



Persistence of apoptosis-resistant T cell-activating dendritic cells promotes T helper type-2 response and IgE antibody production

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Abstract

Antigen-specific T cell-mediated apoptosis of dendritic cells (DCs) represents a unique down-regulatory mechanism that prevents the continuous activation of T cells by antigen-loaded DCs; this regulatory mechanism is impaired in allergy and as a consequence a large proportion of DCs tends to escape apoptosis following cognate interaction with CD4⁺ T cells. However, the biological relevance of greater numbers of apoptosis-resistant DCs to the development of allergic IgE-mediated reactions remained to be determined. Here, we sought to investigate the *in vitro* and *in vivo* regulatory features of apoptosis-resistant DCs and to assess their role in host sensitization. Freshly isolated CD11c⁺ B220⁺ DCs from ovalbumin (OVA)-sensitized, OVA-immunized and naïve Balb/c mice were cultured with OVA-specific T cells and levels of T cell-mediated DCs apoptosis assessed by flow cytometry. Surviving apoptosis-resistant DCs were then recovered and subsequently co-cultured with OVA-specific CD62L^{hi}CD44^{low} naïve T cells or passively transferred into naïve syngenic recipients. *In vitro* profile of DC and T cell lymphokine production, chemokine receptors expression and *in vivo*, post-adoptive DC transfer T helper (T_H) and IgE responses were assessed. Apoptosis-resistant DCs showed differential regulatory properties compared to their freshly isolated counterpart independent of the sensitization status of the donor. When co-cultured with naïve OVA-specific T cells, apoptosis-resistant DCs from either sensitized or immunized mice induced T cells that produced increased levels of IL-4 and reduced levels of IFN- γ and showed increased expression of T_H-2 related CCR4 and CCR8 chemokine receptors. Finally, adoptive transfer of apoptosis-resistant DCs, induced higher levels of OVA-specific IgE responses in absence of antigen challenge in syngenic recipients compared to freshly isolated DCs from both sensitized and immunized mice. These data would suggest that sensitization-associated increased numbers of apoptosis-resistant T cell-activating DCs contribute to the generation/maintenance of IgE-mediated allergic reactions.

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1. Introduction

Apoptosis is one of the most important mechanisms for the maintenance of cellular homeostasis in mammals, and although the mechanism of programmed cell death is relevant to the development of all cell lineages, it is of paramount importance for the generation of immune cell repertoire and responses (Opferman and Korsmeyer, 2003). DCs have a unique ability to present antigen to naïve T cells, however, activation of T cells by DCs has to be kept under strict control to avoid an otherwise uncontrollable reaction. To this end, T cell-mediated DC apoptosis serves as a

down-regulatory mechanism and it exerts a form of control on various subtypes of DCs at least at two different time points during DCs life time. First, T_H-2 cells down-regulated pre-DC type 2 subset through IL-4-mediated killing (Rissoan et al., 1999) and second, T cells induced rapid apoptosis in both DC line and freshly isolated murine DCs in antigen-specific, MHC-restricted manner following DC–T cells cognate interaction (Matsue et al., 1999; Nicoletti et al., 2006). These observations pointed to an important role of T cell-mediated DC cell death in the physiologic regulation of immune responses. Indeed, this mechanism may represent a strategy that allows T cells to control the death or survival of appropriate DC populations in an antigen-specific manner.

Recently, we reported that antigen-specific MHC-restricted T cell-mediated DCs apoptosis was significantly impaired in a mouse model of food allergy (Man et al., 2004). Indeed, large

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proportion of DCs displayed a remarkably higher resistance to T cell-mediated apoptosis compared with their counterpart from non-sensitized mice. This led us to hypothesize that the malfunctioning of this regulatory pathway might have a profound influence on the development of allergic reactions but the biological relevance of these events to the development of IgE-mediated allergic responses remained to be determined. Allergic disease is an increasing problem in Western countries; the prevalence of allergy was found to be doubled in American children (Sicherer et al., 2003) and recently emergency room visits due to allergic reactions have increased by a factor of six in the United Kingdom (de Block et al., 2007). This increased in prevalence is also accompanied by more cases of anaphylaxis (Gupta et al., 2004), which is potentially fatal and although our knowledge of cellular and molecular events underlying allergic reactions has increased markedly over the past few years, many basic aspects of adverse IgE-mediated reactions remain to be fully understood. Here, we sought to investigate the regulatory features of apoptosis-resistant DCs in order to determine their role in the development of allergy-associated T_H -2 responses and subsequent IgE production. We report that apoptosis-resistant DCs, independently of the sensitization status of the host, have acquired greater capability to generate antigen-specific T_H -2 cells *in vitro* and *in vivo* and to induce IgE responses *in vivo* compared to freshly isolated DCs. These data demonstrated that sensitization-associated increased numbers of T_H -2-driving apoptosis-resistant DCs has a significant impact on the development of allergic disease.

2. Materials and methods

2.1. Mouse model of IgE sensitization

Female Balb/c mice, 5 weeks old were maintained in a clean, access-restricted room in conventional conditions throughout the experiments. Experiments were conducted under the current Italian law on animal experimentation. Mice were sensitized as described previously (Blumchen et al., 2004). 5–6 weeks old Balb/c mice were sensitized to ovalbumin (OVA) (Sigma, UK) by six intraperitoneal (i.p.) injections of 10 μ g/mouse of OVA over a period of 3 weeks. Mice injected with PBS are referred as naïve; mice that received a single i.p. injection (10 μ g/mouse) of OVA and produced OVA-specific IgG response but not detectable levels of IgE are referred as immunized. DCs were isolated from all groups 24 h after last injection. Specific IgE and IgG responses were evaluated at serum and cellular level by ELISA and ELISPOT assay respectively as described previously (Chambers et al., 2004).

