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TLR4/MYD88-dependent, LPS-induced synthesis of PGE_2 by macrophages or dendritic cells prevents anti-CD3-mediated CD95L upregulation in T cells

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Antigen-presenting cells (APCs) control T-cell responses by multiple mechanisms, including the expression of co-stimulatory molecules and the production of cytokines and other mediators that control T-cell proliferation, survival and differentiation. Here, we demonstrate that soluble factor(s) produced by Toll-like receptor (TLR)-activated APCs suppress activation-induced cell death (AICD). This effect was observed in non-stimulated APCs, but it was significantly increased after lipopolysaccharide (LPS) treatment. Using different KO mice, we found that the LPS-induced protective factor is dependent on TLR4/MyD88. We identified the protective factor as prostaglandin E_2 (PGE_2) and showed that both APC-derived supernatants and PGE_2 prevented CD95L upregulation in T cells in response to TCR/CD3 stimulation, thereby avoiding both AICD and activated T cell killing of target macrophages. The PGE_2 receptors, EP2 and EP4, appear to be involved since pharmacological stimulation of these receptors mimics the protective effect on T cells and their respective antagonists interfere with the protection induced by either APCs derived or synthetic PGE_2. Finally, the engagement of EP2 and EP4 synergistically activates protein kinase A (PKA) and exchange protein directly activated by cAMP pathways to prevent AICD. Taken together, these results indicate that APCs can regulate T-cell levels of CD95L by releasing PGE_2 in response to LPS through a TLR4/MyD88-dependent pathway, with consequences for both T cell and their own survival.
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Proper activation of T cells is imperative to achieve an efficient immunity against pathogens and tumors and, at the same time, to avoid undesirable collateral effects, such as the appearance of autoimmune diseases. Secondary lymphoid organs provide a particular microenvironment specialized in assembling all the cohorts necessary to perform such a remarkable task.[1] In the presence of antigens, antigen-presenting cells (APCs) and their soluble products, including a variety of cytokines, lipid mediators and chemokines, T cells are activated through the engagement of their T-cell receptors (TCR) and co-stimulatory molecules. As a result, T cells can proliferate, differentiate to effector or memory cells, or eventually die.

Deletion of autoreactive T cells is one of the key mechanisms involved in the process of peripheral tolerance that helps to prevent the appearance of autoimmune diseases.[2] Similarly, elimination of chronically stimulated T cells that can potentially be harmful to the host by the means of the production of abnormal levels of inflammatory cytokines is another feature of an efficient immune system. Finally, death of effector T cells at the end of an immune response is a very important homeostatic mechanism designed to shrink an enlarged, no longer necessary cell population that can compete for growth and survival factors with newly activated antigen-specific T-cell clones. In all three cases, death occurs through a genetically controlled program know as apoptosis.[3] At least in the cases of elimination of autoreactive or chronically stimulated T cells, apoptosis is initiated by the interaction of CD95 (Fas) and CD95L (FasL) in a process known as activation-induced cell death (AICD). In fact, genetic defects in one of these two proteins lead to lymphoproliferative disorders associated with autoimmune syndromes in mice and humans.[4]

Macrophages and dendritic cells (DCs) act as a bridge between innate and adaptive immune systems, given that they function as sensors of microbial products as well as APCs. Stimulation of pattern-recognition receptors by certain pathogen-associated molecular patterns induces macrophage activation, as well as DC maturation and migration from peripheral tissues to draining lymph nodes, where they activate and induce proliferation of antigen-specific T lymphocytes.[5] Among the pattern-recognition receptors, the Toll-like receptor (TLR) family has been described to have an important function in the initiation and development of the

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immune response,[6] as apparently only after TLR activation, DCs become fully mature and capable of conducting T-cell differentiation and shaping the immune responses.[7]

Here, we show that supernatants (sups) from lipopolysaccharide (LPS)-stimulated macrophages or DCs are able to prevent AICD of the DO11.10 T cells. Heat-inactivation or proteinase K treatment does not interfere with the protective ability of the sups, suggesting a function for lipid mediators. Indeed, LPS stimulation of the TLR4/MyD88 pathway leads to the synthesis of prostaglandin E_2 (PGE_2) by macrophages and DCs, which, in turn, inhibits CD3-mediated upregulation of CD95L, thereby avoiding both AICD and activated T-cell killing of target macrophages. Sups or synthetic PGE_2 act through the PGE_2 receptors EP2 and/or EP4 to initiate the activation of PKA (cAMP-dependent protein kinase) and exchange protein directly activated by cAMP (EPAC) signaling pathways. Activation of PKA can prevent AICD by itself, whereas the EPAC pathway can only amplify the protective signal relayed by the PKA pathway. Taken together, our results have shown for the first time that T cells activated in the context of TLR-stimulated APCs are defective in upregulated CD95L and, therefore, unable to undergo AICD (CD95L-dependent suicide) and to promote death of CD95-sensitive targets, such as macrophages.

Results

TLR4/MyD88 stimulation of DC and macrophages cause the release of an inhibitor of AICD. To evaluate the influence of soluble molecules produced by APC on T-cell survival, DO11.10 cells were cultured with immobilized anti-CD3 antibodies for 18 h to mimic AICD. Different concentrations of cell-free sups from macrophage cell lines, adherent peritoneal exudate cells (PEC) or bone marrow-derived DCs (BMDC), activated or not with LPS, were added to anti-CD3-activated DO11.10 cells and apoptosis was evaluated by different criteria. As illustrated in Figure 1a using J774 sups, AICD was inhibited by a dose-dependent concentration of sups from either LPS-stimulated or non-stimulated cells. It can be noted that sups from LPS-stimulated cell cultures significantly increased the protection. It is important to point out that LPS had no direct effect on DO11.10 cells, as they equally died when incubated with anti-CD3 antibodies in the presence or absence of LPS (data not shown). The potency of the sups was also related to the time of culture and LPS exposure. Sups collected at 24 h were protected better than 4h sups (Figure 1b). Again, treatment with LPS increased the protective effect of the sups. The same outcome was observed with sups from PEC or BMDC obtained from C57BL/6 mice (Figure 1c). Together, these results show that soluble factors produced by APCs can protect T cells from AICD, and that LPS treatment potentially upregulates the release of the protective factor(s).

