellular Ca^{2+}-release channels involved in DC activation and/or maturation, Stolk et al. recently showed the RyRs are not indispensable because bone marrow precursors obtained from the liver of RyR1 knockout (KO) mice injected into sublethally irradiated congenic hosts could still be induced to differentiate and mature into normal DCs (Stolk et al., 2006). Notably, however, the Ca^{2+} signaling machinery possesses a vast array of toolkit components, which can be mixed and matched to deliver Ca^{2+} signals (Berridge et al., 2003). Thus, one can assume that in the DC precursors obtained from (lethal) RyR1 KO mice other molecules involved in intracellular Ca^{2+} signaling are compensating for the depletion of RyR1. Clearly, under physiological conditions RyR1 activation, together with signals generated through triggering of Toll-like receptors, generates costimulatory signals involved in the maturation of human DCs.

An interesting observation arising from this study is that addition of KCl to iDCs is accompanied by a rapid and transient increase in $[Ca^{2+}]_i$. In skeletal muscle, depolarization of the plasma membrane is sensed by an L-type Ca^{2+} channel, which undergoes a conformational change allowing it to interact directly with the RyR, thereby activating Ca^{2+} release from the sarcoplasmic reticulum (Sutko and Airey, 1996; Franzini-Armstrong and Protasi, 1997). Although DCs are not electrically excitable, one can envisage that massive release of KCl from cells dying in the vicinity of DCs may activate sensitive channels present on the plasma membrane coupled to the RyR1, thereby leading to RyR1 activation and thus Ca^{2+} release. Such a conclusion is supported by two sets of data: (1) the demonstration that treatment of iDCs with KCl promotes surface expression of CD83, which can be antagonized by dantrolene, and (2) the fact that incubation of iDCs with filtered soluble extracts, but not dialysed extracts, from necrotic cells promoted surface expression of CD83 that could partially be prevented by the addition of dantrolene. Similar results were reported by Sauter et al. (Sauter et al., 2000), who showed that incubation of iDCs with extracts from necrotic cells, but not apoptotic cells, induces DC maturation. Clearly, some of the signals generated by necrotic cells are capable of activating the iDC RyR, providing maturation stimuli. Based on these results, we propose that the intracellular contents released from dying cells in the vicinity of iDCs provide RyR1-dependent costimulatory signal(s), leading to full maturation of DCs even in the presence of suboptimal concentrations of bacterial stimuli. A schematic representation of our hypothesis concerning the signals

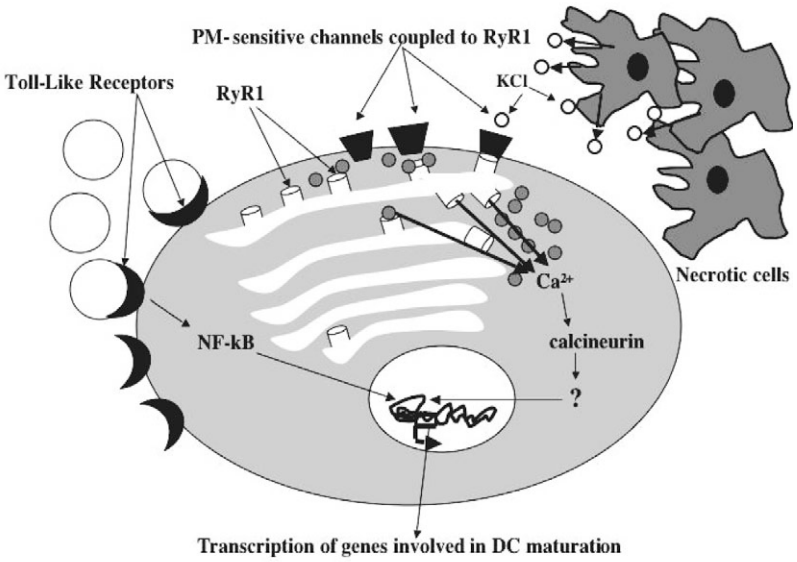

Fig. 6. Cartoon depicting signaling pathways involved in DC maturation. In the presence of sub-optimal Toll-like receptor ligand, the signal(s) generated from cells dying in the vicinity of iDCs bind to receptors that are coupled to the RyR1, activating calcium release. This intracellular pathway leading to DC maturation through RyR1 activation is dependent upon the activity of calcineurin as it could be blocked by cyclosporine A and deltamethrin. PM, plasma membrane.

underlying RyR1 activation and their involvement in the event leading to iDC maturation is illustrated in Fig. 6.