2.2. Cell preparation and DCs apoptosis assay

Splenic CD11c^{+/hi}B220[−]DCs were isolated according to established protocol described in detail elsewhere (Chambers et al., 2004; Man et al., 2004). To generate OVA-T cells mice were immunized by subcutaneous injection of 100 μ g of OVA in CFA into foot pad, and boosted twice at biweekly intervals with OVA (50 μ g/dose). Seven days after the last injection spleens were

removed and cultured in the presence of irradiated syngeneic splenocytes plus OVA (50–100 μ g/ml) and IL-2 (10–30 U/ml) for 10 days. The resulting population was 96–98% CD4⁺ and was cultured for 10 days in the presence of antigen and syngenic splenocytes. T cells harvested showed significant reactivity to autologous OVA-pulsed CD11c^{+/hi}B220[−]DC. Antigen-specific T cells were maintained in culture and fed at 3-week interval with antigen-pulsed splenocytes and IL-2. Similar protocol was used to generate keyhole limpet hemocyanine (KLH)-specific T cells as described (Nicoletti et al., 2006). Apoptosis assay of DCs after co-incubation with antigen-specific T cells was carried out as described elsewhere (Matsue et al., 1999; Man et al., 2004). Phenotypic analysis of all DCs studied was carried out by flow cytometry using fluorescein isothiocyanate (FITC)-labelled antibody anti-Ia, CD80, CD40, CD11b (BD Biosciences), CD86 (AMS Biotechnology), CD8 α (Ebioscience) and DEC205 (NLCD-145) and ICAM-1 (Pharmingen). In some cases, apoptosis of DCs was induced by incubation with ether anti-MHC class II antibody (clone 14-4-44) or treated with carbamoyl cyanide *m*-chlorophenyl-hydrazine (CCCP) to artificially abolish mitochondrial membrane potential ($\Delta\Psi_m$) as described (McLellan et al., 2000); $\Delta\Psi_m$ was determined using the 3,3'-dihexyloxycarbocyanide iodide (DiOC₆) dye (Leverkus et al., 2003).

2.3. Apoptosis-resistant DCs

CD11c⁺/annexin V[−] DCs from both OVA-sensitized and OVA-immunized mice that survived DC–T cell co-culture were sorted and co-cultured with freshly isolated OVA-specific CD62L^{hi}CD44^{low} naïve T. Prior to co-culture with T cells apoptosis-resistant and freshly isolated DCs were subjected to acid extraction to remove MHC class II-bound peptide as described (Sugawara et al., 1987) without altering expression of MHC class II molecules. Both the latter and post-extraction cell viability were evaluated by flow cytometry using specific anti-class II antibody (BD Biosciences) and p.i. up-take, respectively. DCs were then pulsed for 3 h with OVA peptide 323–339 (ISQAVHAAHAEINEAGR) or peptide 947–963 (FNNFTVS-FWLRVPKVSA) from tetanus toxoid (TT) for specificity control.

2.4. DCs–naïve T cell co-cultures

T cells were obtained from splenocytes from 8 to 10 weeks old DO11.10 OVA TCR transgenic mice (Charles River). Following 1 h incubation at 37 °C non-adherent splenocytes were then used to obtain CD4⁺T cells by using CD4⁺T cell negative selection kit (Miltenyi Biotec) according to manufacturer instructions. Naïve CD62L^{hi}CD44^{low} T cells were then sorted. CD4⁺ T cells were 97–99% pure. Co-cultures were carried out at high (T_H -2-inducing) and low (T_H -1-inducing) DC–T cell ratios (1:10 and 1:3, respectively) (Langenkamp et al., 2000). After 24–36 h cells were transferred to 24-well plate and allowed to expand for 3 days; T cells were then cultured on 96-well plate coated with anti-CD3 ϵ (5 μ g/ml) and soluble anti-CD28 (5 μ g/ml) for secondary stimulation as described by

Iwasaki and Kelsall (1999). Supernatant were then collected and levels of IL-4 and IFN- γ determined at various intervals by ELISA. Expression of chemokine receptors by DC-primed T cells was assessed by flow cytometry using PE-labelled antibody anti-CXCR3 (R&D System), anti-CCR5 (Ebioscience), FITC-labelled anti-CCR9 (Ebioscience), anti-CCR4 and anti-CCR8 (Biocompare). Finally, T cell proliferation was monitored by incorporation of [^{3} H]-thymidine.

2.5. Cytokine production

Freshly isolated and apoptosis-resistant DCs were cultured (1×10^5 /ml) alone or in presence of LPS (30–50 ng/ml); production of IL-10, IL-12p70, IL-6, TNF- α and IL-4 was monitored by ELISA in 18–20 h culture supernatant; expression of OX40L was assessed by flow cytometry using specific PE-labelled anti-mouse antibody (clone RM 134L, Ebioscience).

2.6. Adoptive transfer of DCs and ensuing humoral and cellular immune responses

Mice ($n=7$ /group) were injected i.v. with freshly isolated or apoptosis-resistant DCs ($(1-2) \times 10^5$ /mouse) from either OVA-sensitized or OVA-immunized mice. IgE and IgG responses, including IgG1 and IgG2a isotypes, were evaluated at serum and cellular level by ELISA or ELISPOT assay as above. Finally, the number of individual CD4 $^+$ T cells secreting IL-4 or IFN- γ in the spleen of mice undergoing adoptive transfer of DCs was assessed by ELISPOT following *in vitro* antigen recall challenge as described previously (Chambers et al., 2004). Anti-IFN- γ or IL-4 (R4-6A2 or BVD4-1D1, respectively) were used as coating antibody and anti-IFN- γ XMB1.2 or anti-IL-4 BVD6-24G2 (all antibodies were from Pharmingen) were used as detection antibody.

2.6.1. Statistical analysis

Data were analysed by Mann Whitney *W* test and *p* values considered significant at $p < 0.05$. The Pearson correlation coefficient, *r*, between the natural logarithm of the concentrations of IgE and IgG was also determined.