The major LPS receptor is composed of TLR4 and MD-2 heterodimers,[8] and the recognition of LPS by host cells also includes its interaction with LPS-binding protein and CD14.[9] Therefore, as expected, unlike cells carrying the wild-type form of TLR4 (obtained from C3H/HePas mice), TLR4-deficient PEC and BMDC obtained from C3H/HeJ mice did not display any increase in the production of the protective factor after LPS activation (Figure 2a and data not shown). In

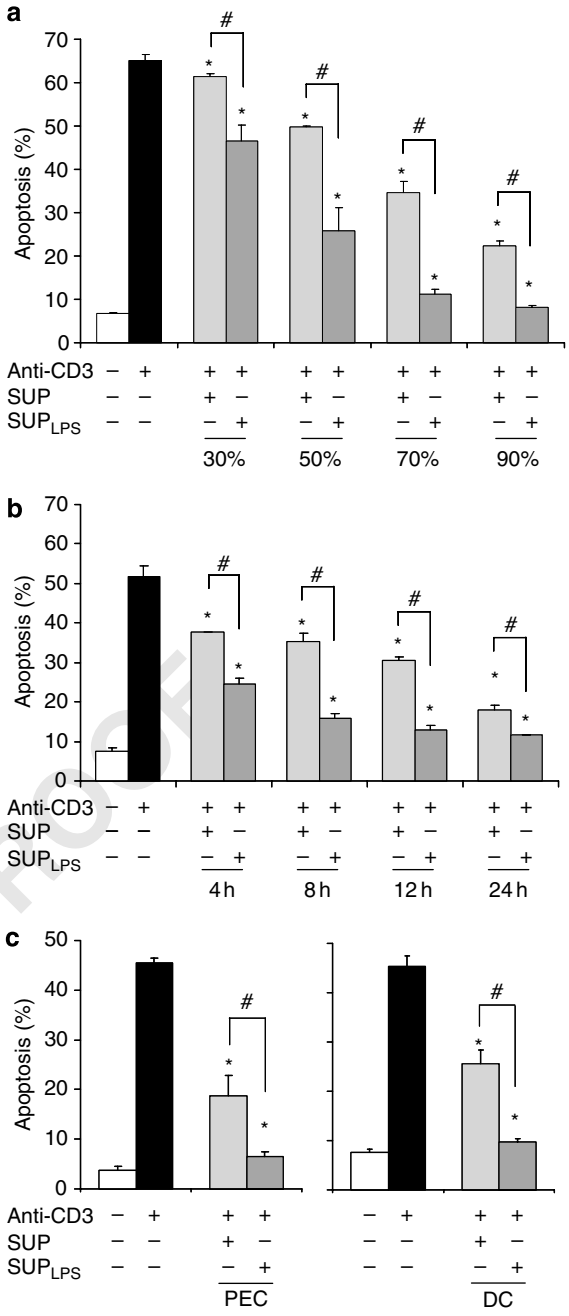

Figure 1 Effect of macrophage/dendritic cell-derived supernatants on AICD of DO11.10 T cell hybridomas. (**a**–**c**) DO11.10 cells were stimulated with or without plate-bound anti-CD3 antibodies for 18 h in the presence of different supernatants. (**a**) The supernatants were generated during 24-h culture of J774 macrophage cells stimulated (SUP_{LPS}) or not (SUP) with 1 μg/ml of LPS and then added to DO11.10 cells in different concentrations. (**b**) Supernatants were obtained after different time points from J774 cells stimulated (SUP_{LPS}) or not (SUP) with 1 μg/ml of LPS. (**c**) Supernatants were obtained from 24-h culture of peritoneal exudate cells (PEC) or dendritic cells (DC) incubated with (SUP_{LPS}) or without (SUP) 1 μg/ml of LPS. Apoptosis was estimated by cell cycle analysis. Numbers represent the average percentage ± S.D. of cells with sub-diploid DNA content. Figure shows representative data of three independent experiments

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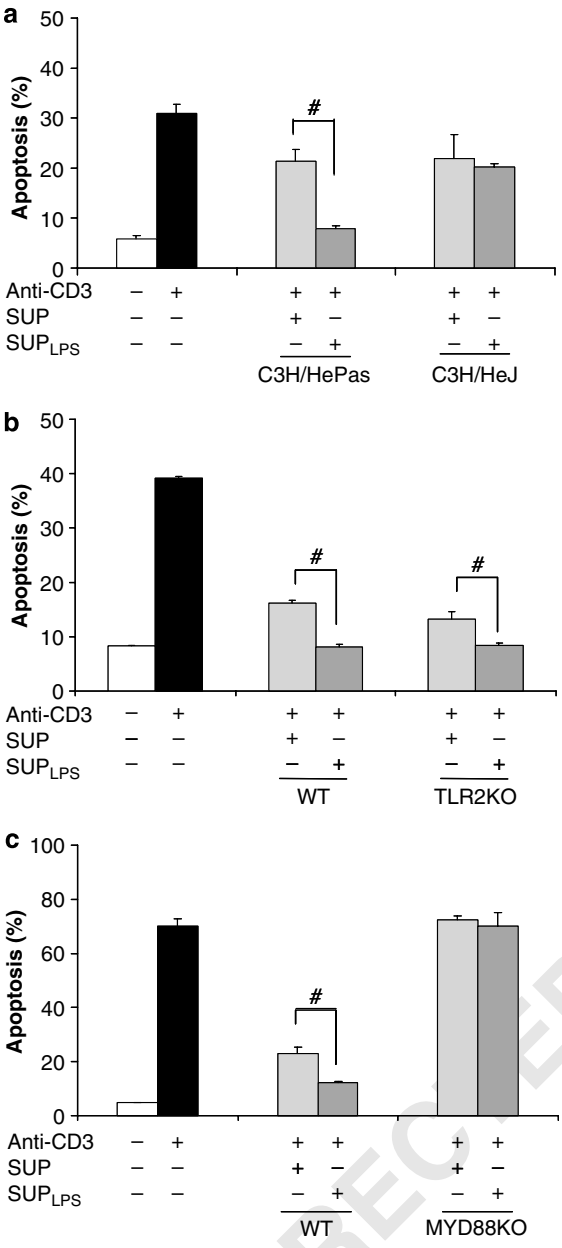

