Chapitre II – Effets de l'activation de CD40 sur la survie des éosinophiles

L'interaction de CD40/CD40L joue un rôle co-stimulateur essentiel dans la réponse immune. Plusieurs types de cellules immunes expriment CD40 et/ou son ligand. La stimulation de CD40 induit différents signaux en fonction du type cellulaire concerné ; signaux d'activation, de survie ou de prolifération (Grewal et Flavell, 1998). En 1996, Ohkawara et collaborateurs, montraient que CD40 était induit à la surface des éosinophiles et que son engagement prolongeait la durée de vie des éosinophiles.

Dans un premier temps, nous avons confirmé les résultats d'Ohkawara et collaborateurs (1996) en mesurant une augmentation significative de l'expression de l'ARNm codant pour CD40, ainsi qu'une augmentation de l'expression membranaire de CD40, détectées lorsque des éosinophiles isolés à partir de sang d'individus sains ou de patients asthmatiques étaient cultivés pendant 48 heures. Nous n'avons pas détecté d'expression de CD40 dans les neutrophiles. Contrairement aux résultats d'Ohkawara et collaborateurs (1996), nous avons montré que l'expression de CD40 à la surface des éosinophiles était spontanée et n'était pas induite ou modulée par un traitement avec des médiateurs pro-inflammatoires tels que le GM-CSF, l'IFN-γ ou les IgA-Ic. Nous avons montré également que les éosinophiles expriment constitutivement CD40L à leur surface. L'expression au cours du temps des ARN messagers codant pour CD40 et CD40L a été mesurée par RT-PCR (*Reverse transcriptase-polymerase chain reaction*) semi-quantitative. La mesure de l'expression des protéines CD40 et CD40L a été réalisée par western blot. Enfin, la mesure au cours du temps de l'expression de CD40 et de CD40L à la surface des éosinophiles a été réalisée par marquage immunologique et par cytométrie en flux.

Dans un second temps, après avoir montré son expression, nous nous sommes intéressés au rôle de l'activation de CD40. Après 48 h de culture, nous avons stimulé le CD40 exprimé à la surface des éosinophiles par une forme soluble recombinante humaine (rh) de CD40L ou par la fixation d'un anticorps agoniste et avons constaté que cette stimulation réduisait de manière statistiquement significative le taux d'apoptose des éosinophiles. Nous avons également montré, par RT-PCR semi-quantitative et western blot, que l'engagement de CD40 induit l'expression génique et protéique de c-IAP2, membre anti-apoptotique de la famille des protéines IAP. Pour démontrer le rôle de cette protéine dans la transmission du signal de survie induit par l'activation de CD40, nous avons empêché l'expression de la protéine c-IAP2 en transfectant des éosinophiles, isolés de la circulation et stimulés par rhCD40L après 48 h de culture, avec de courtes séquences oligodésoxyribonucléiques de séquence inverse (antisens) à la séquence de l'ARNm codant pour c-IAP2. Ce *knockdown* de c-IAP2 a empêché l'augmentation de survie consécutive à l'engagement de CD40 dans les éosinophiles, démontrant le rôle majeur de c-IAP2 dans la transmission de ce signal anti-apoptotique.

Enfin, nous avons montré par western blot qu'il existe une forte corrélation entre le pourcentage d'éosinophiles et le niveau d'expression protéique de c-IAP2 et de CD40 dans les fractions cellulaires de sputums induits chez des patients asthmatiques. Ce résultat suggère que ce mécanisme joue un rôle dans l'inflammation allergique.

CD40 engagement enhances eosinophils survival through induction of c-IAP2 expression :

Possible involvement in allergic inflammation

CD40 engagement enhances eosinophil survival through induction of cellular inhibitor of apoptosis protein 2 expression: Possible involvement in allergic inflammation

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Background: CD40 engagement enhances eosinophil survival, suggesting a role for this receptor in the development of eosinophilia.

Objective: We examined whether CD40 enhances eosinophil survival by inducing the expression of antiapoptotic proteins. Three members of the inhibitor of apoptosis protein (IAP) family, namely cellular (c)-IAP1, c-IAP2, and XIAP, and 2 antiapoptotic proteins of the Bcl-2 family, namely Bcl-x_L and Bfl-1/A1, were investigated.

Methods: Blood and sputum were obtained from healthy subjects and atopic asthmatic patients. Blood eosinophils were isolated by means of magnetic selection. Expression of CD40, IAPs, and Bcl-2 proteins was investigated by using flow cytometry, immunoblotting, or both. CD40 stimulation was achieved with agonistic antibodies or soluble ligands. Apoptosis was assessed by staining with propidium iodide and FITC-conjugated annexin-V. c-IAP2 expression was inhibited with antisense oligonucleotides.

