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Delayed neutrophil apoptosis in chronic periodontitis patients

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Abstract

Background, aims: Neutrophil cells constitute the first defense barrier against the oral bacterial challenge in the periodontium. Reduction of neutrophils could impair this response against periopathogenic bacteria such as *Porphyromonas gingivalis*. Our previous work implicates the apoptosis of neutrophils in the pathogenesis of periodontitis. We now demonstrate that granulocyte monocyte-colony stimulating factor (GM-CSF) present in the gingival crevicular fluid (GCF) and secreted during the immune response reduces the apoptosis of neutrophils.

Method: In this study, the presence of GM-CSF and tumor necrosis factor-alpha (TNF-α) in GCF was determined in samples obtained from adult patients with periodontitis and from control subjects with clinically healthy gingiva. GCF was collected for 30 s using Periopaper® strips, and cytokines were quantified by ELISA. We used ex vivo culture of gingival tissue biopsies for 2 and 4 days in the presence of GM-CSF. Apoptosis was determined using the terminal TdT-mediated dUTP-biotin nick end labeling (TUNEL) technique, and expression of Bax by immunohistochemistry.

Results: The presence of GM-CSF and TNF-α was detected in the majority of sites from periodontal patients (83.3% and 63.3%, respectively), presenting a total amount of 27.65 and 42.38 pg, respectively. GM-CSF reduces the neutrophil apoptosis determined by double staining with TUNEL and myeloperoxidase and by a reduction of Bax expression.

Conclusions: These findings suggest a novel mechanism by which neutrophils specifically accumulate in adult patients with periodontitis.

Key words: apoptosis; periodontitis; neutrophil; bax; GM-CSF

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Adult periodontitis is a chronic inflammatory disease characterized by a constant interaction between pathogenic bacteria and the host defense mechanisms, which eventually leads to periodontal tissue destruction and tooth loss (Seymour 1991, Page et al. 1997, Reynolds & Meikle 1997). In addition to the direct pathological effects of bacteria on periodontal tissues, it has become evident that most of the tissue damage is caused by indirect mechanisms. Bacterial products act upon the cellular constituents of the gingival tissues activating cellular processes that induce the destruction of the connective tissue and bone (Nagase 1997, Havemose-Poulsen et al. 1998, American Academy of Periodontology 1999, Romanelli et al. 1999). Immunohistochemical studies have shown that the majority of mononuclear cells migrate towards the perivascular connective tissue to form an inflammatory infiltrate, mainly composed of neutrophils, T and B lymphocytes and macrophages (Miyasaki 1991, Seymour et al. 1993, Tonetti et al. 1995). The homeostasis of these cell populations, considered as the balance between production and death, maintains a cell number within physiologically appropriate ranges and appropriate for a correct immune response. While the antigenic challenge is responsible for cell growth and clonal expansion, apoptosis is the most common form of physiologic cell death (Savill et al. 1989, Grigg et al. 1991, Gerschenson & Rotello 1992, Cohen 1993). Several authors have demonstrated the presence of apoptotic cells in gingival biopsies from periodontitis patients, showing that apoptotic cells constitute less than 10% of the total cell population (Koulouri et al. 1999, Sawa et al. 1999). Using double staining with a combination of terminal TdT-mediated dUTP-biotin nick end labeling (TUNEL) and different markers for cell populations, we have shown the presence of neutrophils undergoing apoptosis, thus demonstrating that this cell population is also regulated by this process (Gamonal et al. 2001). Neutrophils are the most

abundant immune cells in the gingival inflammatory infiltrate in patients with periodontitis, and their role in its pathogenesis has been clearly demonstrated (Tonetti et al. 1994, Daniel & Van Dyke 1996). Circulating neutrophils have a short half-life and the onset of the apoptotic process is associated with the loss of several important functions, such as adhesion and phagocytosis (Dransfield et al. 1995), which eventually leads to their clearance from the lesion by macrophage ingestion, thus promoting the resolution of the inflammation (Simon 1999). This constitutive tendency to undergo apoptosis prevents neutrophils from lingering at the infection site and limits their proinflammatory potential (Haslett, 1992). However, cytokines such as tumor necrosis factor-alpha (TNF-α), granulocyte monocyte-colony stimulating factor (GM-CSF) (Colotta et al. 1992, Cox et al. 1992, Lee et al. 1993, Dibbert et al. 1999) may delay neutrophil apoptosis by increasing their mitochondrial stability, reducing caspase 3 activity and downregulating the gene expression of Bax, a proapoptotic member of the Bcl-2 family (Tsujimoto & Shimizu, 2000). Recent studies have shown that bacterial products isolated from different strains of *Porphyromonas gingivalis* may also delay neutrophil apoptosis in a dose-dependent fashion (Preshaw et al. 1999).

The objective of this investigation was to identify and measure the presence of TNF-α and GM-CSF in gingival crevicular fluid (GCF) and to correlate it with the presence of apoptotic neutrophils in the gingival tissue of adult periodontitis patients when compared with healthy donors. Furthermore, as a secondary objective we have used ex vivo cultures of human gingival tissue biopsies to study the role of GM-CSF as a potential protective factor that prevents neutrophil apoptosis.