As to the involvement of specific transcription factors in DC maturation and function, NF-kB has been shown to play a role in the regulation of transcription of several genes, including those encoding *IL1A* and *IL1B*, *IL6*, *IL8*, *IL12B*, *TNFα* and *IFNγ* and *CD86* (Ghosh et al., 1998; Grohmann et al., 1998; Lee et al., 1999). Under resting conditions NF-kB forms an inactive complex (NF-kB1+RelA+IkB) detectable in the cytoplasm, which upon activation translocates to the nucleus where it binds to specific promoter sequences and functions as a transcriptional activator. Although it is well-established that the activation of Toll-like receptors by LPS induces nuclear translocation of NF-kB (Ghosh et al., 1998; Lee et al., 1999) (and this study), this does not occur with caffeine, suggesting the involvement of other (Ca^{2+}-dependent) transcriptional regulators. In a previous study we showed that RyR1 activation in human myotubes leads to cyclosporine-A-sensitive IL6 release (Ducreux et al., 2004). In DCs, the effects of cyclosporine A are controversial: one report suggests that pretreatment with cyclosporine A increases maturation and CD80 expression (Ciesek et al., 2005), whereas others report that cyclosporine A inhibits IL12 production and upregulation of costimulatory molecules induced by LPS (Lee et al., 1999; Tajima et al., 2003). Our results support and extend the latter conclusion. The synergistic effects of caffeine on LPS-induced maturation are sensitive to cyclosporine A and deltamethrin, implying the involvement of calcineurin (Barford, 1996; Enan and Matsumara, 1992) in RyR1-induced maturation of DCs. In addition, our data demonstrate that the low-amplitude calcium signal mediated by RyR synergizes with LPS in inducing DC maturation, whereas the large Ca^{2+} transient induced by ATP does not promote the expression of genes associated with DC maturation. On the basis of these data, one may postulate that amplitude modulation of the Ca^{2+} signal is a mechanism contributing to the maturation of DCs. A similar control by amplitude modulation of the Ca^{2+} signal has been proposed to play an important role in B-lymphocyte signaling (Dolmetsch et al., 1997). High LPS concentrations may engage a sufficient number of Toll-like receptors, and thereby the cooperation of additional signaling pathways is not required to fully activate DCs. The model emerging from our data indicates that NF-kB and calcineurin signaling pathways may ultimately converge to regulate the transcription of a set of genes involved in DC maturation. A similar complex regulatory mechanism has been reported for the transcription of the *IL4* gene in T cells, which is under the control of several transcription factors, including NF-kB, NF-AT and NF-IL6 (Li-Weber et al., 2004).

Finally, on a lighter note, based on these findings one may envisage that by drinking caffeine-containing beverages containing up to 85 mg/150 ml caffeine (Barone and Roberts, 1996) (i.e. 3 mM), iDCs residing in the gut may be exposed to concentrations of caffeine sufficient to activate them, especially in the presence of low levels of bacterial derivatives (Iwasaki and Kelsall, 1999), thereby enhancing their antigen-presenting capacity. Thus, relatively small amounts of antigens may be sufficient to induce effective T-cell responses and protection against enteric infections.