Figure 2 Effect of LPS is TLR4- and MyD88-dependent and TLR2-independent. (**a–c**) DO11.10 cells were stimulated or not with immobilized anti-CD3 antibodies for 18 h in the presence of different PEC supernatants. Supernatants were obtained from 24-h culture of (**a**) C3H/HePas or C3H/HeJ PECs; (**b**) TLR2KO or wild-type PECs; and (**c**) MyD88KO or wild-type PECs. In every case, PECs were stimulated (SUP_{LPS}) or not (SUP) with 1 μg/ml of LPS. Apoptosis was estimated by cell cycle analysis. Numbers represent the average percentage ± S.D. of cells with sub-diploid DNA content. Figure shows representative data of three independent experiments

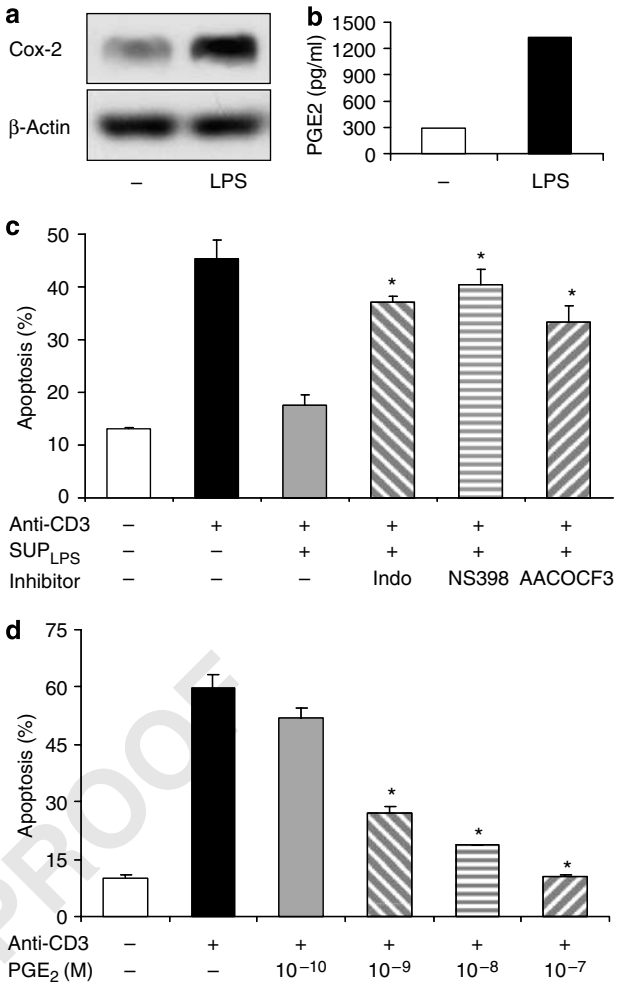

Figure 3 Prostaglandin E_2 is the protective factor found in LPS-stimulated supernatants. (**a**) Detection of Cox-2 expression by western blot analysis after 24-h culture of J774 stimulated or not with 1 μg/ml of LPS. β-Actin expression was used as loading control. (**b**) Measurement of PGE_2 secretion by ELISA after 24-h incubation of J774 cells with or without 1 μg/ml of LPS. (**c**) DO11.10 cells were stimulated or not with plate-bound anti-CD3 antibodies for 18 h in the presence of different J774 supernatants. J774 cells were pretreated with 10 μg/ml of indomethacin, 10 μM NS398 or 10 μM AACOCF3 for 30 min, stimulated with 1 μg/ml of LPS and then incubated for 24 h to generate the supernatants. (**d**) DO11.10 cells were stimulated or not with plate-bound anti-CD3 antibodies for 18 h in the presence of different concentrations (10^{-10} to 10^{-7} M) of exogenous PGE_2. Apoptosis was estimated by cell cycle analysis. Numbers represent the average percentage ± S.D. of cells with sub-diploid DNA content. Figure shows representative data of three independent experiments

comparison, PEC obtained from $TLR2^{-/-}$ mice respond to LPS at the same extent as PEC obtained from their wild-type counterparts (Figure 2b).

TLR4 signals through two distinct pathways, a MyD88-dependent response and a TRIF-dependent, MyD88-independent pathway.[8] As illustrated in Figure 2c, sups from LPS-stimulated MyD88KO PEC did not protect DO11.10 cells from AICD. Moreover, even the basal production of the protective factor was abrogated in the MyD88KO PEC, suggesting that wild-type cells produced the protective factor in response to trace amounts of TLR ligands present in the culture medium. Similar results were obtained with MyD88KO BMDC (data not shown).

PGE_2 released by APCs upon LPS stimulation protects DO11.10 from AICD. To gain insight into the molecular nature of the protective factor, we heat-inactivated LPS-stimulated, APC-derived sups for 15 min at 70°C and observed that they retain their ability to rescue DO11.10 cells

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from AICD (data not shown). The same results were obtained using proteinase K-treated sups (data not shown). Therefore, we deduced that it was unlikely that a protein was responsible for the protection and assumed that the best candidates were lipid mediators. Cyclooxigenase-2 (Cox-2) is one of the main target genes that are activated by TLR4/MyD88-dependent pathway.[10] This enzyme participates in the arachidonic acid conversion into PGE_2 that, in turn, has a well-known immunomodulatory activity.[11] Unstimulated J774 cells expressed detectable levels of Cox-2 protein (Figure 3a) and released a reasonable amount of PGE_2 to sup (Figure 3b), both of them were considerably increased upon LPS stimulation (Figure 3a and b). Treatment of these cells with indomethacin (indo), an inhibitor of Cox-1 and Cox-2, as well as with NS398, a Cox-2-specific inhibitor, significantly reduced the ability of the LPS-stimulated sups to protect DO11.10 cells from AICD (Figure 3c). Similar results were obtained using AACOCF3, an inhibitor of phospholipase A2, which acts on the arachidonic acid pathway upstream of Cox-2 activity (Figure 3c). Moreover, the addition of exogenous PGE_2 to anti-CD3-stimulated DO11.10 cells induced a dose-dependent protection from AICD (Figure 3d), reminiscent of the protection observed with sups from PEC/BMDC cultures. These data indicate that PGE_2 is most likely the relevant factor secreted by APCs upon LPS stimulation that protects T cells from AICD.