Material and Methods

Patient population

The study group consisted of 15 adult patients (six men and nine women; 35–66 years old; mean age 46.02 ± 8.45 years) with moderate to advanced periodontitis selected from the Graduate Periodontal Clinic at the Faculty of Odontology of the University Complutense, Madrid, Spain. Patients were included in this study if they had at least 14 natural teeth excluding third molars, and including at least 10 posterior teeth; had 5–6 teeth with probing depth (PD) $\geqslant 5$ mm and with attachment loss $\geqslant 3$ mm and showed extensive radiographic bone loss; and had received no treatment at the time of examination. Subjects were excluded if they suffered from systemic illness or if they had received antibiotics or non-steroid anti-inflammatory therapy in the 6-month period prior to the study. Eight periodontally healthy subjects were recruited for the control group (three men and five women; 35–48 years old; mean age 40.56 ± 4.48 years). Subjects in the control group were healthy, had no prior history of periodontal disease, and exhibited PD < 3 mm and no attachment loss, clinical inflammation, sulcular bleeding or radiographic bone loss. Prior to the study, all subjects received a supragingival prophylaxis to remove gross calculus and allow probing depth measurement. The protocol was clearly explained to all patients and Institutional Review Board-approved informed consents were signed. Within 2 weeks of the detection of disease, all patients were rendered appropriate periodontal treatment.

Clinical measurements

Clinical parameters were evaluated by a single calibrated investigator in all teeth (excluding third molars) and they included PD, relative attachment loss and dichotomous measurements of supragingival plaque accumulation (PI) and bleeding on probing to the base of the crevice (BOP). Six sites were examined for each tooth: mesiobuccal, buccal, distobuccal, distolingual, lingual and mesiolingual positions. The Florida Disk Probe was used for relative attachment level recordings and the Florida Probing Depth for PD recording (Florida Probe Corporation, Gainesville, FL, USA).

Collection of GCF

After isolating the tooth with a cotton roll, supragingival plaque was removed with curettes (Hu Friedy, Gracey, USA), avoiding touching the marginal gingiva. The crevicular site was then gently dried with an air syringe and the GCF was collected with Periopaper® filter paper strips (ProFlow, Amityville, NY, USA). The strips were placed into the sulcus/pocket until mild resistance was sensed and left in place for 30 s. Those strips contaminated by saliva or blood were excluded from the sampled group and new sites were selected. Sites were selected randomly with a PD > 5 mm and > 3 mm of attachment loss, in periodontitis patients. A calibrated Periotron-6000® (ProFlow, Amityville, NY, USA) was used for volume determination of the strips. Then, the Periopaper® strips were immediately placed inside a sterile vial and stored at -70°C until further analysis. From the 15 patients with periodontitis, the GCF samples were obtained from two sites with PD > 5 mm (30 samples). GCF samples were also obtained from two sites in the eight healthy subjects (16 samples) at the mesiobuccal gingival sulci (teeth 16 and 26).

Analysis of GCF

Once the GCF was collected, its volume on the Periopaper® strips was measured using a calibrated Periotron-6000®. A standard curve correlating digital readout to volume was constructed for each calibration with standard human serum. The readings from the Periotron-6000® were converted to an actual volume (μl) by reference to this curve. After GCF volume measurements, strips were placed in Eppendorf vials with 50 μl of phosphate-buffered saline with 0.05% Tween-20 (PBS-T). GCF was extracted by centrifugation at $10{,}000 \times g$ for 5 min at 4°C (Biofuge 17RS, Heraeus, Sepatech, Germany) and the procedure was repeated 3 times (Gamonal et al. 2000).

Quantification of cytokines

Aliquots of each GFC sample were assayed by an enzyme-linked immunosorbent assay (ELISA) to determine the levels of TNF-α and GM-CSF using a commercial kit and according to the manufacturer's recommendations (Endogen Inc., Cambridge, MA, USA). Briefly, 96-well microplates were coated with 100 μl of GCF in duplicate and incubated for 2 h at room temperature (RT). Plates were washed 4 times and incubated with 200 μl HRP-conjugated streptavidin (Endogen) for 2 h at RT. After extensive washing, 200 μl of substrate solution was added (Endogen). The reaction was stopped after 30 min by the addition of 50 μl stop solution and color was measured at 450 nm using an automated microplate spectrophotometer (Labsystems Multiskan, Bichro-

matic, Finland). The amount of each cytokine was determined in picograms (pg) using a standard curve (7.8–1000 pg) obtained with a standard recombinant cytokine (Endogen Inc., Cambridge, MA, USA). Cytokine values lower than the detection limit in our assay (below 7.8 pg) were considered undetectable. Cytokine concentration (pg/μl) was calculated from the volume of GCF estimated from the Periotron-6000® reading, according to the following formula: cytokine concentration (pg/μl) = total cytokine (pg)/volume (μl).