Materials and Methods

DC generation and stimulation

iDCs were generated from human peripheral blood mononuclear cells (PBMC) as previously described (Schnurr et al., 2004). Briefly, monocytes were purified by positive sorting using anti-CD14-conjugated magnetic microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany). The recovered cells (95-98% purity) were cultured for 5 days at 3×10^5/ml in differentiation medium containing: RPMI, 10% FCS glutamine, non-essential amino acids and antibiotics (all from Invitrogen, Basel, Switzerland), supplemented with 50 ng/ml GM-CSF (Laboratory Pablo Cassarà, Buenos Aires, Argentina) and 1000 U/ml IL4 (a gift from A. Lanzavecchia, Institute for Research in Biomedicine, Bellinzona, Switzerland). Maturation was induced by an 18-hour culture, unless otherwise stated, in the presence of the Toll-like receptor-4 agonist LPS (from *Salmonella abortus equi*; Sigma Chemicals, St Louis, MO) at the doses indicated in the different experiments. The phenotype of the cells was evaluated prior to and after maturation by flow cytometry. Briefly, cells were washed and resuspended in phosphate-buffered saline (PBS). They were then incubated for 30 minutes at 4°C in the presence of 1:20 dilutions of fluorochrome-labeled commercial monoclonal antibodies recognizing the following surface markers: CD1a, CD14, CD40, CD80, CD86, CD83, CD54, HLA-ABC, HLA-DR, CD3, CD19 and CD56, or isotype-matched controls (BD Pharmingen, Basel, Switzerland). After two washes, specific fluorescence was evaluated by flow cytometry, by using a FACSCalibur instrument equipped with Cell Quest software (Becton Dickinson, San Josè, CA). Plasmacytoid DCs were magnetically isolated from peripheral blood using BDCA-2 DC isolation kits (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The number and viability of DCs was determined by trypan blue exclusion and purity was assessed by flow cytometry. For functional experiments, in vitro-derived iDCs were cultured

in the presence of the indicated concentration of LPS ± 10 mM caffeine ± 20 μM dantrolene or a standard, optimal concentration of LPS (1 μg/ml) as control for the indicated time period. In some experiments, iDCs were stimulated with 100 μM ATP as indicated or with 100 mM KCl plus 1 ng/ml LPS for 30 minutes, followed by removal of KCl and addition of differentiation medium containing 1 ng/ml LPS for 4 hours. When the latter protocol was applied, NaCl was substituted for KCl in order to maintain osmolarity of the medium. For experiments in which iDCs were incubated with the supernatant of necrotic cells, experiments were performed essentially as described by Sauter et al. (Sauter et al., 2002); briefly, 6×10^6 HEK293 cells were rinsed twice with PBS, resuspended in 3 ml PBS and subjected to five cycles of freeze-thawing. Viability was assessed by trypan blue exclusion and was >95%. Cells were centrifuged, the resulting supernatant was filtered through a 0.2 μm Millipore filter and one half was added to iDC cultures (0.8×10^6 cells) in 1 ml differentiation medium plus a final concentration of 1 ng/ml LPS; the other half was added to iDCs that had been pretreated with 20 μM dantrolene for 30 minutes and then processed as described above. Cells were incubated for 4 hours at 37°C. For experiments in which iDCs were incubated with the dialysed supernatant of necrotic cells, experiments were performed as described above, except that the supernatants were dialysed overnight against 1×PBS, filtered and then added to iDCs.

Ryanodine receptor isoform expression

Total RNA was isolated from leukocyte populations and reverse transcribed into cDNA as previously described (Girard et al., 2001). The expression of genes encoding ryanodine receptor isoforms was investigated by PCR analysis using previously reported specific primers and conditions (Hosoi et al., 2001).

Immunofluorescence analysis and single-cell intracellular Ca^{2+} measurements

Immunofluorescence analysis was performed on paraformaldehyde-fixed DCs using allophycocyanine-conjugated anti-CD1a (BD Pharmingen), or on acetone:methanol (1:1)-fixed iDCs, using a goat anti-RyR raised against the NH_2-terminus and recognizing the three RyR isoforms (Santa Cruz Biotech), followed by Alexa Fluor-555-conjugated donkey anti-goat antibodies (Molecular Probes) or rabbit anti-NF-kB (sc-109; Santa Cruz Biotech), followed by Alexa Fluor-488-conjugated chicken anti-rabbit antibodies (Molecular Probes), as previously described (Ducreux et al., 2004). Fluorescence was visualized using a 100× Plan Neofluar oil immersion objective (NA 1.3) mounted on a Zeiss Axiovert 100 confocal microscope.