3. Results

3.1. T cell-mediated DC apoptosis in OVA-sensitized mice

We previously showed that T cell-mediated DC apoptosis is impaired in an oral mouse model of food allergy (Man et al., 2004). Thus, we assessed these events in a different mouse model of sensitization to OVA. We observed that rapid T cell-mediated DC apoptosis took place only in complete co-culture where DCs, OVA-specific T cells and OVA were simultaneously present (Fig. 1) and also that levels of apoptosis were significantly lower ($p < 0.001$) in CD11c $^+$ DCs from OVA-sensitized ($37.5 \pm 8\%$) compared to OVA-immunized mice ($68 \pm 9\%$) or naïve ($67 \pm 7\%$). Culture of DCs in the presence of OVA and KLH-specific T cells or of anti-class II antibody completely abolished T cell-mediated apoptosis. Thus, increased resistance

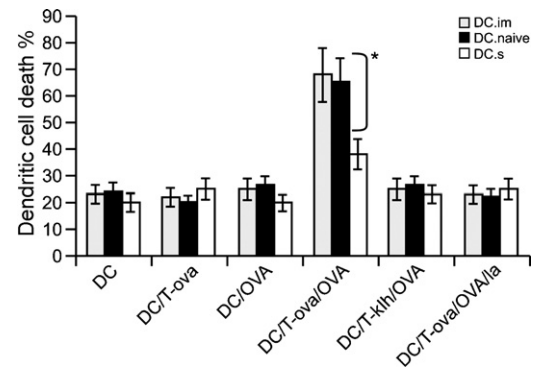
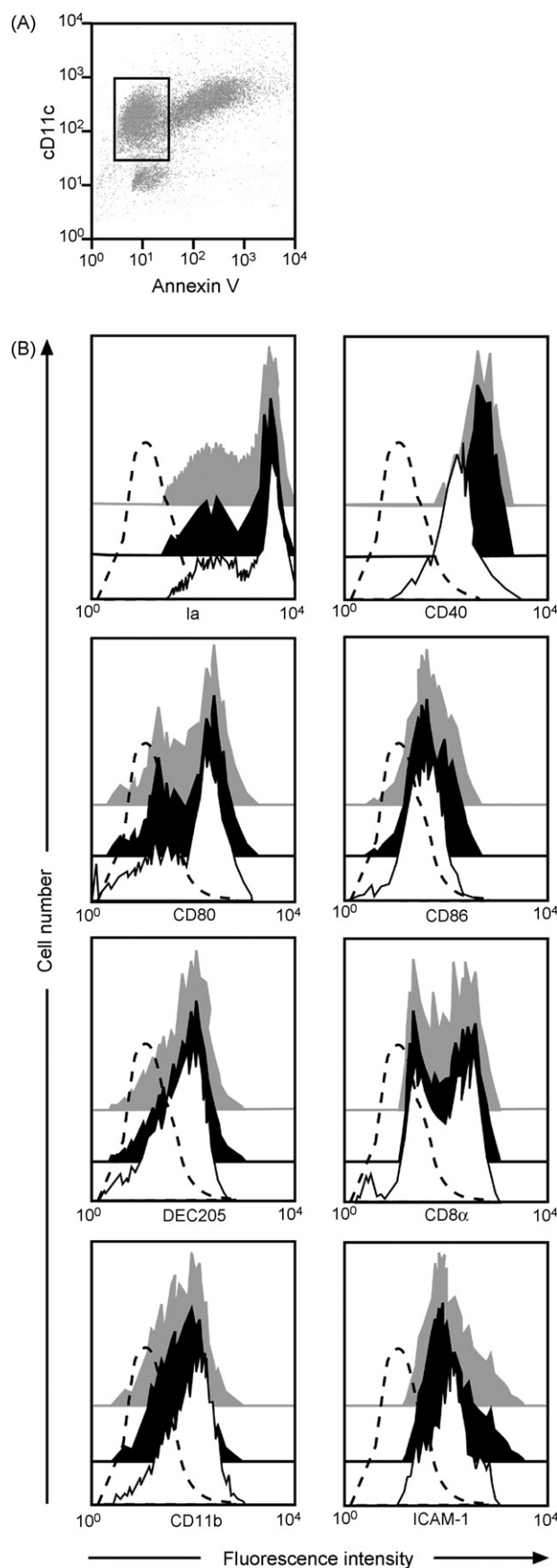


Fig. 1. T cell-mediated apoptosis of splenic DCs from immunized (DC.im, shaded bar) naïve (DC.naive, black bar) or sensitized (DC.s, empty bar) mice. High levels of DC apoptosis were seen when these were cultured in the presence of both OVA and OVA-specific T cells (DC-T/OVA-OVA). Number of DCs undergoing apoptosis were significantly ($*p < 0.001$) reduced in sensitized mice. Co-culture in presence of KLH-specific T cells (DC-T-klh/OVA) and anti-class II antibody (DC-T-ova/OVA/Ia) abolished DC apoptosis. Data represent means $\pm$ S.D. of two independent experiments.

to T cell-mediated apoptosis of DCs appeared to be a feature of the sensitized immune system independent of both the nature of the allergen and sensitization protocol used.

3.2. Apoptosis-resistant DCs showed differential lymphokine production

Cells from DC-T cell co-cultures were pooled and the surviving apoptosis-resistant CD11c $^+$ annexinV $^-$ DCs (Fig. 2A) from both OVA-sensitized (Fig. 2B, black histogram) and OVA-immunized, mice (Fig. 2B, shaded histogram) were analysed for the expression of DC surface markers and compared with freshly isolated CD11c $^+$ B220 $^-$ DCs from OVA-sensitized mice (Fig. 2B, empty histogram). Expression of CD40 was increased in apoptosis-resistant DCs while the other markers were expressed at similar levels in all populations tested. Freshly isolated DCs from naïve and OVA-immunized mice did not differ from the freshly isolated DCs from OVA-sensitized mice (data not shown). Freshly isolated DCs from immunized (F.I.im) or sensitized (F.I.s) or apoptosis-resistant and from immunized (APO-R.im) or sensitized (APO-R.s) mice were then cultured in presence of bacterial LPS and levels of regulatory cytokines measured (Fig. 3). All groups of cells produced very low levels of IL-10 (Fig. 3A); instead levels of both TNF- α (Fig. 3B) and IL-12 (Fig. 3C) were significantly higher in freshly isolated DCs. Levels of IL-6 (Fig. 3D) appeared to be higher, although not significantly different in apoptosis-resistant DCs. Production of IL-4 was not detected in none of the DC groups (Fig. 3E) and similar levels of expression of OX40L was seen in freshly isolated DCs from sensitized mice compared to APO-R.s (Fig. 3F) suggesting that these DCs have not acquired an active TH2-inducing phenotype; finally, we observed a reduced expression of CD86 on APO-R.s DCs compared to freshly isolated DC from sensitized mice (F.I.s) (Fig. 3G). The latter would indicate an exhausted phenotype (Kajino et al., 2006).