Results: Freshly isolated eosinophils from healthy and asthmatic patients did not express CD40. Conversely, eosinophils expressed CD40 spontaneously when cultured for 48 hours. At this time point, CD40 stimulation significantly delayed eosinophil apoptosis. Inhibition of eosinophil apoptosis was accompanied by induction of c-IAP2 but not c-IAP1, XIAP, Bcl-x_L, or Bfl-1/A1 expression. Antisense knockdown of *c-iap2* abolished CD40-induced enhancement of eosinophil survival. Sputum cells from asthmatic patients, unlike those from healthy subjects, substantially expressed CD40 and c-IAP2. Moreover, a strong correlation was found between the percentage of eosinophils in the sputum from asthmatic patients and the sputum level of CD40 and c-IAP2 expression.

Conclusion: The results demonstrate that CD40 engagement enhances eosinophil survival through induction of c-IAP2 expression and suggest a role for this mechanism in allergic inflammation. (J Allergy Clin Immunol 2002;110:443-9.)

Allergy is characterized by the accumulation of activated eosinophils at the site of inflammation. Inhibition of eosinophil apoptosis has been proposed as a key mechanism for the development of tissue eosinophilia in allergic disorders.[1] IL-5, IL-3, and GM-CSF, 3 T_H2 cell-derived cytokines, dramatically increase the life span of purified eosinophils by inhibiting their spontaneous apoptosis in vitro.[2-4] Moreover, these cytokines are overexpressed in inflamed allergic tissue[5] and have been shown to increase eosinophil survival in vivo, thereby crucially contributing to the accumulation of eosinophils in allergic inflammation.[6,7]

CD40, a 50-kd member of the TNF receptor superfamily, is expressed on many different cell types, including B cells, macrophages, monocytes, dendritic cells, endothelial cells, epithelial cells, fibroblasts, and carcinoma cells.[8] CD40 ligand (CD40L, also known as gp39 and CD154) is a protein with homology to TNF.[8] CD40L is expressed on many cell types, such as activated T cells, dendritic cells, natural killer cells, monocytes, mast cells, and eosinophils, and can engage CD40 as a membrane-bound or soluble ligand.[8] CD40-CD40L interaction provides critical signals regulating diverse cellular responses, ranging from proliferation and differentiation to growth suppression and cell death.[8] Although CD40 induces apoptosis in some cells, such as hepatocytes and carcinoma cells,[9,10] it conveys antiapoptotic signals in other cells, including B cells.[11] CD40 ligation rescues B cells from IgM- or Fas-induced apoptosis by means of upregulating expression of Bcl-x_L and Bfl-1/A1,[12-14] 2 antiapoptotic members of the Bcl-2 family of proteins. In B cells CD40 stimulation also triggers expression of the cellular inhibitor of apoptosis protein 2 (c-IAP2, also referred to as HIAP-1 and MIHC),[15] a member of the inhibitor of apoptosis (IAP) family. However, whether CD40-induced c-IAP2 expression is sufficient to protect B cells from apoptosis has never been demonstrated.

Abbreviations used
AS: Antisense
CD40L: CD40 ligand
DMRIE-C: 1,2-Dimyristyloxypropyl-3-dimethyl-hydroxy
ethyl ammonium bromide-cholesterol
IAP: Inhibitor of apoptosis protein
Ic: Immune complexes
ODN: Oligodeoxyribonucleotide
rhCD40L$_T$: Trimeric recombinant human CD40L

A few years ago, Ohkawara et al[16] reported that eosinophils isolated from the blood or tissue of allergic subjects express CD40 and that CD40 stimulation results in enhanced survival of cultured blood eosinophils. These findings suggest that CD40 ligation, like IL-5, IL-3, and GM-CSF stimulation, might enhance eosinophil survival in allergic disorders and therefore participate in the development of eosinophilia. However, although potentially relevant in the pathophysiology of allergy, CD40-mediated delay of eosinophil apoptosis has not been further investigated.

Here we confirm that eosinophils may express CD40 and that CD40 engagement delays eosinophil apoptosis. Furthermore, we show that CD40-induced enhancement of eosinophil survival results from induction of c-IAP2 expression and provide in vivo evidence that this mechanism might be involved in allergic inflammation.

METHODS
Subjects

Fifteen patients with atopic asthma (age, 35 ± 18 years) and 10 healthy subjects (age, 42 ± 19 years) participated in the study. All subjects were steroid naive. Asthma was diagnosed on the basis of a clinical history of recurrent wheeze, breathlessness, or cough associated with either significant reversibility of FEV_1 (>15% from baseline and ≥ 200 mL) after inhalation of 400 µg of salbutamol when baseline FEV_1 was less than 80% of predicted value or bronchial hyperresponsiveness to methacholine (methacholine dose producing a 20% fall in FEV_1 [PC_{20M}] <16 mg/mL when baseline FEV_1 was >70% of predicted value). Atopy was defined by positive skin prick test responses (wheal diameter of ≤ 3 mm when compared with that produced with control saline) to common aeroallergens (Stallergènes). The protocol of the study was approved by the local ethical committee.