Single-cell intracellular calcium measurements were performed on fura-2-loaded iDCs attached to poly-L-lysine-treated glass coverslips mounted onto a 37°C thermostated chamber that was continuously perfused with Krebs-Ringer medium containing 1 mM $CaCl_2$. Individual cells were stimulated with the indicated agonist in Krebs-Ringer (plus 1 mM $CaCl_2$) by way of a 12-way 100 mm diameter quartz micromanifold computer-controlled microperfuser (ALA Scientific, Westbury, NY), as previously described (Ducreux et al., 2004). Online (340 nm, 380 nm and ratio) measurements were recorded using a fluorescent Axiovert S100 TV inverted microscope (Carl Zeiss, Jena, Germany) equipped with a 40× oil immersion Plan Neofluar objective (0.17 NA), filters (BP 340/380, FT 425, BP 500/530) and attached to a Hamamatsu multiformat CCD camera. The cells were analyzed using an Openlab imaging system and the average pixel value for each cell was measured at excitation wavelengths of 340 and 380 nm, as previously described (Ducreux et al., 2004).

Quantitative gene expression analysis

Eighteen hours after stimulation, total RNA was extracted from DCs (Qiagen, Basel, Switzerland) and treated with Deoxyribonuclease I (DNase I) (Invitrogen, Carlsbad, CA) to eliminate contaminant genomic DNA. After reverse transcription using the Moloney MurineLeukemia Virus Reverse Transcriptase (M-MLV RT) (Invitrogen), cDNA was amplified by quantitative real-time PCR in the ABI Prism™ 7700 using the TaqMan® technology. Commercially available exon-intron junction-designed primers for GAPDH, CD83, IFNα, IL12B and IL23A (Applied Biosystems, Forster City, CA) were used. Gene expression was normalized using self-GAPDH as reference.

Allostimulatory properties of DCs

Allostimulatory capacity of DCs treated with different concentrations of LPS alone or with 100 μM ATP, 10 mM caffeine plus or minus 20 μM dantrolene, was assayed by standard mixed lymphocyte reaction (MLR) tests by culturing cells in the presence of allogenic PBMC in RPMI medium supplemented with 5% pooled human serum at a 10:1 ratio (Mohty et al., 2002). Lymphocyte proliferation was evaluated by [^{3}H]-thymidine incorporation.

Activation of intracellular signaling pathways

NF-kB translocation was monitored by indirect immunofluorescence on DCs treated with LPS and/or caffeine (10 mM) as indicated, for 5 or 60 minutes at 37°C. Cells were fixed with acetone:methanol and processed as described above for RyR immunofluorescence, using rabbit anti-NF-kB [anti-p65 (RelA) polyclonal antibodies; Santa Cruz Biotech], followed by Alexa Fluor-488-conjugated chicken anti-rabbit antibodies (Molecular Probes).

Involvement of the calcium/calmodulin-dependent calcineurin signaling pathway was determined by studying the sensitivity of CD83 surface expression to cyclosporine A or deltamethrin. Briefly, iDCs were pretreated with carrier, cyclosporine A (2 μM) (Sigma Chemicals) or deltamethrin (10 μM) (Fluka Chemicals, Buchs, Switzerland) for 30 minutes, followed by incubation for 4 hours with 1 μg/ml LPS or sub-optimal concentrations of LPS in the presence or absence of 10 mM caffeine. Cells were harvested and surface expression of CD83 was investigated by flow cytometry using FITC-labeled anti-CD83 monoclonal antibodies (BD Pharmingen).

Statistical analysis and software programs

Statistical analysis was performed using the Student's *t*-test for unpaired samples; means were considered statistically significant when the P value was <0.05. The Origin computer program (Microcal Software, Northampton, MA) was used to generate graphs and for statistical analysis; figures were assembled using Adobe Photoshop.

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