3.3. Apoptosis-resistant DCs promoted the differentiation of T_H-2 cells

Levels of IFN γ produced by T cells following secondary stimulation with CD3 ϵ and CD28 significantly declined when these were primed with apoptosis-resistant DCs from either sensitized (APO-R.s) or immunized (APO-R.im) mice (Fig. 4A). In contrast, these T cell produced significantly higher levels of IL-4. Freshly isolated DC from immunized (F.I.im.) and sensitized (F.I.s) did not differ in their ability to polarize T helper responses. T cell primed with apoptosis-resistant DCs expressed diverse CRs repertoire (Fig. 4B). While expression of CCR9 and CXCR3 by T cells did not vary according to the type of DC used for *in vitro* priming, differences were observed in regards to expression of CCR4, CCR8 and CCR5. The first two were up-regulated ($p < 0.01$ and $p < 0.001$, respectively) following priming with apoptosis-resistant from both immunized (APO-R.im) and sensitized (APO-R.s) DCs. Expression of CCR8 was also increased ($p < 0.01$) in naïve T cells primed with freshly isolated DCs from sensitized mice while, CCR5 expression was higher ($p < 0.01$) in T cells primed with freshly isolated DC from immunized mice (F.I.im.) compared to T cells exposed to the other DC populations tested. Also, apoptosis-resistant DCs did not differ from their freshly isolated counterpart in stimulating T cell proliferation (Fig. 4C). Thus, induction of T cells that produced high levels of IL-4 and low levels of IFN- γ and expressed the T_H2-associated chemokine receptors CCR4 and CCR8 (Bonecchi et al., 1998; Zingoni et al., 1998) suggested that apoptosis-resistant DCs may participate in T_H2-skewing.

3.4. Adoptive transfer of apoptosis-resistant DCs induced high levels of specific IgE antibody

Adoptive transfer of freshly isolated DCs from sensitized mice (F.I.s) induced a significant increase ($p < 0.001$) of OVA-IgE specific antibody forming cells (AFC) (Fig. 5A) compared to freshly isolated DCs from immunized mice (F.I.im). The number of OVA-specific IgE AFC increased significantly ($p < 0.001$) when apoptosis-resistant DCs from both sensitized (APO-R.s) and immunized (APO-R.im) were used in the adoptive transfer. At serum level a significant increase of OVA-specific IgE was observed on day 21 following transfer of freshly isolated sensitized DCs (88.8 ± 43 ng/ml) compared to immunized DCs (< 10 ng/ml) ($p < 0.001$). Also in this case apoptosis-resistant DCs from either immunized (182 ± 63 ng/ml) or sensitized (178 ± 66 ng/ml) showed superior ability to induce specific IgE compared to freshly isolated sensitized DCs ($p < 0.001$).

Fig. 2. Phenotypic analysis of apoptosis-resistant DCs that survived T cell-mediated apoptosis. CD11c⁺Annexin V⁻ DCs were sorted (Fig. 2A, boxed area) and then levels of expression of DC markers were assessed (Fig. 2B) in apoptosis-resistant DCs from both OVA-sensitized (black histograms) and OVA-immunized (shaded histograms) and freshly isolated DCs from OVA-sensitized mice (empty histograms). Expression of CD40 was increased on apoptosis-resistant DCs irrespective of the sensitization status of the donor, while the other markers tested were expressed at similar levels. The data depicted here represents one of three independent experiments producing similar results.