Sputum induction and processing

Sputum induction and processing were performed as previously described.[17] Sputum cell counts were performed on cytospin preparations stained with Diff-Quick (Dade Berhing). In all cases 500 nonsquamous nucleated cells were counted by an experienced observer blind to the clinical details.

Cell sorting and culture

Neutrophils were isolated from the blood of healthy subjects. Granulocytes were separated from mononuclear cells by means of density centrifugation (Histopaque, Sigma). Contaminating erythrocytes were removed from the granulocyte fraction by means of hypotonic lysis. The resulting granulocyte population contained mainly neutrophils (>95%), as determined by counting of cytospin preparations stained with Diff-Quick. Eosinophils were isolated from the blood of healthy and asthmatic subjects by incubating the

granulocyte population with microbeads coated with anti-CD16 and anti-CD3 antibodies (CD16 and CD3 MicroBeads, Miltenyi Biotec). CD16-positive neutrophils and residual CD3-positive T cells were depleted by passing the cells through a magnetic cell-separation system (VarioMacs, Miltenyi Biotec). CD16-negative eosinophils were collected, and eosinophil purity was controlled by staining with Diff-Quick. The resulting cell population always contained at least 99% eosinophils. Blood neutrophils and eosinophils were cultured at 2×10^6/mL in RPMI-1640 medium supplemented with 1% glutamine, 10% FCS, 50 µg/mL streptomycin, and 50 IU/mL penicillin (all from GIBCO BRL).

Antibodies and cytokines

In flow cytometric experiments FITC-conjugated anti-CD40 (Serotec) and anti-CD40L (Calbiochem) mAbs and isotype-matched control antibodies conjugated to FITC (Serotec) were used. In immunoblot experiments anti-CD40, anti-c-IAP1, anti-c-IAP2, anti-Bfl-1/A1 (all from Santa Cruz Biotechnology), and anti-XIAP (R&D Systems) polyclonal antibodies; anti-Bcl-x$_L$ (Transduction Laboratories) and anti-α-tubulin (Santa Cruz Biotech) mAbs; and control rabbit serum (Serotec) were used. CD40 stimulation was obtained by using agonistic anti-CD40 mAbs (Becton & Dickinson Biosciences) or trimeric recombinant human CD40L (rhCD40L$_T$; Bender MedSystems). CD40L inhibition was obtained with neutralizing anti-CD40L mAbs (Becton & Dickinson Biosciences). Recombinant human GM-CSF and IFN-γ were purchased from Roche. IgA-immune complexes (IgA-Ic) were obtained by incubating 15 µg of human IgA (Sigma) with 20 µg of anti-human IgA (Sigma) per milliliter of culture medium.

Analysis of CD40 and CD40L protein expression by means of flow cytometry

Cells were washed, stained with FITC-conjugated anti-CD40 (1:10 dilution) or anti-CD40L (1:50 dilution) antibodies for 30 minutes at 4°C, washed again, and assessed for CD40 or CD40L expression by means of flow cytometry with a FACStar Plus (Becton & Dickinson).

Apoptosis-necrosis assays

Apoptosis and necrosis were assessed by staining with annexin-V-FITC and propidium iodide with the Annexin-V-FLUOS staining kit (Roche), according to the recommendations of the manufacturer. Flow cytometric analyses were performed with a FACStar Plus (Becton & Dickinson).

Immunoblots

Immunoblots were performed as previously described.[18] The membranes were incubated for 1 hour with the first antibody (1:200 dilution) and then incubated for 45 minutes with peroxidase-conjugated goat anti-rabbit IgG (1:5000 dilution, Kirkegaard & Perry Laboratories) or peroxidase-conjugated rabbit anti-mouse IgG for Bcl-x$_L$ and α-tubulin (1:1000 dilution, Dako). The intensity of specific signals for c-IAP2 and CD40 was assessed by using photodensitometry of the autoradiography (Gel Doc 2000, Bio-Rad). Equal loading of proteins on the gels was always confirmed by probing the blots for α-tubulin (data not shown).

RT-PCR

RNA extraction, reverse transcription, and amplification were performed as previously described.[18] cDNA products were amplified by using primers specific for cd40 (5' primer, CTG TTT GCC ATC CTC TTG GT; 3' primer, CGA CTC TCT TTG CCA TCC TC) and the gene encoding glyceraldehyde 3-phosphate dehydrogenase (5' primer, ACT GGC ATG GCC TTC CGT GT; 3' primer, TTA CTC CTT GGA GGC CAT GT).