Ex vivo culture of gingival tissues

A gingival biopsy sample was taken from each patient with periodontitis ($n = 15$). The base incisions were made 1–2 mm subgingivally; therefore, the specimens consisted of gingival margin, sulcular epithelium and gingival connective tissue. As controls, one biopsy was collected from the control group. Tissues were cut and thoroughly washed in a phosphate buffer (PBS pH 7.2) containing ampicillin and streptomycin in an Eppendorf tube. The sample was then transferred to a 24-well/plate containing RPMI medium 1640 supplemented with 10% fetal calf serum (complete medium). Biopsies were cultured for 2 and 4 days in complete culture medium in the presence of 100 μg/ml of recombinant GM-CSF (RyD Systems, Abingdon, UK). After the specific time in culture, the biopsies were paraffin-embedded and analyzed by immunohistochemistry.

Immunohistochemistry

A standard three-stage immunoperoxidase avidin–biotin–peroxidase complex technique (ABC) was used to detect Bax (Pharmingen, San Diego, CA, USA) and myeloperoxidase (Dako, Glostrup, Denmark). Briefly, nonspecific tissue binding was blocked by incubation with 1% horse serum for 30 min. Specimens were then incubated for 60 min with 40 μl of diluted primary antibody. All rinsing steps were performed with Tris-buffered saline (TBS pH 7.2–7.6). Biotinylated horse anti-mouse IgG secondary antibodies were incubated at 1:200 dilutions for 30 min (Dako, Glostrup, Denmark). Endogenous peroxidase activity was quenched by exposure for 30 min to 0.3% H_2O_2 in methanol. Sections were then incubated for 45 min with preformed avidin biotin–horseradish peroxidase macromolecular complexes (Dako). A black color was developed by exposure for 6–8 min to 0.5 mg/ml of the chromogen 3′,3′ -diaminobenzidine tetrahydrochloride (DAB) (Dako). Sections were counterstained with hematoxylin-eosin, dehydrated and permanently mounted. Positive and negative controls were processed with each series.

DNA nick end labeling of tissue sections

The presence of death-associated DNA fragmentation was assessed in situ by TUNEL using a commercial kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's recommendations and as previously described in detailed (Gamonal et al. 2001). Three experimental, positive and negative control slides were prepared for each biopsy. Positive control slides consisted of paraffin-embedded tissue sections of rat mammary gland provided by the manufacturer. For negative controls, TdT was omitted during incubation.

Data analysis

The clinical parameters and the total amount and concentration of each cytokine at healthy and diseased sites were calculated as subject mean $\pm$ standard deviation. Sites of patients were used as the experimental unit of observations and statistical data were analyzed considering subjects as the experimental unit. Clinical measurements and the total amount and concentration of each cytokine were compared between healthy and diseased sites using unpaired Student's t-test. The significance of differences within each group was assessed using the ANOVA test. The significance ($\alpha = 0.05$) of differences was assessed using the Tukey test. The correlation of cytokine levels with the presence of apoptotic cells and Bax proteins was calculated using Pearson's correlation. All the slides were coded by the same person (A.S.) and analyzed by another person (J.G.), making the measurements double blind. A second examiner (A.A.) verified the results. Analysis of immunochemistry biopsies was measured considering a range between 0 and 4, to establish the degree of positive cells present in the inflammatory infiltrate: 0 = no detectable positive cells; 1 = less than 10%; 2 = approximately 25%; 3 = approximately 50%, 4 = more than 50%. The number of TUNEL-positive cells was determined by counting 100 cells, evaluated for each single determination and reported as the mean of analysis from three sections cut and mounted on treated slices for each patient (Gamonal et al. 2001).

Results

The clinical characteristics of the population included in this study are grouped in Table 1. Fifteen periodontitis patients and eight healthy subjects were studied, with ages ranging from 35 to 66 years (mean age 46.02 ± 8.45 years for the periodontitis group; mean age 40.56 ± 4.48 years for the control group).

No statistically significant differences in age or gender existed between the two groups. Significantly lower amounts of GCF were obtained from control subjects as compared with periodontitis patients ($p = 0.0001$).

Most sites analyzed in periodontitis patients had detectable levels of TNF-α (83.3%, 25/30) and GM-CSF (63.3%, 19/30), whereas only 37.5% (6/16) and 43.7% (7/16) had detectable TNF-α and GM-CSF, respectively, in healthy sites. The total amount and concentration of the different cytokines are shown in Table 2 and no statistically significant differences were found when using subjects as the experimental unit. The total amount of TNF-α was higher in periodontitis sites than in control (42.38 versus 13.21 pg; $p < 0.05$), although with a similar cytokine concentration (36.5 versus 39.8; $p > 0.05$). In contrast, GM-CSF was always present, although its concentration was lower in sites with periodontitis than in healthy sites (23.6 versus 92.5 pg/μl; $p < 0.05$).

Neutrophil destruction mediated by apoptosis was detected ''in situ'' by double staining using TUNEL and myeloperoxidase labeling (Fig. 1).