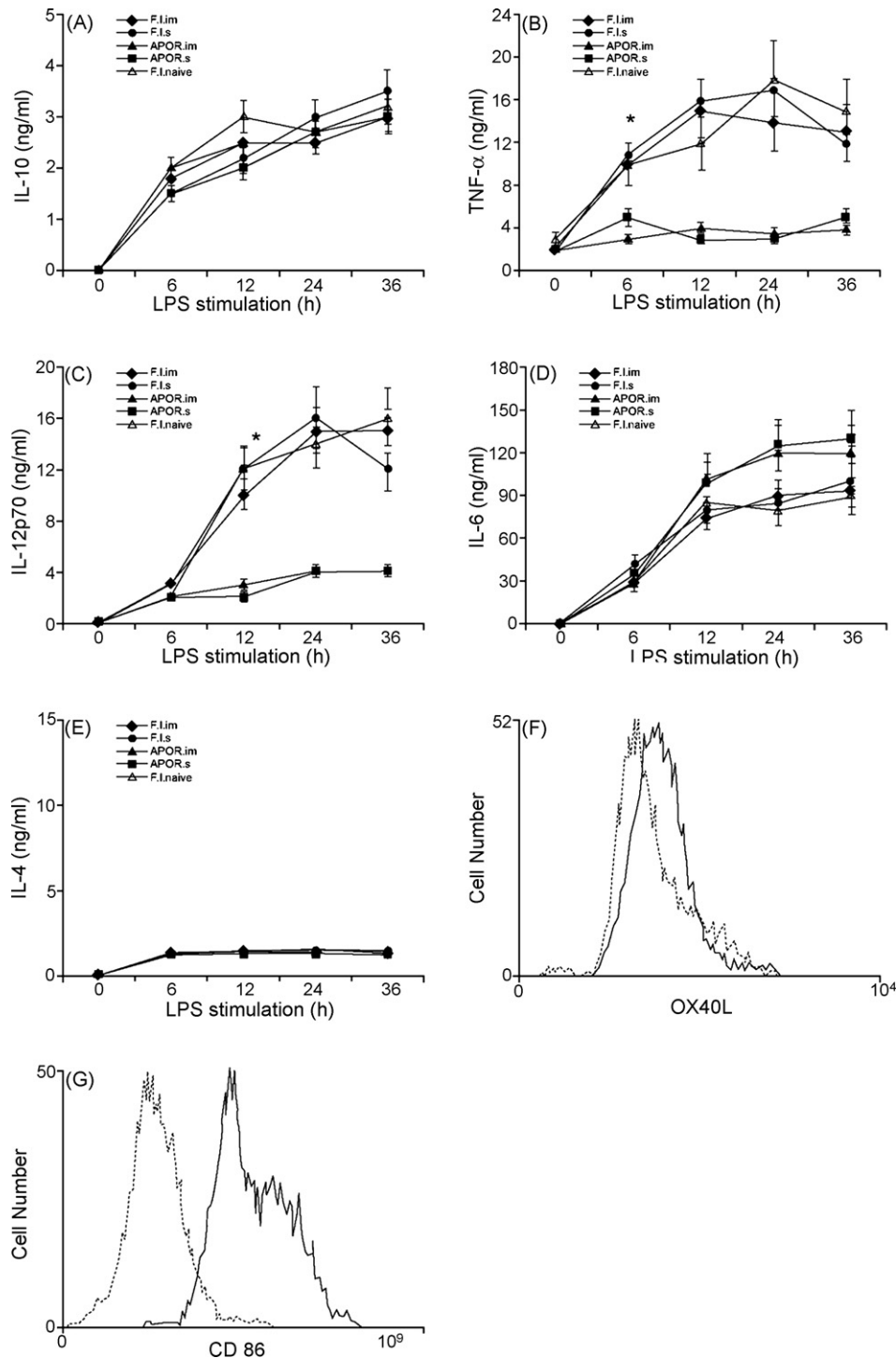


Fig. 3. Profile of lymphokines production by apoptosis-resistant (APO-R) and freshly isolated (F.I.) DCs from OVA-immunized (im), sensitized (s) and naïve mice. DCs were cultured in presence of LPS and levels of TNF- α (Fig. 3A), IL-10 (3B), IL-6 (Fig. 3C), IL-12p70 (Fig. 3D) and IL-4 (Fig. 3E) determined by ELISA at several time points. Asterisks (*) indicate when p values started to be significantly different. Data represent means $\pm$ S.D. of three independent experiments. Also, no difference was observed in the expression of OX40L between freshly isolated DC from sensitized mice (dotted line) compared to APO-R.s DCs (continuous line); in contrast, the latter showed a reduced expression of CD86, thus suggesting an exhausted phenotype (Fig. 3G).

Levels of serum IgG were also analyzed (Fig. 5C). No significant difference was observed between IgG responses elicited by freshly isolated immunized (562 ± 101 ng/ml) and sensitized (430 ± 116 ng/ml) DCs. However, apoptosis-resistant DCs from both sensitized (337 ± 100 ng/ml) and immunized

(338 ± 121 ng/ml) mice showed a reduced ability to induce OVA-specific IgG compared with freshly isolated DCs from immunized mice ($p=0.001$) but not from sensitized mice ($p=0.1$). Levels of IgE and IgG were inversely correlated ($r=-0.61$; $p=0.0005$) (Fig. 5D). In Fig. 5E we show that

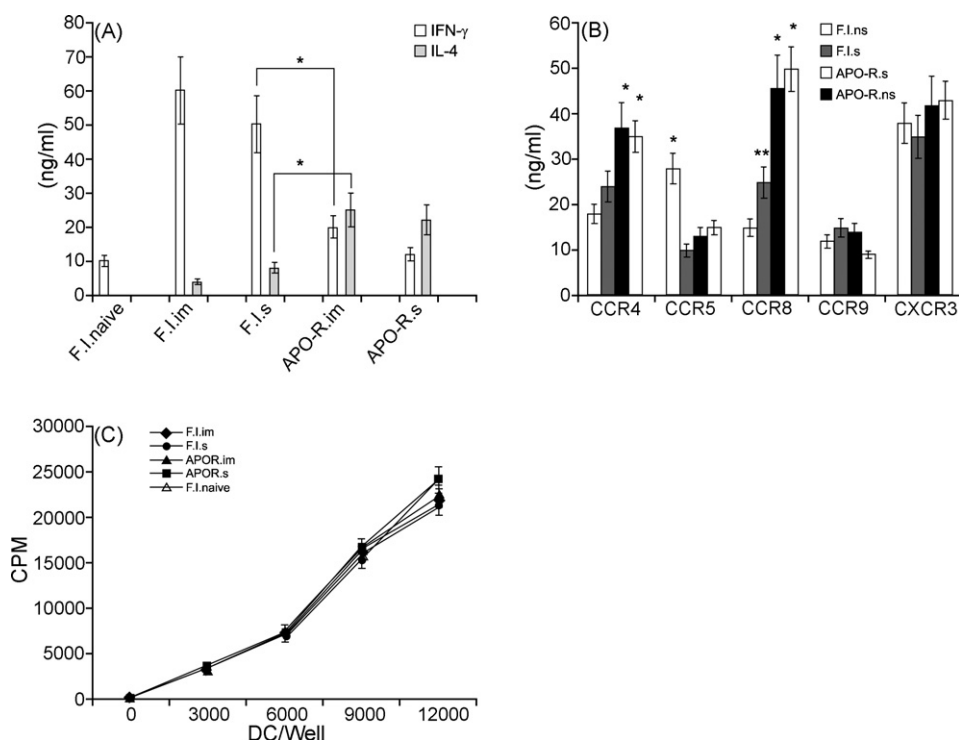


Fig. 4. Apoptosis-resistant DCs from either OVA-sensitized (APO-R.s) or OVA-immunized (APO-R.im) mice differed from their freshly isolated counterpart in polarizing T helper response. OVA-specific naïve T cells (Fig. 4A) produced high levels of IFN- γ when primed with freshly isolated DCs from both immunized (F.I.im) and sensitized (F.I.s) mice. Instead, they produced higher levels of the T_H2 lymphokine IL-4 when primed with apoptosis-resistant DCs irrespective of the sensitization status of the donor. Asterisks (*) indicate statistically significant difference between two representative groups. Fig. 4B shows the expression of chemokine receptors on naïve OVA-specific T cells following co-culture with DCs. Apoptosis-resistant DCs from both OVA-immunized and sensitized mice increased the expression of CCR4 and CCR8 (*), while co-culture with freshly isolated DCs from immunized or sensitized mice induced increase of CCR5 (*) and CCR8 (**), respectively. All DC populations used induced similar levels of T cells proliferation (Fig. 4C). Data represent means $\pm$ S.D. of three to four independent experiments.

non-apoptotic DCs (APO-R. DC-T) that were recovered from co-culture with OVA-specific T cell alone after 16–20 h cultures, did not induce *in vivo* IgE responses following adoptive transfer into naïve syngenic recipients (5/6 mice groups) compared to APO-R.s DCim. This would suggest that the ability of apoptosis-resistant DCs to induce T_H2 is either T cell-dependent or it appears at later stage in culture. Finally, the importance of APO-R DCs in shaping T_H2 responses was also confirmed by the observations that their adoptive transfer was accompanied by alteration of IgG1 (T_H2-associated) and IgG2a (T_H1-associated) ratio (Table 1) and an increase in, the numbers of IL-4-producing CD4⁺T cells (Table 2) as determined following antigen recall challenge *in vitro*.