APC-derived sups inhibit CD95L upregulation induced by anti-CD3 stimulation. CD95L is rapidly upregulated in T cells upon TCR re-stimulation and the interaction between CD95L and its receptor CD95 is required for AICD.[12,13] As CD95L is mainly regulated at the transcriptional level, we examined whether LPS-stimulated, APC-derived sups could modulate CD95L expression. DO11.10 cells were stimulated with plate-bound anti-CD3 antibodies in the presence or absence of BMDC-, PEC- and J774-derived sups or exogenous PGE_2. After 4 h, total mRNA was extracted, converted to cDNA and RT-PCR for CD95L mRNA was performed. Although sups from non-stimulated APCs were capable of interfering with anti-CD3-mediated CD95L mRNA upregulation, sups from LPS-stimulated cells, which were shown to contain higher amounts of PGE_2, displayed a superior inhibitory effect (Figure 4a and data not shown). A dose-dependent inhibition of anti-CD3-mediated CD95L mRNA upregulation was also observed using exogenous PGE_2 (Figure 4b). In addition, flow cytometry analysis revealed that both sups and PGE_2 were capable of preventing the expression of CD95L at the cell surface (Figure 4c).

PGE_2 signals through EP2 and/or EP4 receptors to protect DO11.10 cells from AICD. The family of PGE_2 receptors comprises four G-protein-coupled receptors, namely EP1, EP2, EP3 and EP4, each one displaying distinct biochemical properties depending on the G-protein associated. EP1 usually couples to G_q and induces the elevation of free Ca^{2+} levels. EP2 and EP4 are coupled to G_s proteins and when activated raises the cAMP levels through adenylate cyclase activity. On the other hand, EP3 is often coupled to G_i proteins and present the opposite effect,

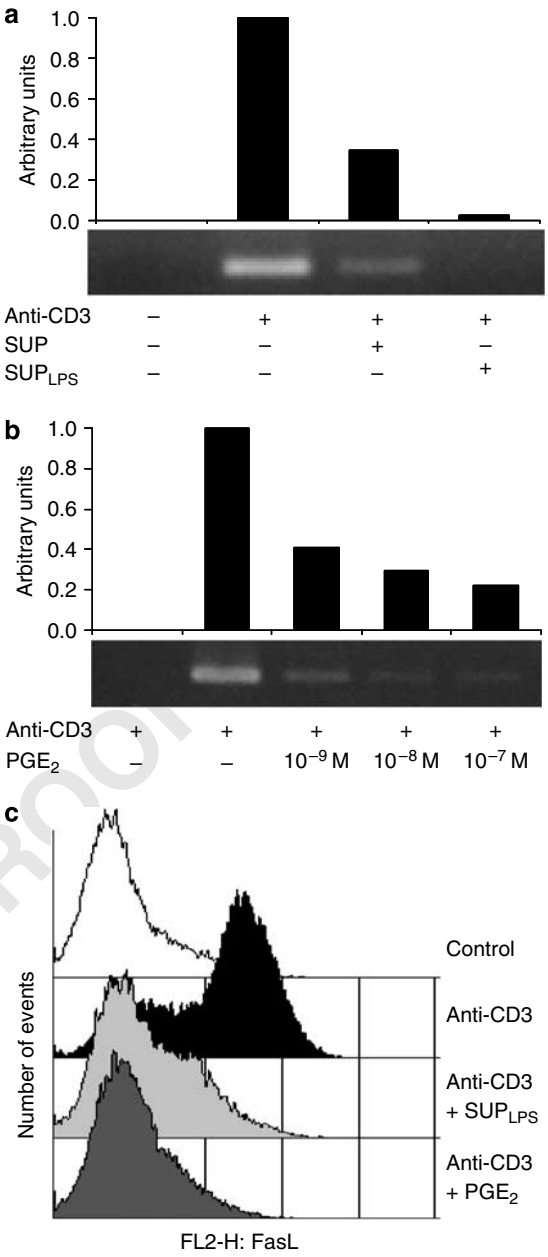

Figure 4 Effect of PGE_2 and macrophage-derived supernatants on the CD95L expression. (**a** and **b**) RT-PCR for CD95L in DO11.10 cells. Total mRNA was extracted from cells stimulated or not with anti-CD3 antibodies for 4 h in the presence or absence of (**a**) supernatants derived from 24-h culture of J774 cells stimulated (SUP_{LPS}) or not (SUP) with 1 μg/ml of LPS or (**b**) different concentrations (10^{-9} to 10^{-7} M) of exogenous PGE_2. (**c**) CD95L protein expression in DO11.10 cells stimulated or not with anti-CD3 in the presence of 90% supernatant of LPS-stimulated J774 cells or 10^{-7} M PGE_2 for 8 h. The expression was measured by flow cytometry using anti-Cd95L-PE antibodies

that is the inhibition of cAMP production.[14] To identify the receptor(s) associated with PGE_2-induced protection from AICD, we first analyzed the expression of the EP proteins by western blot. With the exception of EP1, all PGE_2 receptors were expressed in DO11.10 cells, and none of them were modulated by CD3 stimulation (Figure 5a). Forskolin, a direct activator of adenylate cyclase, inhibited AICD in a dose-

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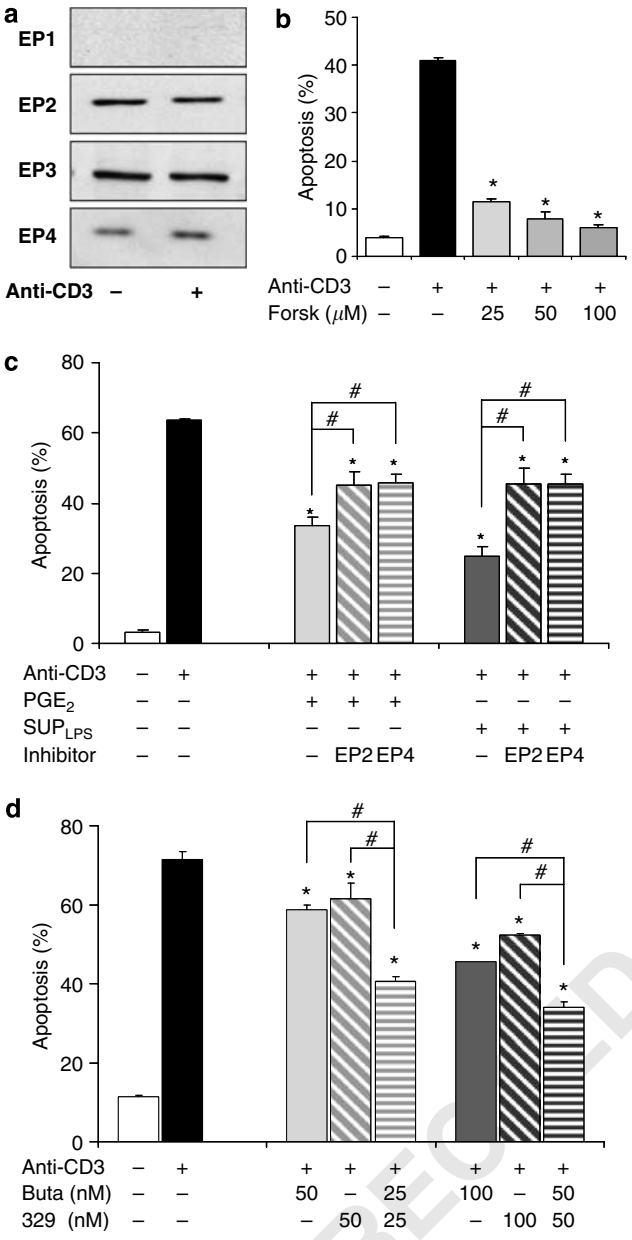