Culture of small gingival biopsies (less than 2 mm) in RPMI medium containing 10% fetal calf serum with 100 μg/ml of recombinant GM-CSF and subsequent immunohistochemistry allowed the sequential analysis of the neutrophil apoptosis process during 4 days. General microscopic changes observed in the hematoxylin and eosin stained sections are shown in Fig. 2. There is an increasing level of spontaneous apoptosis, measured as TUNEL-

Table 1. Clinical characteristics of periodontitis and control groups (mean ± SD)

	Periodontitis group ($n = 15$)	Control groups ($n = 8$)
age (years)	46.02 ± 8.45	40.56 ± 4.48
% males	60.0	62.5
mean probing depth (mm)	3.62 ± 0.42	0.84 ± 0.2
mean relative attachment level (mm)	10.64 ± 0.38	0.30 ± 0.1
mean probing depth at sample sites	5.76 ± 0.54	0.8 ± 0.2
% sites with plaque	79.50 ± 9.8	30.60 ± 7.2
% sites with bleeding on probing	48.50 ± 9.8	0
GCF volume (μl)		
mean volume	1.19 ± 0.27*	0.27 ± 0.14*
range	0.73–1.60	0.10–0.67

*GCF volume periodontitis versus control groups, $p = 0.0001$.

Table 2. TNF-α and GM-CSF in GCF from periodontitis and control patients (mean ± SD)

	TNF-α		GM-CSF	
	periodontitis	control	periodontitis	control
total amount (pg)	41.2 ± 17.8*	13.0 ± 3.0*	27.4 ± 14.4	26.6 ± 7.1
range	10.3–135.4	9.0–18.1	8.7–95.9	8.7–56.9
concentration (pg/μl)	35.3 ± 18.4	36.6 ± 11.7	21.7 ± 14.7#	84.1 ± 35.8#
range	8.9–113.9	19.2–56.0	6.0–102.1	26.0–167.3
number of subjects	15	8	15	8

*Total amount TNF-α periodontitis versus control, $p < 0.05$.

#Concentration GM-CSF periodontitis versus control, $p < 0.05$.

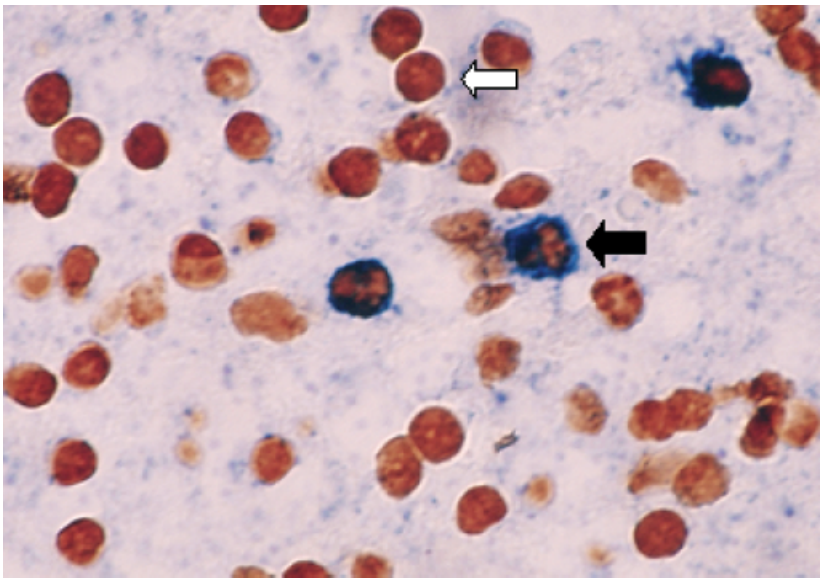

Fig. 1. TUNEL detection and immunohistochemical in tissue-frozen sections of biopsies from periodontitis patients. Double-staining TUNEL/myeloperoxidase-positive cells (filled arrow) of a gingival biopsy from periodontitis patients stained using a DAB chromogen and fast blue. Cells TUNEL-negative are shown with an empty arrow (original magnification × 100).

positive cells, detected in tissue sections after 2 and 4 days in culture with RPMI complete media, indicating that most of the infiltrating inflammatory cells in human gingiva may undergo apoptosis (Fig. 3). The incubation of gingival biopsies with 10 μg/ml of recombinant GM-CSF, however, always delayed the presence of apoptotic cells as measured by immunohistochemistry (Fig. 3, panel C versus panel D and Table 3).

The presence of apoptotic cells in tissue-cultured biopsies was confirmed by studying Bax protein localization, by means of immunohistochemistry using an anti-Bax monoclonal antibody. As shown in Fig. 4, fresh biopsies derived from normal human gingival did not express Bax or its expression was reduced. In contrast, by days 2 and 4, the expression of the proapoptotic protein was detectable in all investigated biopsies. The incubation with GM-CSF correlated with a lower number of apoptotic cells in all tissue sections from periodontitis patients ($r = 0.34$, $p > 0.05$) (Table 3).