3.5. DCs from sensitized and immunized mice did not differ in their susceptibility to MHC-mediated apoptosis induced by anti-MHC antibody

MHC class II-mediated apoptosis has been investigated mainly by using specific anti-class II antibody (Leverkus et al., 2003; Bertho et al., 2002). Thus, we determined levels of apoptosis (Fig. 6A) and changes of mitochondrial membrane potential ($\Delta\Psi_m$) (Fig. 6B and C) in DCs when cultured in the presence of various concentration of anti-class II antibody (clone 14-4-44). In contrast to what we observed in DC-T cell co-cultures, DCs from immunized and sensitized donors did not show differential

Table 1
Serum levels of OVA-specific IgG1 and IgG2a following adoptive transfer of DCs

Adoptive transfer	IgG1 (μ g/ml)	IgG2a (μ g/ml)	<i>r</i> (IgG1/IgG2a)
F.I.im	155 $\pm$ 56	250 $\pm$ 35	0.62
F.I.s	130 $\pm$ 60	155 $\pm$ 30	0.83
APO-R.im	100 $\pm$ 25	89 $\pm$ 15	1.23
APO-R.s	125 $\pm$ 20	90 $\pm$ 27	1.38

Levels of OVA-specific IgG1 (T_H2-associated) and IgG2a (T_H1-associated) and their ratio (*r*) were determined by ELISA in sera of recipient Balb/c (7 mice/group) on day 21 post-adoptive transfer of freshly isolated DCs from OVA-immunized (F.I.im) or OVA-sensitized (F.I.s) or apoptosis-resistant DCs from OVA-immunized (APO-R.im) or sensitized (APO-R.s) mice. IgG1/IgG2a ratios varied according to the type of DCs transferred with APO-R DCs from either immunized or sensitized mice favouring the production of T_H2-associated IgG1 isotype

susceptibility to anti-class II antibody-induced apoptosis. These data showed that DC apoptosis induced by the engagement of MHC II by monoclonal antibody does not mimic faithfully the full range of events taking place during DC-T cell cognate interaction.

4. Discussion

Allergic responses are linked to T_H2 cells; (Turcanu et al., 2003; Romagnani, 2000); however, neither T_H1 nor T_H2 orig-

Table 2
IL-4 and IFN γ -producing T cells following adoptive transfer of DCs

Adoptive transfer	Day 0			Day 7			Day 21		
	IL-4	IFN γ	<i>r</i>	IL-4	IFN γ	<i>r</i>	IL-4	IFN γ	<i>r</i>
F.I.im	195 $\pm$ 2	98 $\pm$ 10	1.9	178 $\pm$ 15	90 $\pm$ 14	1.9	210 $\pm$ 15	101 $\pm$ 8	2.0
F.I.s	220 $\pm$ 1	80 $\pm$ 12	2.7	195 $\pm$ 25	85 $\pm$ 9	2.1	195 $\pm$ 20	92 $\pm$ 10	2.1
APO-R.im	190 $\pm$ 1	95 $\pm$ 18	2.0	*380 $\pm$ 40	100 $\pm$ 20	3.8	*715 $\pm$ 112	125 $\pm$ 40	5.7
APO-R.s	185 $\pm$ 18	85 $\pm$ 21	2.1	*410 $\pm$ 42	112 $\pm$ 30	3.6	*660 $\pm$ 126	108 $\pm$ 35	6.1

Splenocytes from naïve Balb/c that had received adoptive transfer of freshly isolated DCs from OVA-immunized (F.I.im) or OVA-sensitized (F.I.s) or apoptosis-resistant DCs from OVA-immunized (APO-R.im) or sensitized (APO-R.s) mice were cultured *in vitro* in antigen (OVA) recall challenge at various times following adoptive transfer. Following their isolation T cells were used to determine the number of IL-4 and IFN γ -producing T cells by ELISPOT in duplicate. Values represent the mean $\pm$ S.D. On Days 7 and 21 numbers of IL-4 producing cells significantly (*) increased in mice that underwent adoptive transfer of both APO-R.im and APO-R.s compared to mice that had received both F.I.im and F.I.s DCs. Means were used to calculate IL-4/IFN γ ratio (*r*).