Antisense knockdown of *CIAP2*

Antisense phosphorothioate oligodeoxyribonucleotides (AS ODNs) directed to *c-iap2* messenger (m)RNA and negative control ODNs have been designed and manufactured by BIOGNOSTIK (Germany). Their sequences were as follows: *c-iap2* AS ODNs, 5′-TAT GTT CAT ATG GTT TAG-3′; control ODNs, 5′-ATA TCC TTC CAG TAC AG-3′. The ODNs were delivered to the cells in the form of complexes with a liposome formulation of the cationic lipid 1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide (DMRIE) and cholesterol (DMRIE-C Reagent, Gibco BRL). One milliliter of OPTI-MEM I Reduced Serum Medium (Gibco BRL) containing 24 µL of DMRIE-C and 1 mL of OPTI-MEM I containing 8 µg of DNA were mixed and allowed to complex for 45 minutes at room temperature. Eosinophils (2×10^6) from healthy patients cultured for 40 hours were then suspended in 0.25 mL of serum-free medium, added to the transfection medium, and incubated for 4 hours. Afterward, 2 mL of growth medium containing 20% FCS was added, and the cells were cultured for another 4 hours before stimulation with rhCD40L$_T$ or agonistic anti-CD40 antibodies. Forty-eight hours later, eosinophils were assessed for c-IAP2 expression and viability.

Statistical analysis

Standard least-square linear regressions were carried out to study the correlation between the amount of c-IAP2 and CD40, as assessed by means of photodensitometry, and the percentage of eosinophils in sputum from asthmatic patients. Coefficients of correlation (*r*) were presented as measures of linear association for regression relationships. Other numeric data, except sputum cell counts, were presented as means ± SDs. The differences between mean values were estimated by using a Student *t* test for unpaired data. Sputum cell counts were expressed as medians and compared between atopic asthmatic patients and healthy subjects by using the Mann-Whitney test. In any statistical analysis, *P* values of less than .05 were considered significant.

RESULTS
Eosinophils spontaneously express CD40 when cultured

Blood neutrophils and eosinophils from healthy subjects were purified and cultured for 0, 24, or 48 hours before being assayed for CD40 expression by using immunoblotting (Fig 1, *A*, data obtained at 24 hours are not shown) and flow cytometry (Fig 1, *B*, data obtained for neutrophils are not shown) to examine the potential of granulocytes to express CD40. Freshly isolated blood granulocytes did not express CD40. Conversely, CD40 was detectable in a few eosinophils cultured for 24 hours and was strongly expressed in eosinophils cultured for 48 hours. CD40 was undetectable in neutrophils cultured for 24 or 48 hours.

RT-PCR analyses were performed to determine whether induction of CD40 expression in cultured eosinophils is due to transactivation of the *cd40* gene (Fig 1, *C*). Freshly isolated eosinophils expressed only low levels of *cd40* mRNA. These levels were dramatically increased in eosinophils cultured for 36 hours, indicating that increased CD40 expression in these cells is due to *cd40* upregulation.

IFN-γ, GM-CSF, and IgA-Ic have been reported to induce or enhance CD40 expression in certain cell types.[16,19,20] Blood eosinophils and neutrophils have
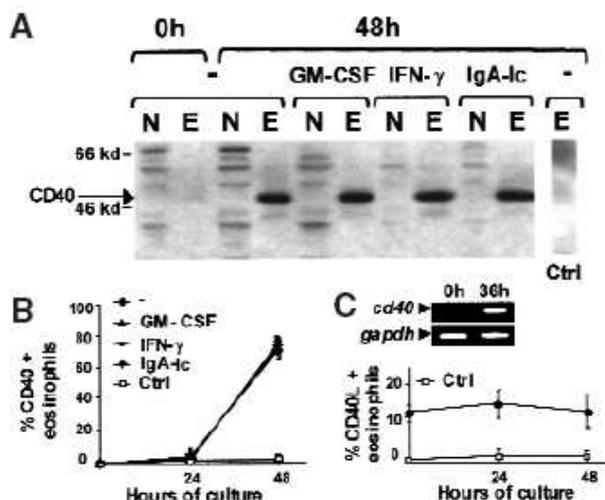

FIG 1. Eosinophils spontaneously express CD40 when cultured. A, Blood eosinophils (E) and neutrophils (N) from healthy subjects were cultured in the presence or absence of IFN-γ, GM-CSF, or IgA-Ic for 0, 24, 36, or 48 hours. Both cell types were assayed for CD40 expression by means of immunoblotting. *Ctrl*, Immunoblot performed with a control rabbit serum. B, Eosinophils were assayed for CD40 or CD40L expression by using flow cytometry (B; *Ctrl*, results obtained with isotype-matched control antibodies) and analyzed by means of RT-PCR for *cd40* expression (C; as a control for quantification, glyceraldehyde 3-phosphate dehydrogenase [*gapdh*] was also amplified).

been cultured in the presence of these compounds for 24 or 48 hours before being assayed for CD40 expression to determine whether they affect CD40 expression in granulocytes. Neither IFN-γ (10 µg/mL), GM-CSF (500 U/mL), nor IgA-Ic induced CD40 expression in neutrophils, as determined by means of immunoblotting (Fig 1, *A*) and flow cytometry (data not shown). Similarly, these compounds were unable to enhance spontaneous expression of CD40 in eosinophils (Fig 1, *A* and *B*).