Figure 5 PGE_2-mediated protection of DO11.10 cells from AICD occurs through EP2/EP4 receptors. (**a**) Detection of EP1–EP4 receptors expression by western blot after 8-h incubation of DO11.10 cells stimulated or not with plate-bound anti-CD3 antibodies. (**b**) DO11.10 cells were stimulated with plate-bound anti-CD3 antibodies for 18 h in the presence of different concentrations (25, 50 and 100 μM) of forskolin, an activator of cAMP production. Apoptosis was estimated by cell cycle analysis. (**c**) Pretreatment of DO11.10 for 30 min with inhibitors of EP2 (10 μM AH6809) or EP4 (10 μM ONO-AE3-208) were capable of reverting the protection against anti-CD3-induced death conferred by 10^{-7} M PGE_2 or LPS-stimulated J774-derived supernatant (SUP_{LPS}). Apoptosis was estimated after 18 h of incubation by cell cycle analysis. (**d**) DO11.10 cells were stimulated with plate-bound anti-CD3 antibodies for 18 h in the presence of butaprost (EP2- specific agonist), ONO-AE1-329 (EP4-specific agonist) or a combination of both agonists. Apoptosis was estimated by cell cycle analysis. Numbers represent the average percentage ± S.D. of cells with sub-diploid DNA content. Figure shows representative data of three independent experiments

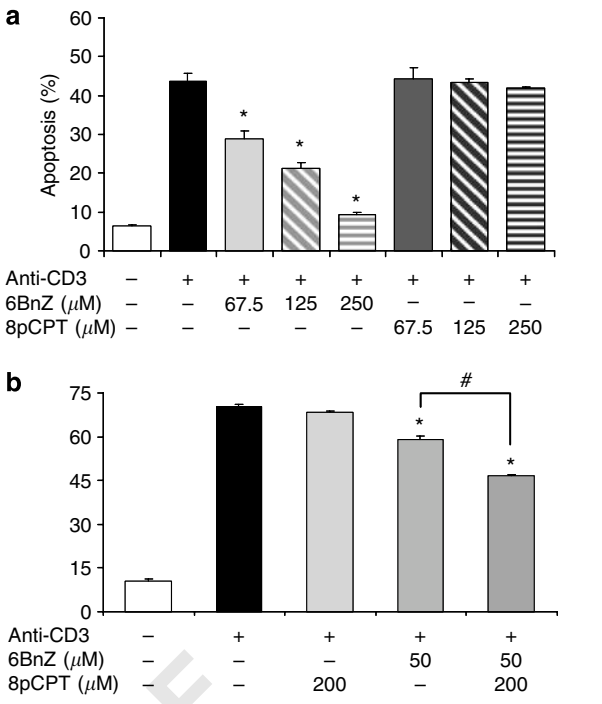

Figure 6 Effect of PKA- and EPAC-specific agonists on AICD of DO11.10 hybridomas. (**a**) DO11.10 cells were stimulated with plate-bound anti-CD3 antibodies for 18 h in the presence of different concentrations (67.5, 125 and 250 μM) of 6-Bnz-cAMP, a specific activator of PKA pathway, or 8-pCPT-2′-*O*-Me-cAMP, a specific activator of EPAC pathway. (**b**) DO11.10 cells were stimulated with plate-bound anti-CD3 antibodies for 18 h in the presence of 50 μM 6-Bnz-cAMP, 200 μM 8-pCPT-2′-*O*-Me-cAMP or a combination of both compounds. Apoptosis was estimated by cell cycle analysis. Numbers represent the average percentage ± S.D. of cells with sub-diploid DNA content. Figure shows representative data of three independent experiments

dependent manner (Figure 5b), suggesting that G_s-protein-coupled EP receptor(s) could be responsible for the protection of AICD by PGE_2. The employment of EP2 and EP4 antagonists (AH6809 and ONO-AE3-208, respectively) revealed that both receptors appear to participate in the protective effect of PGE_2-and APC-derived sups (Figure 5c). In fact, direct stimulation of either EP2 or EP4 receptors by their specific agonists, butaprost free acid (EP2) and ONO-AE1-329 (EP4), resulted in protection of DO11.10 cells from AICD (Figure 5d). Moreover, when combined in half-doses, these agonists induced a significantly higher protection, indicating synergism between these two receptors.

EPAC pathway enhances the PKA-mediated AICD suppression induced by PGE_2. After the EP2/EP4 engagement, endogenous cAMP levels are raised, activating two major signaling pathways, namely the classical PKA pathway and, an alternative, recently described EPAC pathway.[15] To determine the relevance of these two pathways in the inhibition of AICD, DO11.10 cells were stimulated with plate-bound anti-CD3 antibodies in the presence of specific and highly selective agonists for either PKA (6-Bnz-cAMP) or EPAC (8-pCPT-2′-*O*-Me-cAMP). The sole activation of PKA strongly prevented AICD in a dose-

dependent manner, whereas the exclusive activation of EPAC had no significant effect (Figure 6a). However, it is important to emphasize that the activation of EPAC amplified the effect of suboptimal doses of the PKA agonist (Figure 6b).