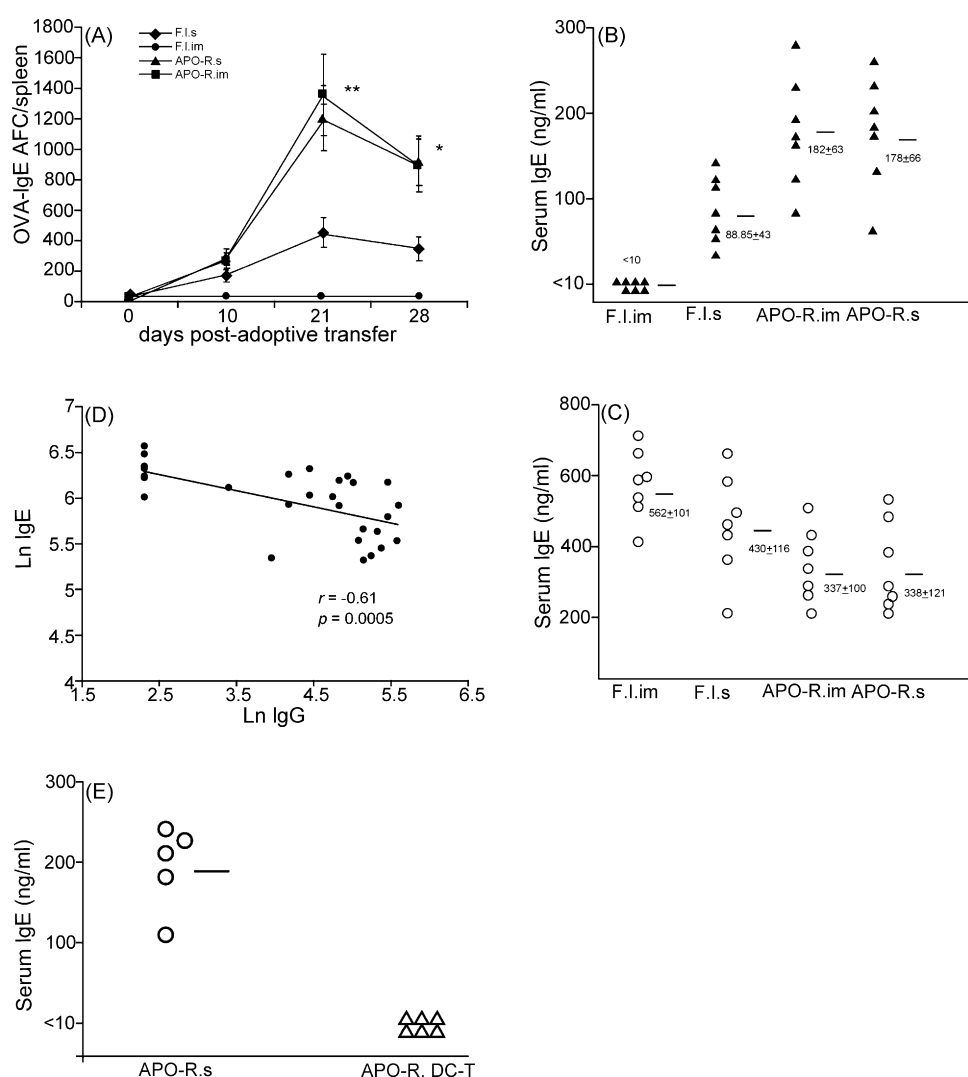


Fig. 5. Apoptosis-resistant DCs from either OVA-sensitized (APO-R.s) or OVA-immunized (APO-R.im) mice differed from their freshly isolated counterpart in inducing OVA-specific IgE and IgG responses following adoptive transfer into naïve recipients. Numbers of IgE-antibody forming cells (AFC) were enumerated by ELISPOT at various intervals following the transfer (Fig. 5A). Freshly isolated DCs from sensitized mice induced a significant increased of OVA-specific AFC (*) compared to their counterpart from immunized mice. Numbers of IgE-specific AFC were higher when apoptosis-resistant DCs from either sensitized or immunized mice were used in the transfer (**). Serum levels of IgE (Fig. 5B) and IgG (Fig. 5C) OVA-specific antibody were also evaluated. Finally, levels of IgE and IgG were inversely correlated (Fig. 5D). Finally, non-apoptotic DCs recovered from co-culture with OVA-specific T cells alone after 16–20 h culture did not induce serum IgE *in vivo* after their transfer into naïve syngenic recipients (Fig. 5E).