Discussion

Apoptosis, or programmed cell death, plays a critical role in the regulation of inflammation and the host immune response (Cohen 1991). The apoptotic process includes a series of coordinated morphological and biochemical events in the affected cell, resulting in its death and removal by scavenger phagocytes (Cohen 1991). Apoptosis of neutrophils in vitro can be modulated by survival (Colotta et al. 1992; Lee et al. 1993) and death factors (Liles et al. 1996, Murray et al. 1997). It has been detected by expression of members of the Bcl-2 family, such as Bax in tissue sections (Iwai et al. 1994, Weinmann et al. 1999). However, its possible role in the pathogenesis of periodontitis has not been investigated. With this purpose, we have shown the expression of Bax- and DNA fragmentation-positive neutrophil cells in tissue sections from patients with periodontitis. Furthermore, we have tried to correlate the presence of these cells with the presence of TNF-α and GM-CSF in GCF. This study shows how these soluble factors may play a role in delaying apoptosis of the infiltrating inflammatory cells in periodontitis. The role of TNF-α to induce, delay or be ineffective on neutrophil apoptosis has been controversial (Akgul et al. 2001). While TNF-α rapidly accelerates apoptosis in some neutrophils, it also generates an antiapoptotic signal in the remaining neutrophils (Murray et al. 2001). Considering periodontitis as a long-term disease, the finding of high levels of TNF-α in the GCF of periodontitis subjects suggests a protective role in neutrophil apoptosis. The levels of total TNF-α (pg) are strongly reduced when the

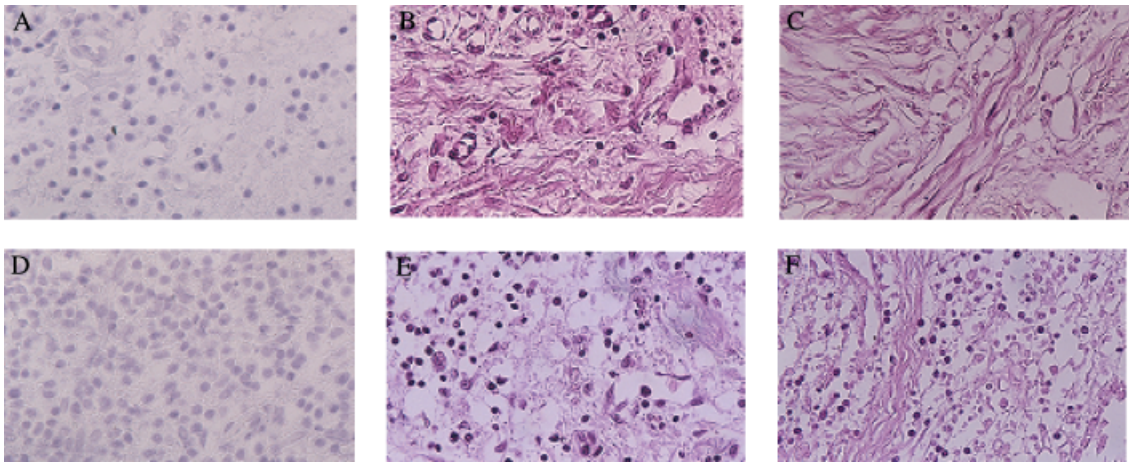

Fig. 2. Microscopy changes in ex vivo cultures of human gingival biopsy stained with hematoxylin and eosin. Ex vivo cultures of small gingival biopsies, less than 2 mm, in RPMI/FCS medium with 100 μg/ml of recombinant GM-CSF were stained with hematoxylin and eosin after 0 (A, D), 2 (B, E) and 4 days (C, F) from control (A, B, C) and periodontitis patients (D, E, F) (original magnification × 40).

Table 3. Bax-positive and TUNEL-positive cells in periodontal patients

Tissue sections	Bax-positive			TUNEL-positive (mean ± SD)				
	Day 0	Day 2	Day 4	Day 0	Day 2		Day 4	
					GM-CSF (+)	GM-CSF (−)	GM-CSF (+)	GM-CSF (−)
Disease	0	< 10%	> 25%	5.6 ± 2.7	9.6 ± 2.2	10.8 ± 2.1	16.7 ± 5.0	21.9 ± 7.2*

*TUNEL-positive cells in the absence versus presence of GM-CSF, $p < 0.05$.

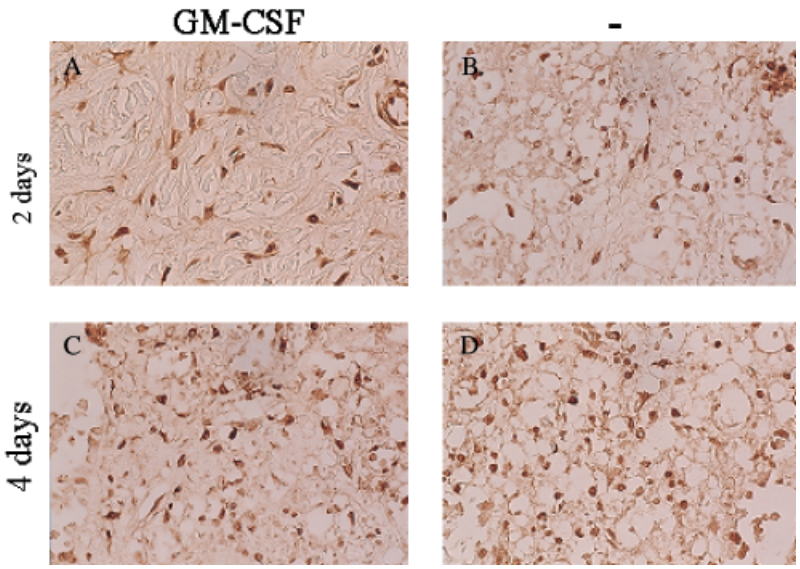

Fig. 3. GM-CSF-induced protection of spontaneous apoptosis on ex vivo sections of periodontitis biopsies. Ex vivo cultures of gingival biopsies maintained for 2 (A and B) and 4 (C and D) days in RPMI/FCS medium in the presence (A and C) or absence (B and D) of 100 μg/ml recombinant GM-CSF were analyzed for apoptosis using TUNEL assay (original magnification × 40).