APC-derived sups and PGE_2 inhibit CD95L upregulation and cell death in primary T cells. Because all our experiments were performed in DO11.10 hybridoma cells, we wanted to check whether the effect of APC-derived sups and PGE_2 also occurs with primary T cells. Incubation of

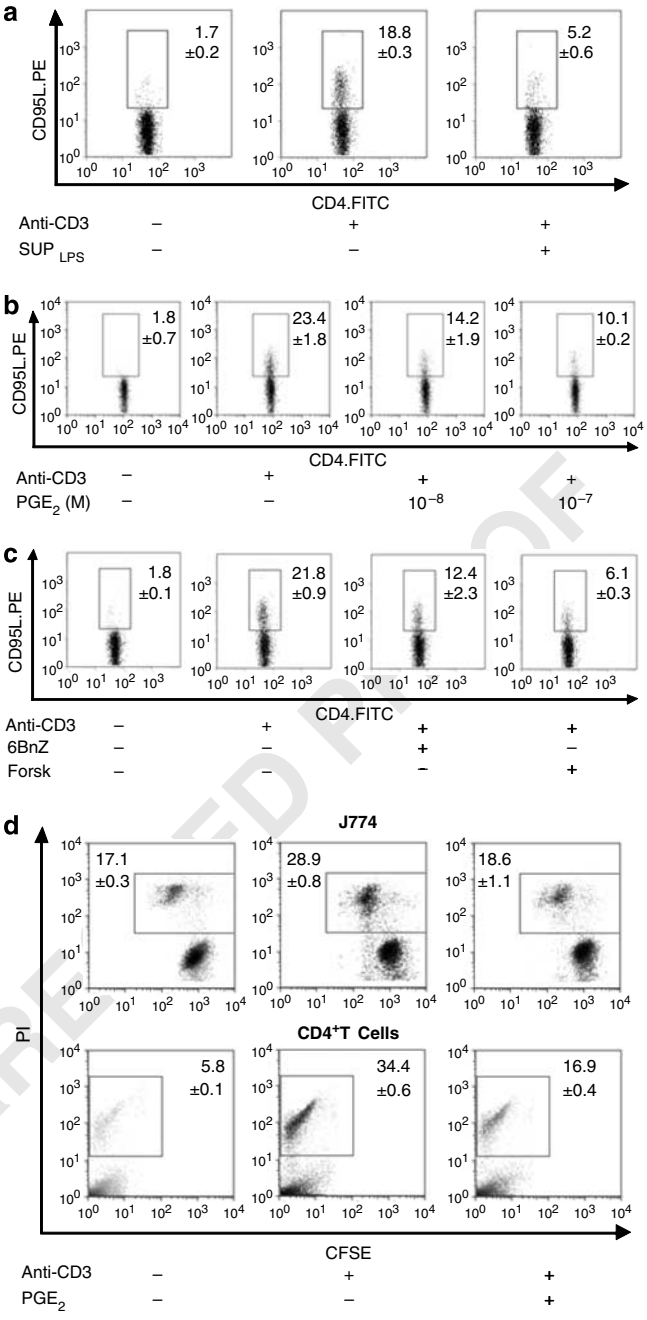

Figure 7 APC-derived sups and PGE_2 inhibit CD95L upregulation and cell death in primary T cells and in CFSE-labeled target macrophages. After eliminating red cells by ammonium chloride lyses and depleting adherent cells by 2-h incubation, total BALB/c spleen cells were incubated with or without plate-bound anti-CD3 for 12 h alone or in the presence of (**a**) supernatants derived from 24-h culture of J774 cells stimulated with 1 μg/ml of LPS (SUP_{LPS}) or (**b**) different concentrations (10^{-8} to 10^{-7} M) of exogenous PGE_2 or (**c**) 10 μM of forskolin or 125 μM of 6-Bnz-cAMP. After staining with anti-CD4.FITC and anti-CD95L.PE antibodies, $CD4^+$ T-cell blasts were analyzed for CD95L expression by flow cytometry. Numbers represent the average percentage ± S.D. of cells within the gated region. Figure shows representative data of five independent experiments. To assess CD95L-mediated cell death, J774 macrophages were stained with CFSE and co-cultured with T-cell blasts in a proportion of three blasts for each macrophage. Cells were incubated with or without plate-bound anti-CD3 for 24 h in the presence or absence of 10^{-7} M PGE_2 (**d**). Separate analysis of death rates was performed for each CFSE-positive (J774 macrophages; upper panel) and -negative (T-cell blasts; lower panel) populations by PI incorporation. Numbers represent the average percentage ± S.D. of cells within the gated region. Figure shows representative data of three independent experiments

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splenocytes (Figures 7a and b, and Supplementary Figure S4) or 5- to 7-day T-cell blasts (Supplementary Figure S6 and data not shown) with APC-derived sups or PGE_2 prevented anti-CD3-mediated CD95L upregulation in gated $CD4^+$ T lymphocytes. Moreover, in accordance with the results obtained in DO11.10 hybridoma cells, both the direct activator of adenylate cyclase, forskolin and, the specific activator of PKA, 6-Bnz-cAMP blocked anti-CD3-mediated induction of CD95L in freshly isolated $CD4^+$ splenocytes (Figure 7c and Supplementary Figure S4) or 5- to-7-day T-cell blasts (Supplementary Figure S6 and data not shown).

Finally, APC-derived sup, PGE_2 or forskolin inhibited anti-CD3-mediated cell death, as observed by changes in scattering properties or PI incorporation (Figure 7d; Supplementary Figures S5 and S7 and data not shown). In addition to the death of $CD4^+$ T cells, we also observed death of CFSE-labelled target J774 macrophages when co-incubated with anti-CD3-treated freshly isolated splenocytes or T-cell blasts (Figure 7d; Figure S7 and data not shown). Most importantly, J774 cell death was inhibited by the treatment of primary T cells with PGE_2, a condition that prevented the upregulation of CD95L in these cells.

Together, our data provide novel evidence that APCs are able to modulate T cell as well as its own survival by releasing PGE_2 in response to LPS through a TLR4/MyD88-dependent mechanism. PGE_2, in turn, initiates EP2 and EP4 signaling pathways in T cells, culminating in the activation of PKA, and perhaps EPAC, thereby preventing anti-CD3-mediated CD95L upregulation and their subsequent suicide (AICD) and killing of target cells.