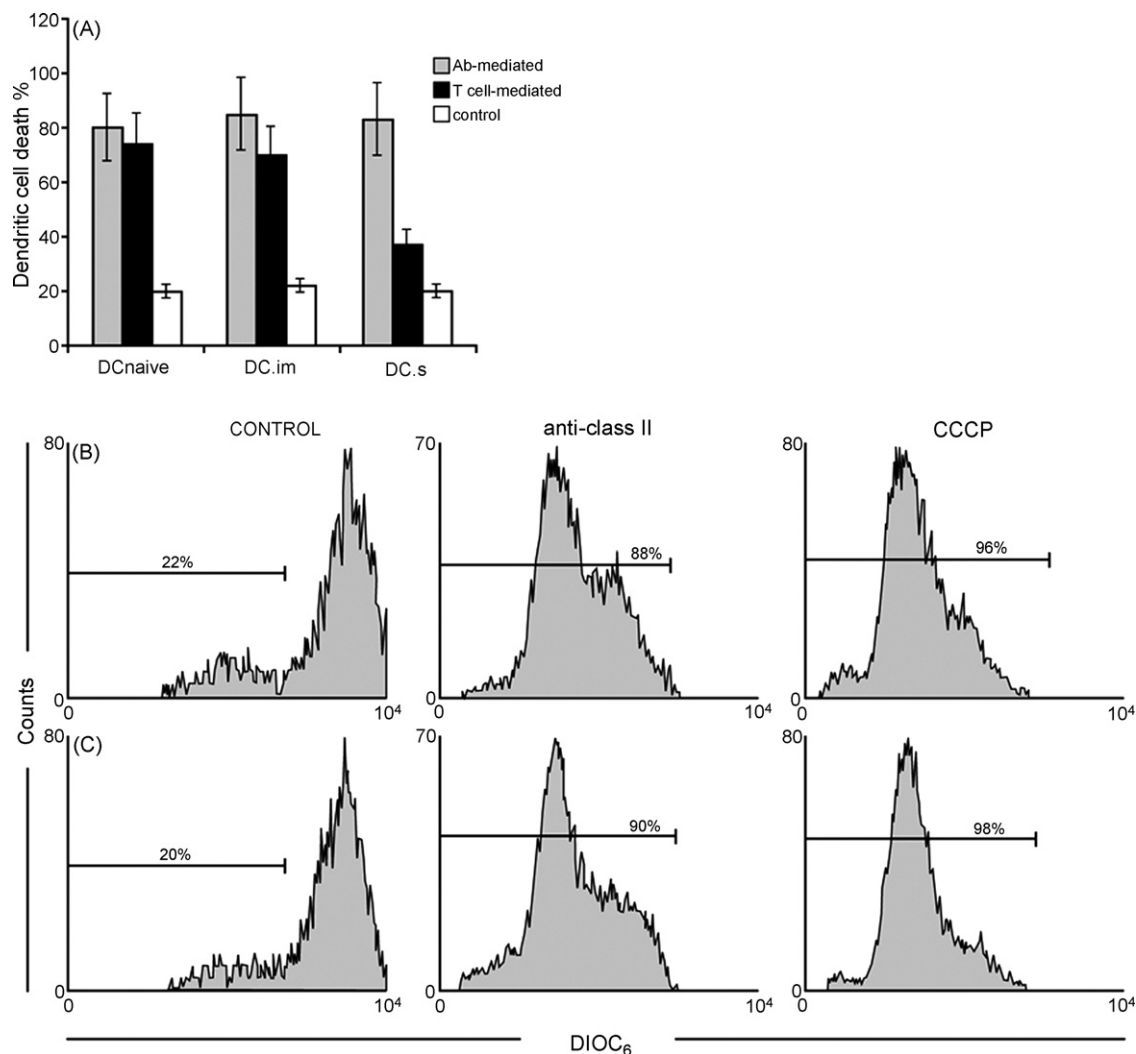


Fig. 6. DCs from naïve (DCnaive), immunized (DC.im) and sensitized (DC.s) did not differ in their susceptibility to MHC class II-mediated apoptosis (Fig. 6A) induced via direct binding of anti-class II antibody for 10–12 h (shaded bars). These values are compared, for each DC population with levels of apoptosis induced by antigen-specific T cells (black bars). Lack of differential susceptibility to MHC II-mediated apoptosis induced in DC from immunized (Fig. 6B) or sensitized (Fig. 6C) by anti-class-II antibody was confirmed by determining the mitochondrial membrane potential ($\Delta\psi_m$) by DiOC₆ staining in DC left untreated (control), cultured with either anti-class II antibody (anti-class II) or with carbamoyl cyanide *m*-chlorophenyl-hydrazone (CCCP).

inate from distinct precursor; instead these develop from a common progenitor following cognate interaction with DCs at the time of antigen presentation. We previously found that the finely balanced interplay between DCs and T cells was altered in sensitized mice (Man et al., 2004). DCs from sensitized mice had acquired an increased resistance to antigen-specific T cell-mediated apoptosis, a mechanism that is thought to be important in down-regulating DC-mediated activation of T cells (Rissoan et al., 1999; Matsue et al., 1999; Yokota et al., 2003; Nat et al., 2002). The notion that in allergic mice DCs evaded T cell-mediated apoptosis prompted us to test the hypothesis that the survival of DCs following cognate T cell interaction could lead to a sustained and prolonged T cell activation that favoured T_H2 responses and IgE production. This hypothesis stemmed from the notion that kinetics of DCs activation is important for the polarization of T helper responses (Langenkamp et al., 2000). Although experimental evidence support the existence of

two separate DC lineages, DC type 1 (DC1) T_H1-inducer and DC type 2 (DC2) T_H2-inducer, at an early stage of activation, DCs challenged with appropriate stimuli displayed a remarkable capacity to induce a T_H1 response; however, after 48 h, when they became exhausted these DCs had acquired the capacity to polarize T_H2 response. Thus, it was plausible to hypothesize that apoptosis-resistant DCs had acquired T_H2 inducing properties of exhausted cells, thus promoting T_H2 allergic responses. We found that apoptosis-resistant DCs promoted T_H2 polarization of antigen-specific naïve T cells. The lack of production of IL-4, low expression of OX40L and reduced expression of CD86 (Kajino et al., 2006) strongly suggested that these cells are exhausted DCs rather than DCs that have acquired an active T_H2-inducing phenotype. The observation that splenic DCs have acquired DC2 features is even more remarkable in consideration of their intrinsic ability to stimulate T_H1 responses as opposed to gut-derived T_H2-inducing DCs (Iwasaki and Kelsall, 1999). T

cell-mediated DC apoptosis is strictly MHC class II-dependent. Although this form of apoptosis required cytoskeleton motion, divalent cations and phosphatase activity neither caspase activation, cellular respiration, RNA or protein synthesis, nitric oxide production nor CD95:CD95L interactions were required (McLellan et al., 2000). However, the same study showed that CD40 counter-acted the pro-apoptotic effect of MHC class-II, thus exerting a protective effect and it would be tempting to speculate that the higher levels of CD40 seen in apoptosis-resistant DCs may contribute to the acquisition of superior resistance to MHC II-mediated apoptosis. Within this scenario the CD40-CD154 (CD40L) interaction may play an important role. The expression of CD154 on T cells is transient, reaching its peak within 2 h and then being markedly reduced after 24 h (Spriggs et al., 1992); this led to hypothesize that the withdrawal of the CD154 signal could lead to an increase susceptibility to MHC II-dependent apoptosis. Thus, the increased expression of CD40 on apoptosis-resistant DCs may compensate the reduced levels of CD154 and in so doing it might contribute to maintaining high resistance to MHC II-mediated apoptosis.

Most, if not all data on the mechanisms underlying MHC II-mediated apoptosis have come from study that involved the use of anti-class II antibodies (Leverkus et al., 2003; Bertho et al., 2002; McLellan et al., 2000). However, in contrast to what observed during DC–T cell co-culture experiments the levels of MHC II-mediated apoptosis induced via antibody did not differ in DCs from either immunized or sensitized donors. This notion suggested that the engagement of MHC II is not the sole event leading to DC apoptosis and that other molecules (cytokine/co-stimulatory) are involved in these events. Apoptosis-resistant DCs showed a differential profile of cytokine production. Reduced levels of TNF- α and IL-12p70 and slightly increased levels of IL-6 were detected following LPS challenge *in vitro* compared to their freshly isolated counterpart. In particular, variations of the levels of both IL-12 and IL-6, although the latter not being statistically significant, by DCs could play an important role in the development of food allergy and in the increased resistance of DCs to T cell-mediated apoptosis, respectively. First, lack of DC-derived IL-12 may contribute to IgE production either directly, by not counter-balancing T_H2 response, or indirectly by not providing the required support to other cell type, such as cytotoxic type 1 and 2 CD8⁺T cell necessary to suppress antigen-specific IgE (Thomas et al., 2002). Second, it is possible that even minor increase of levels of IL-6 may have a direct impact on these events given its powerful anti-apoptotic activity (Leu et al., 2003; Cavarretta et al., 2007).

The potential role of long-term stimulation of T_H2 cells by allergen-loaded DCs has been previously addressed in allergic reactions to airborne antigens (Julia et al., 2002). A specific subset of CD11c⁺CD11b⁺DCs that captured *Leishmania* LACK antigens and remained able to stimulate the expansion of antigen-specific T_H2 long after antigen exposure was described in the bronchoalveolar lavage in mice. In this case it was not established whether these cells had survived T cell-mediated apoptosis but it was suggested that the persistence of T_H-2 activating DCs within the airways could play a major role in

atopic asthma. The finding that apoptosis-resistant DCs have the ability to promote T_H2 responses and robust allergen-specific IgE responses *in vitro* and *in vivo* would suggest that the sensitization-associated persistence of greater numbers of apoptosis-resistant T_H2-inducing DCs contribute to generate IgE-mediated allergic reactions.

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