concentration is measured (total quantity per volume, pg/μl) due to the presence of a larger volume of GCF in periodontitis patients as compared with healthy subjects (Table 1). From our data we conclude that neutrophil-delayed apoptosis may be associated with the production of TNF-α and GM-CSF, since intracellular Bax levels were markedly reduced in the presence of these soluble factors.

Apoptosis is being recognized as playing a major role in the inflammatory process. Aberrant apoptosis regulation is considered to contribute to autoimmune disorders such as systemic lupus erythematosus (Georgescu et al. 1997), rheumatoid arthritis (Zhang et al. 2001), viral diseases including AIDS (Ikuta et al. 1997) and bacterial infection (Zychlinsky & Sansonetti 1997). Therefore, abnormalities in the regulation of cell homeostasis may contribute to a number of different pathogenic processes. Certain cytokines increase the lifespan of the neutrophil by inhibiting their apoptotic cell death in vitro (Brach et al. 1992), and this prolonged neutrophil survival has been associated with an enhanced inflammatory response (Dibbert et al. 1999). Direct evidence for apoptosis delay in periodontal tissue has not been shown in any previous investigation. To demonstrate this event, we used cultures of human gingival biopsies obtained from periodontitis subjects. These cultures may mimic the in vivo microenvironment but without the capacity of new neutrophil recruit-

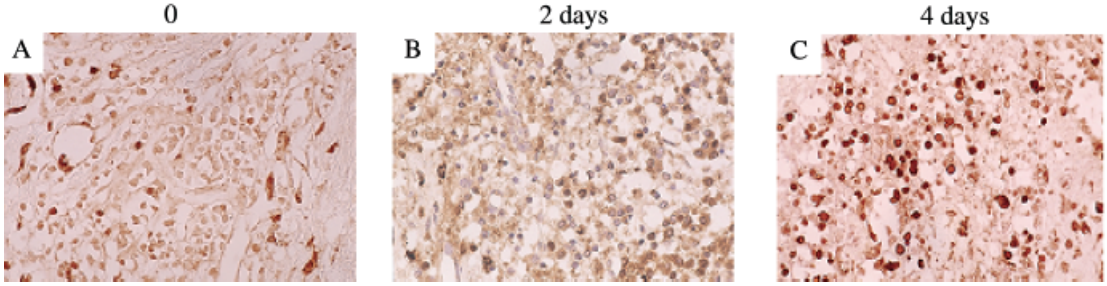

Fig. 4. Bax expression levels in the presence of GM-CSF from ex vivo sections of periodontitis biopsies. Ex vivo cultures of gingival biopsies maintained for 0 (A), 2 (B) and 4 (C) days in RPMI/FCS medium in the presence of 100 μg/ml recombinant GM-CSF were analyzed for Bax expression (original magnification $\times 40$).

ment. Thus, this ex vivo system allowed us to study the onset of the apoptotic process in the tissue and the possible role played by GM-CSF (Simon et al. 1997). Sequential immunohistochemistry analysis from these biopsies allowed us to identify cellular DNA fragmentation on tissue sections and therefore to study the apoptosis at a single cell level. Our findings show that ex vivo biopsies could be maintained in culture for a few days. During this time, an increase in cellular death was evidenced by an increase in the number of Bax-positive cells. However, when the sample was embedded in complete medium containing GM-CSF, the total number of TUNEL-positive cells was reduced, therefore showing its delaying effect on the apoptotic process. Owing to the impossibility of administrating systemic GM-CSF, the use of short ex vivo cultures might constitute a useful tool to analyze the effect of certain regulatory factors on the gingival tissues.

Our results demonstrate that cytokines GM-CSF and TNF-α are present in the GCF of patients with periodontitis and in control subjects. GM-CSF was detected in 63.3% of the GCF samples from periodontal patients whereas only 43.7% had detectable GM-CSF in healthy sites, and little variation was observed with respect to the total amount. Our data show that TNF-α was present in the GCF of periodontitis (total amount 42.3 pg). Therefore, the delay of neutrophil apoptosis could be related, at least partially, to the presence of factors such as GM-CSF (Dibbert et al. 1999) and TNF-α (Colotta et al. 1992). The role of TNF-α expression in periodontitis has already been reported (Rossomando et al. 1990). The levels of TNF-α are significantly higher in inflamed periodontal tissues than in healthy tissues (Stashenko et al. 1991). TNF-α induces the local upregulation of polymorphonuclear function by enhancing (priming) the oxidative and secretory response to stimuli found at infection sites (Steinbeck & Roth 1989). Furthermore, this study suggests that its presence together with GM-CSF in GCF may be involved in the homeostatic control of the neutrophil and may thus delay its death and increase its inflammatory potential in periodontitis (Haslett 1992). GM-CSF can be produced by a number of different cell types, including T lymphocytes, macrophages, endothelial cells, fibroblasts and stromal cells (Quesniaux & Jones 1998). Its production often requires a prior stimulation of the producer cells by antigens such as lipopolysaccharides (LPS), IL-1 and TNF-α in macrophages, fibroblasts and endothelial cells (Rasko & Gough, 1994). It has been recognized that GM-CSF prolongs the survival of neutrophil by significantly delaying the constitutive apoptosis, resulting in their enhanced survival and increased phagocytic function (Coxon et al. 1999). This effect has been attributed to the upregulation of Bcl-xL levels induced by GM-CSF (Ochiai et al. 1997, Iwai et al. 1994).