Discussion

Signals such as 'danger molecules,' pathogen-associated molecular patterns and inflammatory mediators are necessary to drive the expansion and differentiation of antigen-specific T cells toward an effector phenotype capable of providing immunity.[6] In the absence of such signals, in most cases, the fate of T lymphocyte is anergy or death. Our data here provide evidence that the incubation of APCs with LPS, a TLR4-interacting pathogen-associated molecular pattern, also considered a 'danger signal' and a recognized pro-inflammatory molecule, induces a potent release of a soluble factor that protect T cells from AICD. Heat inactivation and proteinase K treatment of the sups did not interfere with the protection, suggesting that the AICD-inhibitory factor is not a protein. Among all the possibilities, we considered that lipid mediators derived from the arachidonic acid metabolism were good candidates, as multiple immunomodulatory activities have been ascribed to leukotrienes, thromboxanes and prostaglandins, and these molecules are known to be produced during inflammation and infection.[16]

Because our results clearly showed that (a) LPS induced the expression of COX-2 and subsequent synthesis of PGE_2 by macrophages and DCs; (b) COX-2 inhibitors, such as indomethacin and NS398, inhibited the appearance of the protective factor in sups from LPS-treated APCs; and (c) synthetic PGE_2 mimics all the effect of our LPS-stimulated sups, including the inhibition of CD95L upregulation by anti-CD3 antibodies. We conclude that the protection from AICD that we observed with our sups is to a large extent because of PGE_2.

PGE_2 exhibits a vast and diverse range of effects on the immune system, including the inhibition of proliferation[11,17] and the induction of apoptosis.[18–21] Interestingly, one of the mechanisms described to mediate PGE_2-mediated apoptosis is the upregulation of CD95L.[22] However, in agreement with our results, data in the literature also point out to a protective effect of PGE_2. High levels of PGE_2 was associated with prolonged survival of $CD45RO^+$ T cells in the inflamed human pleural space,[23] human $CD4^+CD8^+$ lymphoblasts could be rescued from ConA- or PHA-induced death by PGE_2[24] and AICD was shown to be prevented by PGE_2 through blockage in the expression of CD95L.[25] Curiously, lung fibroblast-derived PGE_2 was shown to block AICD in a CD95L-independent way.[26] It is important to mention that neither sups derived from LPS-treated APCs nor PGE_2 interfered with the proliferation of untreated or anti-CD3-treated DO11.10 cells (Supplementary Figure S1). These results are in agreement with the report showing that in the absence of TLR ligands, activated T cells divide well but accumulate poorly, largely due to a higher death rate.[27]

There are two possible signaling pathways triggered by the LPS receptor TLR4. One is dependent on MyD88 and the other uses TRIF as the adaptor molecule.[8] As Cox-2 expression is controlled by NF-κB activation through the MyD88 pathway,[10] it was not surprising that MyD88-deficient cells failed to produce the protective factor upon LPS stimulation. Importantly, even the basal level of protection seen in sups obtained from different APCs was completely dependent on the expression of MyD88, suggesting that other TLR ligands that initiated an MyD88 pathway can also provide survival ability to APC-interacting T lymphocytes.

LPS-stimulated APC-derived sups as well as PGE_2 prevented the CD3-mediated upregulation of CD95L by the PGE_2 receptors EP2 and/or EP4. These receptors are coupled to G_s proteins responsible for the augmentation of cAMP levels through adenylate cyclase activity.[14] Downstream of these events are the activation of two major biochemical pathways controlled by PKA and EPAC, respectively.[15] Although our data support the notion that the activation of PKA has a major function in holding back the expression of CD95L after PGE_2 stimulation, it became obvious to us that the EPAC pathway can cooperate with PKA signaling. Although PKA-mediated downregulation of CD95L has already been shown,[28] the involvement of EPAC in apoptosis inhibition is rather new.

Importantly, we also observed that $CD4^+$ T cells that upregulated their CD95L are capable of killing target macrophages. If similar phenomenon occurs *in vivo* during the initiation of the immune response, then CD95L-mediated killing of APCs by activated T cells would abrogate the presentation of antigen and the proper induction of a protective immune. PGE_2 would then have an important function in this scenario by avoiding the CD95L upregulation in antigen-specific T cells, and consequently preventing the premature death of APCs.

We propose a new mechanism that helps the organism to avoid the appearance of autoimmune diseases. In this scenario, deficiency in the production of PGE_2 should hamper

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the appearance of T cells specific for fighting against pathogens. Similarly, overproduction of PGE_2 should be associated with better immunity or with some forms of autoimmune diseases. In this regard, Perkins and collaborators[29] showed a positive correlation between elevated levels of PGE_2 and better protection against infant severe malaria. Also, a non-obese-diabetes mouse, a well-known model of autoimmune disease, displays an augmented level of circulating PGE_2,[30] reduced levels of CD95L expression[31] and an AICD-resistant phenotype.[32] In addition, elevated expression of Cox-2, higher levels of circulating PGE_2 and defective AICD have also been associated with autoimmune diabetes in humans.[33,34] Another form of autoimmune disorder associated with higher levels of PGE_2 is rheumatoid arthritis, in both animal models and human patients.[35] Altogether, these results, along with ours, suggest a potential for genetic and/or pharmacological manipulation of PGE_2 synthesis to improve immunity and control autoimmune responses.

Materials and Methods

Mice. Six-to 8-week-old C57BL/6, BALB/c, BALB/c Tg DO11.10, 129/Sv, C3H/HeJ, C3H/HePas, TLR2$^{-/-}$ and MyD88$^{-/-}$ female mice were bred in our animal facilities at the University of São Paulo. TLR2$^{-/-}$ and MyD88$^{-/-}$ mice were kindly provided by Dr. Bernard Ryffel (Centre National de la Recherche Scientifique, Orléans, France).

Cell lines and reagents. DO11.10 T-cell hybridoma cell lines were a gift of Dr. Douglas Green. J774 macrophage cell line was obtained from ATCC (Manassas, VA, USA). Cells were regularly maintained at 37°C in 5% CO_2 in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 10 mM HEPES, 2 mM L-glutamine, 100 μg/ml of streptomycin and 100 U/ml of penicillin.