Blood eosinophils have been demonstrated to constitutively bear CD40L.[21] In our study the percentage of freshly isolated blood eosinophils expressing CD40L was 12.9% ± 2.1% (Fig 1, *C*). This percentage remained constant over a culture period of at least 48 hours (Fig 1, *C*). Freshly isolated and cultured blood neutrophils did not express CD40L (data not shown).

CD40 ligation potently enhances survival of cultured blood eosinophils

We next investigated the effects of CD40 stimulation on the survival of cultured blood eosinophils. Blood eosinophils from healthy donors cultured for 48 hours were treated with rhCD40L$_T$ or agonistic anti-CD40 antibodies. Forty-eight hours later, eosinophil viability was evaluated by using dual-color annexin-V-FITC/propidium iodide staining and flow cytometric analyses. Because eosinophils themselves express CD40L, we also examined whether CD40-CD40L interactions involved in autocrine or paracrine modes of stimulation might modulate eosinophil viability. In this case CD40L inhibition was obtained with neutralizing anti-CD40L antibodies.
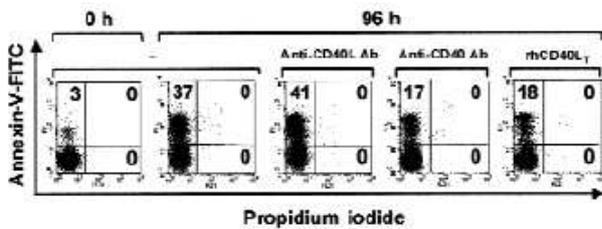
FIG 2. CD40 ligation enhances survival of cultured blood eosinophils. Blood eosinophils from healthy subjects were cultured for 48 hours before treatment with either neutralizing anti-CD40L antibodies (1:200 dilution), agonistic anti-CD40 antibodies (1:200 dilution), or rhCD40L$_T$ (2 µg/mL). Forty-eight hours later, eosinophils were assayed for apoptosis by using flow cytometry.
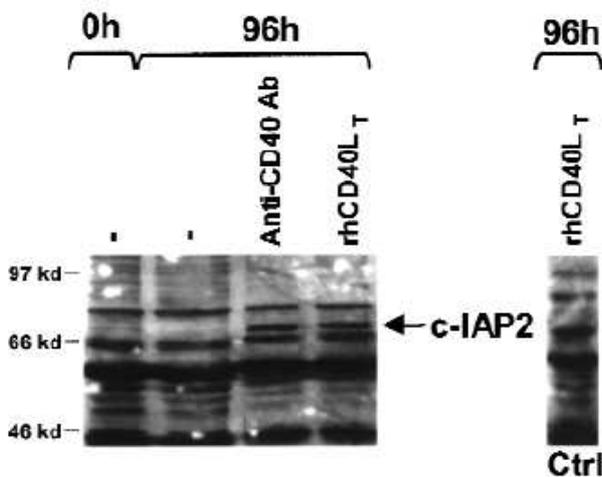

FIG 3. CD40 engagement induces c-IAP2 expression in cultured blood eosinophils. Blood eosinophils from healthy subjects were cultured for 48 hours before treatment with either agonistic anti-CD40 antibodies (1:200 dilution) or rhCD40L$_T$ (2 µg/mL). Forty-eight hours later, the cells were assayed for c-IAP2 expression by using immunoblotting. *Ctrl*, Immunoblot performed with a control rabbit serum.

CD40L neutralization did not affect the rate of eosinophil death (Fig 2). Conversely, the percentage of apoptosis in CD40-activated eosinophils was significantly reduced (16% ± 3% in eosinophils treated with anti-CD40 antibodies and 19% ± 4% in eosinophils treated with rhCD40L$_T$) compared with the percentage of apoptosis in untreated eosinophils (39% ± 7%, Fig 2). Further experiments showed that rhCD40L$_T$ and anti-CD40 antibodies inhibited eosinophil apoptosis in a dose-dependent manner, with peak effects reached at 4 µg/mL rhCD40L$_T$ and 1:200 vol/vol anti-CD40 antibodies, and that their prosurvival effects were still detectable 5 days after treatment (as in eosinophils cultured for 7 days, data not shown).