Since Bcl-X_L opposes the activity of Bax, an increased Bcl-X_L/Bax ratio may contribute to delaying eosinophil apoptosis in allergic and other eosinophilic diseases (Dibbert et al. 1998). The mammalian Bcl-2 family of apoptosis-associated proteins consists of members that inhibit apoptosis (Bcl-2, Bcl-xL, etc.) and others that induce apoptosis (Bax, Bak, etc.), and the balance between these proapoptotic and antiapoptotic members determines the fate of cells in many systems (Kroemer 1997). Bax is a member of the Bcl-2 family and functions as a death agonist within apoptotic pathways (Knudson et al. 1995, Marzo et al. 1998). It has been shown that Bcl-2 antagonizes the proapoptotic activity of Bax by forming heterodimers with it (Oltvai et al. 1993). Since Bax (Weinmann et al. 1999), but not Bcl-2 (Iwai et al. 1994) was reported to be expressed in neutrophil, it is likely that neutrophils contain functional Bax homodimers, which are, at least partially, responsible for the short lifespan of these cells. The proapoptotic activity of Bax might be mediated by the release of cytochrome c from mitochondria (Marzo et al. 1998), with subsequent activation of the caspase cascade (Green & Reed 1998). Clearly, high levels of Bax may contribute to, but may not be the only mechanism responsible for the short lifespan of neutrophils. Our research group has previously demonstrated elevated Bcl-2 expression in periodontitis subjects, which together with the low expression of Bax may suggest that Bcl-2 protects against the apoptosis induced by Bax (Gamonal et al. 2001). GM-CSF, IL-3, G-CSF and IFN-γ markedly downregulated the expression of Bax in normal neutrophils. This downregulation has been associated with a cytokine-mediated prevention of neutrophil apoptosis (Dibbert et al. 1999).

Since the majority of apoptotic cells in our tissue sections were neutrophils (Gamonal et al. 2001) and their relative number was low, we can speculate that the protective mechanism mediated through GM-CSF described above operates in the pathogenesis of periodontitis. The localized accumulation of functional neutrophils at sites of inflammation is pivotal in the host defense against infection and the orderly elimination of neutrophils is equally important in the resolution of the inflammatory response.

In summary, our data show a positive correlation between the delay in the apoptotic process of neutrophils in periodontal tissues with an increase in

the levels of TNF-α, GM-CSF in GCF and a low expression of Bax, thus suggesting a possible role of these soluble mediators in the pathogenesis of periodontitis.

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Zusammenfassung

Verzögerte Apoptose neutrophiler Granulozyten bei chronischer Parodontitis

Hintergrund: Neutrophile Granulozyten stellen die erste Verteidigungslinie gegen die orale bakterielle Exposition des Parodonts dar. Eine Reduktion der Zahl der neutrophilen Granulozyten könnte die Reaktion auf parodontalpathogene Bakterien wie *Porphyromonas gingivalis (Pg)* schwächen. Eine vorangegangene Arbeit hatte die Bedeutung der Apoptose neutrophiler Granulozyten für die Parodontitispathogenese gezeigt.

Zielsetzung: Nachweis, dass der Granulozyten-Monozytenkolonie-stimulierende Faktor (GM-CSF), der sich in der Sulkusflüssigkeit (SF) findet und während der Immunantwort gebildet wird, die Apoptose der neutrophilen Granulozyten reduziert.

Methode: Die Anwesenheit von GM-CSF und TNFα in der SF wurde in Proben von erwachsenen Patienten mit Parodontitis und von Kontrollprobanden mit klinisch gesunder Gingiva bestimmt. SF wurde 30 s lang mit Periopaper®-Streifen gesammelt und die Zytokine mittels ELISA quantifiziert. *Ex-vivo*-Kulturen von Biopsien gingivalen Gewebes wurden für 2 und 4 Tage mit GM-CSF inkubiert. Die Apoptose wurde mittels der "terminal TdT-mediated dUTP-Biotin nick end labeling" (TUNEL) Technik und durch die Expression von Bax immunhistochemisch bestimmt.

Ergebnisse: GM-CSF und TNFα wurden in der Mehrzahl der Stellen von Parodontitispatienten nachgewiesen (83,3% bzw. 63,3%), mit einer Gesamtmenge von 27,65 pg bzw. 42,38 pg. GM-CSF reduzierte die Apoptose der neutrophilen Granulozyten.