Indomethacin, NS398, synthetic PGE_2, Butaprost free acid and H6809 were purchased from Cayman Chemicals Co. (Ann Arbor, MI, USA). LPS from *Escherichia coli* was purchased from Sigma-Aldrich (St Louis, MO, USA) and AACOCF3 from Biomol International (Plymouth Meeting, PA, USA). 6-Bnz-cAMP and 8-pCPT-2′-*O*-Me-cAMP were from Biolog LSI (Bremen, Germany). Anti-CD3 (clone 2C11), anti-CD4 (clone H129.19), anti-CD28 (clone 37.51) and anti-CD95L (clone MFL3) are from BD Pharmingen (San Diego, CA, USA) and anti-EP1-4 is from Cayman Chemicals. ONO-AE1-329 and ONO-AE3-208 were provided by ONO Pharmaceuticals (Osaka, Japan). Recombinant Annexin V-FITC was produced as described earlier.[36]

Isolation of adherent peritoneal cells. Four to six mice were injected intraperitoneally with 1 ml of thioglycolate (Sigma-Aldrich), and after 5 days, cells were obtained by peritoneal lavage with chilled RPMI-1640 medium.[37] Peritoneal cells (1×10^6) were incubated for 4 h in RPMI-1640 medium supplemented with 3% heat-inactivated FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), sodium pyruvate (1 mM), L-glutamine (2 mM) and 2-mercaptoethanol (2-ME; 50 μM). All supplements were purchased from Life Technologies (Rockville, MD, USA). Non-adherent cells were removed by three vigorous washes with medium, resulting in a population that comprises 96–98% of F4/80$^+$ cells (data not shown).

Generation of DCs. DCs were generated *in vitro* from bone marrow cells as described earlier.[38] Briefly, cells removed from the femurs were cultured with 20 ng/ml of recombinant granulocyte–macrophage colony-stimulating factor (rGM-CSF, a gift from Dr. Brian Kelsall) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 μM 2-ME, 100 U/ml penicillin and 100 μg/ml streptomycin. The medium with rGM-CSF was replaced on the fourth day. Cells were harvested on day 7 with flushes of cold DMEM. DCs were matured with 1 μg/ml of LPS from *E. coli* (Sigma-Aldrich) for 18 h, resulting in a population that comprises around $>$95% of CD11c$^+$ cells (data not shown).

Activation of primary T cells and generation of T-cell blasts. Spleens or lymph nodes were removed aseptically from BALB/c or BALB/c Tg DO11.10 cells and teased into single-cell suspension. Red blood cells were lysed with ammonium chloride solution and adherent cells were removed by 2 h incubation. For activation, 2.5×10^5 cells were stimulated with 1 μg/ml plate-bound anti-CD3 in a flat-bottomed 96-well plates in a final volume of 100 μl for 8–12 h.

For generation of T-cell blasts, 1×10^6 cells were stimulated for 48 h with 1 μg/ml of plate-bound anti-CD3 and 1 μg/ml of soluble anti-CD28 in a flat-bottomed six-well plates in a final volume of 1 ml. Cells were washed and cultured for 4 more days with 100 U/ml of recombinant human IL-2 (Proleukin – Zodiac Prod. Farm. – Brazil). Later, dead cells and cell debris were eliminated by centrifuging cells over Ficoll-Hypaque (GE HealthCare).

During all steps, cells were cultured at 37°C in 5% CO_2 in DMEM supplemented with 10% FCS, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium piruvate, 100 μM non-essential amino acids, 100 μM vitamins, 10 μM 2-ME, 100 μg/ml streptomycin and 100 U/ml penicillin. All supplements were purchased from Life Technologies.

CFSE staining protocol. For CFSE labeling, a final concentration of 1 μM CFSE was added to a cell suspension of 5×10^6 cells per ml in PBS. After 5 min of incubation in room temperature, staining was terminated with the addition of 10 vol PBS/5% FCS and immediate centrifugation. At least three extra washes with PBS/5% FCS were performed before re-suspending cells in culture medium.

Assessment of apoptosis. Apoptosis was quantified by different criteria. Using a FACScalibur flow cytometer (Becton-Dickinson), flow cytometric analysis of DNA content and externalization of phosphatidylserine residues were performed as described earlier.[39] The results represent the average ± S.D. in triplicate samples. Every experiment was repeated at least three times. Some samples were also analyzed morphologically or based on changes of light-scattering properties of the dead cells.[40]

SDS-PAGE and western blot. Cells were harvested, washed once in ice-cold PBS, lysed directly in SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol and 2.5% 2-ME) and boiled for 5 min. Samples were resolved under reducing conditions for 2 h at 80 V in SDS-polyacrylamide gels. Separated proteins were then blotted onto PVDF membranes at 80 mA overnight. Blots were blocked for 2 h in TBST (10 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.05% Tween) containing 0.1% sodium azide and 5% nonfat dried milk, and then probed for 2 h with an appropriate dilution of the primary antibody. Reactions were detected with suitable secondary antibody conjugated to horseradish peroxidase (Jackson Laboratory, Bar Harbor, ME, USA and Amersham, Arlington, IL, USA) using enhanced chemiluminescence solution (Pierce, Rockford, IL, USA).[41]

Detection of PGE_2 in culture sups. The concentration of PGE_2 in the culture sups was determined by ELISA using a commercially available kit (Cayman Chemicals). Briefly, 100 μl of each sample was incubated with the eicosanoid conjugated with acetylcholinesterase and the specific antiserum in 96-well microtitration plates, coated with anti-IgG immunoglobulins. After the addition of the enzymatic substrate, the optical density of the samples was determined at 412 nm in a microplate reader, and the eicosanoid concentration was calculated from standard curves.

Quantification of CD95L expression. CD95L mRNA expression was detected by RT-PCR with specific primers (sense: 3′-CAGCAGTGCCA CTTCATCTTGG-5′; antisense: 3′-TTCACTCCAGAGATCAGAGCGG-5′) for the mouse CD95L transcript (accession no. NM010177). The total amplification product (471 bp) was subjected to a 1% Tris-borate-EDTA agarose gel. β-Actin amplification for each sample (sense: 3′-TGGAATCCTGTGGCATCCATGAAAC-5′; antisense: 3′-TAAAACGCAGCTCAGTAACAGTCCG-5′; amplification product 349bp) was used as a loading control.

CD95L protein expression was measured by flow cytometry. DO11.10 cells were immunostained with anti-CD95L.PE (clone MFL3; BD Pharmingen) after the appropriated treatment periods. Cell Quest software was used to analyze the samples and to generate the histogram graphs.

Statistical analysis. Experiments were performed always in triplicates and at least three times. Data are presented as mean values ± S.D. Statistical analysis of the data was carried out using one-way ANOVA and Tukey as a post-test.

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Differences between experimental groups were considered significant for $P < 0,01$. All statistic tests were performed using Prism v4 software (GraphPad Software Inc.).

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)