Freshly isolated blood eosinophils were also cultured for 96 hours in the presence of 125 U/mL GM-CSF, a potent inhibitor of spontaneous granulocytic apoptosis.[2] At 96 hours, GM-CSF–stimulated eosinophils showed a significant decrease in apoptosis (14% ± 4%) compared with that seen in untreated cells (data not shown). However, at this time point, the rate of apoptosis in GM-CSF–treated and CD40-activated eosinophils was com-

parable, indicating that CD40 activation is as potent as GM-CSF stimulation in protecting eosinophils from spontaneous apoptosis.

CD40 engagement induces c-IAP2 expression in cultured blood eosinophils

The expression levels of Bcl-x$_L$ and Bfl-1/A1 and 3 representative members of the IAP family, namely XIAP, c-IAP1, and c-IAP2, were assessed by means of immunoblotting in untreated eosinophils and in eosinophils treated with agonistic anti-CD40 antibodies or rhCD40L$_T$ to address the question of whether CD40 stimulation protects eosinophils from apoptosis through the control of Bcl-2 family proteins or IAPs.

XIAP, c-IAP1, and c-IAP2 (Fig 3) were undetectable in freshly isolated eosinophils and in eosinophils cultured for 96 hours, whereas Bcl-x$_L$ and Bfl-1/A1 were weakly expressed in these cells (data not shown). Neither XIAP, c-IAP1, Bcl-x$_L$, nor Bfl-1/A1 protein levels were modified after CD40 engagement (data not shown). Conversely, both anti-CD40 antibodies and rhCD40L$_T$ induced c-IAP2 expression in cultured eosinophils (Fig 3).

Induction of c-IAP2 expression contributes to the reduction in eosinophil apoptosis after CD40 stimulation

To confirm that induction of c-IAP2 expression observed in CD40-activated eosinophils contributes to the delayed apoptosis in these cells, we determined the effects of selectively inhibiting c-IAP2 expression after CD40 stimulation on eosinophil longevity. *c-iap2* knockdown was obtained by using *c-iap2* AS ODNs. Exposure of blood eosinophils from healthy subjects cultured for 40 hours to an optimal dose of DMRIE-C/*c-iap2* AS ODN complexes for 8 hours before CD40 stimulation with rhCD40L$_T$ or anti-CD40 antibodies resulted in an approximately 4-fold decrease in c-IAP2 expression, as determined by means of immunoblotting (Fig 4, *A*) and photodensitometry (data not shown) performed 48 hours after stimulation. Conversely, DMRIE-C alone and DMRIE-C/control ODN complexes had no effect on CD40-induced c-IAP2 expression (Fig 4, *A*).

The ability of *c-iap2* AS ODNs to specifically down-regulate c-IAP2 allowed exploration of its role in enhanced eosinophil survival after CD40 engagement. Apoptosis assays were performed on eosinophils cultured for 40 hours that were first treated for 8 hours with DMRIE-C alone or in combination with *c-iap2* AS or control ODNs and then treated for 48 hours with rhCD40L$_T$ (Fig 4, *B*) or anti-CD40 antibodies (data not shown). Inhibition of c-IAP2 expression by *c-iap2* AS ODNs significantly increased the rate of apoptosis in CD40-activated eosinophils (Fig 4, *B*). Indeed, although DMRIE-C alone induced eosinophil apoptosis (compare Fig 4, *B*, with Fig 2), the percentage of apoptosis was greater in eosinophils treated with AS ODNs (87% ± 6%) than in eosinophils treated with control ODNs or with DMRIE-C alone (60% ± 8% and 65% ± 6%, respectively).
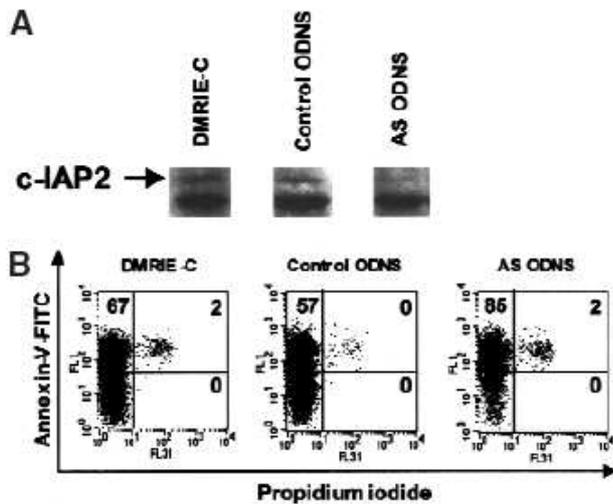
FIG 4. AS knockdown of *c-iap2* increases the rate of apoptosis in CD40-activated eosinophils. Blood eosinophils from healthy subjects were cultured for 40 hours and then treated for 8 hours with DMRIE-C alone or combined with *c-iap2* AS or control ODNs before stimulation with rhCD40L$_T$ (2 µg/mL). Forty-eight hours later, the cells were assayed for c-IAP2 expression by using immunoblotting (A) or for apoptosis by using flow cytometry (B).
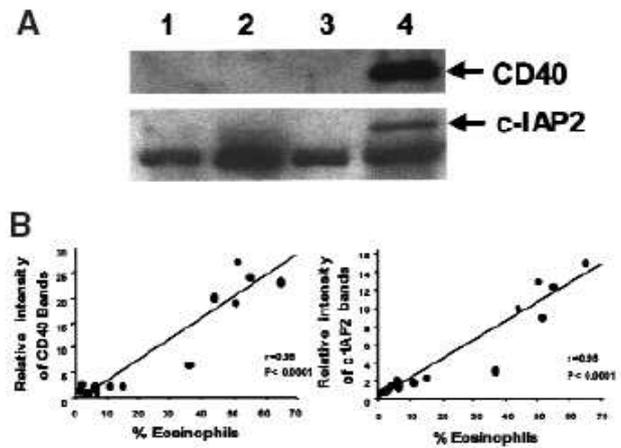

FIG 5. Evidence for CD40 and c-IAP2 expression in sputum eosinophils from atopic asthmatic patients. A, Blood eosinophils and sputum cells from healthy subjects (*lanes 1 and 2*, respectively) and atopic asthmatic patients (*lanes 3 and 4*, respectively) were isolated and assayed for CD40 and c-IAP2 expression by means of immunoblotting. B, Relationships between levels of CD40 and c-IAP2 expression in sputum cells from asthmatic patients, as assessed by means of photodensitometry, and sputum relative eosinophil counts.

Evidence for expression of CD40 and c-IAP2 in sputum eosinophils from patients with atopic asthma

Blood and sputum samples were taken from patients with atopic asthma to determine whether eosinophils from allergic patients express CD40 and c-IAP2. When compared with healthy subjects, atopic asthmatic patients had significantly increased sputum relative eosinophil counts (25% vs 0%) and reduced sputum relative macrophage counts (30% vs 58%).

Freshly purified blood eosinophils from atopic asthmatic patients neither expressed CD40 nor c-IAP2 (Fig 5, A, lane 3). However, when cultured, blood eosinophils from asthmatic patients, like blood eosinophils from healthy subjects, spontaneously expressed CD40. Moreover, CD40 stimulation also induced c-IAP2 expression in these cells (data not shown).

Sputum cells from asthmatic patients substantially expressed both CD40 and c-IAP2 (Fig 5, A, lane 4), whereas these proteins were undetectable in sputum cells from healthy subjects (Fig 5, A, lane 2). The levels of c-IAP2 and CD40 expression in sputum cells from asthmatic patients, as assessed by means of photodensitometry, were strongly correlated with sputum relative eosinophil counts, with very important levels of CD40 and c-IAP2 expression in sputum samples containing more than 40% eosinophils (Fig 5, B). Altogether, these results suggest that CD40 and c-IAP2 are expressed in eosinophils present at the site of allergic inflammation.

DISCUSSION

Enhanced eosinophil survival crucially contributes to the accumulation of these effector cells at the site of inflammation in allergic diseases.[1,6,7] Despite their important role in the pathophysiology of allergy, the precise mechanisms by which eosinophils escape spontaneous apoptosis in allergic disorders have not been completely elucidated. In the present study we confirmed a previous report by Ohkawara et al[16] that eosinophils may express CD40 and that CD40 activation delays eosinophil apoptosis as potently as GM-CSF stimulation. We also demonstrated that CD40 mediates enhancement of eosinophil survival at least partly through induction of c-IAP2 expression and provided in vivo evidence that CD40-mediated c-IAP2 expression occurs in eosinophils at the site of allergic inflammation.

Ohkawara et al[16] reported that freshly isolated blood eosinophils from allergic patients express CD40 and that IgA-Ic enhance the basal expression of CD40 in these cells. Regarding these specific results, our data contrast with those of Ohkawara et al. First, in our study CD40 protein was undetectable in blood eosinophils from both healthy and allergic subjects, as demonstrated by means of immunoblotting and flow cytometric experiments. Although Ohkawara et al clearly showed that blood eosinophils from allergic patients express *cd40* mRNA, their flow cytometric analyses indicated that CD40 protein is hardly detectable at the surface of these cells. It is therefore possible that *cd40* transcription occurs at low levels in blood eosinophils but that posttranscriptional events prevent CD40 protein expression in these cells. Second, our data show that IgA-Ic are unable to affect CD40 expression in blood eosinophils. In the study of Ohkawara et al,[16] CD40 expression was substantially increased in blood eosinophils cultured for 18 hours in the presence of IgA-Ic when compared with freshly isolated eosinophils; however, comparison with control

eosinophils cultured for 18 hours in the absence of IgA-Ic was not made. Our data indicate that cultured blood eosinophils spontaneously express CD40 and that IgA-Ic are unable to modulate this expression. Together, these observations suggest that spontaneous CD40 expression, rather than IgA-Ic–mediated CD40 expression, might have occurred in the study of Ohkawara et al.

IFN-γ and GM-CSF are potent inducers of CD40 expression in certain cell types.[19,20] Eosinophils bear receptors for IFN-γ and GM-CSF.[22,23] However, these cytokines failed to enhance CD40 expression in eosinophils, suggesting that the molecular mechanisms by which IFN-γ and GM-CSF mediate CD40 expression are deficient in eosinophils. An interesting question therefore concerns the stimuli capable of inducing CD40 expression in eosinophils. Our findings, in addition to those of Ohkawara et al,[16] clearly indicate that such stimuli are present in the medium of cultured eosinophils and at the site of allergic inflammation. Further studies are needed to identify them.

The data presented herein confirm previous findings of Ohkawara et al[16] that CD40 engagement induces delayed apoptosis in eosinophils. Interestingly, inhibition of eosinophil CD40L had no effect on eosinophil life span, indicating that the interaction between eosinophil CD40 and CD40L does not contribute to enhanced eosinophil survival. CD40 stimulation has been demonstrated to enhance B-cell survival through induction of several antiapoptotic proteins, including Bcl-x$_L$ and Bfl-1/A1.[12-14] In B cells CD40 activation also results in c-IAP2 expression.[15] However, whether induction of c-IAP2 expression participates in CD40-induced enhancement of B-cell survival has not been established. IAP family members may suppress apoptosis induced by a variety of stimuli in different cell types.[24] These proteins appear to interfere with cell death, triggering cascade at different levels. For example, c-IAP1 and c-IAP2 can bind to the TNF receptor–associated factor 2, which is essential for the activation of nuclear factor κB, a transcription factor that induces the expression of genes that counteract apoptotic signals and prevent cell death.[25] Moreover, c-IAP1 and c-IAP2 have also been shown to be direct inhibitors of the terminal effector caspases 3 and 7.[26] Here, using immunoblotting and AS knockdown of *c-iap2*, we unambiguously showed that CD40 engagement protects eosinophils from apoptosis at least partly by inducing c-IAP2 expression. Moreover, by showing a convincing correlation between c-IAP2 expression and sputum eosinophil percentage, we provide strong evidence that c-IAP2 is expressed in eosinophils present at the site of inflammation and that c-IAP2 might contribute to the persistence of eosinophilic inflammation.

The contribution of CD40-CD40L interactions to allergen-induced airway inflammation has been investigated in mice lacking CD40 or CD40L.[27-29] These studies generated contradictory conclusions. Indeed, although Lei et al[28] concluded that CD40-CD40L interactions play a crucial role in the development of allergic inflammation, Hogan et al[27] and Mehlop et al[29] stated that CD40-CD40L interactions are not required for allergen-induced inflammatory response. It is possible that the discrepancy between these 2 conclusions might have arisen from protocol differences. Hogan et al[27] and Mehlop et al[29] have assessed the magnitude of tissue eosinophilia within 1 day of allergen exposure. At this time point, CD40$^{-/-}$ and CD40L$^{-/-}$ mice displayed high levels of lung eosinophilia similar to that observed in wild-type mice. Conversely, Lei et al[28] measured the percentage of lung eosinophils 72 hours after allergen challenge. At this time point, CD40L-deficient mice exhibited a significant decrease in the percentage of lung eosinophils when compared with wild-type mice. Because CD40 engagement enhances eosinophil survival, as demonstrated in the present study and in the previous report of Ohkawara et al,[16] the results obtained in CD40 and CD40L knockout mice are all consistent with a model in which CD40-CD40L interactions do not interfere with the early development of tissue eosinophilia after allergen exposure but are rather critically required for the maintenance of eosinophilic inflammation.

It has been established that GM-CSF–, IL-5–, and IL-3–mediated enhancement of eosinophil survival is a key contributory event leading to the accumulation of eosinophils at the site of inflammation in allergic disorders.[6,7] Thus it was assumed that therapeutic inhibition of this mechanism could be useful in the resolution of allergic inflammation.[1,30] Similarly, our data, in addition to the finding that CD40 ligation is essential for Ig isotype switching to IgE in B cells,[31] prompt us to postulate that CD40 and CD40L, as well as c-IAP2, could be important pharmacologic targets in controlling allergic inflammation.

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