Schlussfolgerung: Diese Ergebnisse legen einen neuen Mechanismus nahe, der zur spezifischen Ansammlung von neutrophilen Granulozyten bei erwachsenen Patienten mit Parodontitis führt.

Résumé

Apoptôse des neutrophiles retardée chez les patients avec parodontite chronique

Les neutrophiles constituent la première ligne de défense de l'organisme contre les bactéries dans le parodonte. La réduction des neutrophiles pourrait diminuer cette réponse contre les bactéries parodontales pathogènes tels que le *Porphyromonas gingivalis* (*P.g.*). L'apoptôse des neutrophiles dans la pathogenèse de la parodontite a été étudié. Le facteur stimulant la colonie-monocyte-granulocyte (GM-CSF) présent dans le fluide créviculaire gingival (GCF) et secrété durant la réponse immunitaire réduit l'apoptôse des neutrophiles. Dans cette étude la présence de GM-CSF et TNFalpha dans le GCF a été déterminée dans des échantillons obtenus d'adultes avec parodontite et de contrôles avec gencive cliniquement saine. Le GCF a été prélevé durant 30s à l'aide de bandelettes Periopaper® et les cytokines ont été quantifiées par ELISA. La culture *ex vivo* a été utilisée pour l'analyse de biopsies des tissus gingivaux durant deux et quatre jours en présence de GM-CSF. L'apoptôse a été déterminée par la technique du TUNEL et l'expression de Bax par immunohistochimie. La présence de GM-CSF et TNFalpha a été détectée dans la majorité des sites des patients avec parodontite (respectivement 83,3 et 63,3%), présentant une quantité totale de respectivement, 27,7 et 42,4 pg. GM-CSF réduit l'apoptôse des neutrophiles déterminée par double coloration avec TUNEL et myéloperoxidase et par une réduction de l'expression Bax. Ces découvertes suggèrent un mécanisme nouveau par lequel les neutrophiles s'accumulent spécifiquement chez les adultes avec parodontite.

References

Akgul, C., Moulding, D. A. & Edwards, S. W. (2001) Molecular control of neutrophil apoptosis. *FEBS Letters* **487**, 318–322.

American Academy of Periodontology (1999) The pathogenesis of periodontal diseases. *Journal of Periodontology* **70**, 457–470.

Brach, M. A., deVos, S., Gruss, H. J. & Herrmann, F. (1992) Prolongation of survival of human polymorphonuclear neutrophils by granulocyte–macrophage colony-stimulating factor is caused by inhibition of programmed cell death. *Blood* **80**, 2920–2924.

Cohen, J. J. (1991) Programmed cell death in the immune system. *Advances in Immunology* **50**, 55–85.

Cohen, J. J. (1993) Apoptosis. *Immunology Today* **14**, 126–130.

Colotta, F., Re, F., Polentarutti, N., Sozzani, S. & Mantovani, A. (1992) Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products. *Blood* **80**, 2012–2020.

Cox, G., Gauldie, J. & Jordana, M. (1992) Bronchial epithelial cell-derived cytokines (G-CSF and GM-CSF) promote the survival of peripheral blood neutrophils in vitro. *American Journal of Respiratory Cell and Molecular Biology* **7**, 507–513.

Coxon, A., Tang, T. & Mayadas, T. (1999) Cytokine-activated endothelial cells delay neutrophil apoptosis in vitro and in vivo: a role for granulocyte/macrophage colony-stimulating factor. *Journal of Experimental Medicine* **190**, 923–933.

Daniel, M. A. & Van Dyke, T. E. (1996) Alterations in phagocytes function and periodontal infection. *Journal of Periodontology* **67**, 1070–1076.

Dibbert, B., Daigle, I., Braun, D., Schranz, C., Weber, M., Blaser, K., Zangemeister-Wittke, U., Akbar, A. N. & Simon, H. U. (1998) Role for Bcl-xL in delayed eosinophil apoptosis mediated by granulocyte–macrophage colony-stimulating factor and interleukin-5. *Blood* **92**, 778–783.

Dibbert, B., Weber, M., Nikolaizik, W. H., Vogt, P., Schoni, M. H., Blaser, K. & Simon, H. U. (1999) Cytokine-mediated Bax deficiency and consequent delayed neutrophil apoptosis: a general mechanism to accumulate effector cells in inflammation. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 13330–13335.

Dransfield, I., Stocks, S. C. & Haslett, C. (1995) Regulation of cell adhesion molecule expression and function associated with neutrophil apoptosis. *Blood* **85**, 3264–3273.

Gamonal, J., Acevedo, A., Bascones, A., Jorge, A. & Silva, A. (2000) Levels of interleukin-1b, -8, -10 and RANTES in gingival crevicular fluid and cell populations in adult patients with destructive periodontitis and the effect of periodontal treatment. *Journal of Periodontology*.

Gamonal, J., Bascones, A., Acevedo, A., Blanco, E. & Silva, A. (2001) Apoptosis in chronic adult periodontitis analysed by in situ DNA breaks, electron microscopy and immunohistochemistry. *Journal of Periodontology* **72**, 517–525.

Georgescu, L., Vakkalanka, R., Elkon, K. & Crow, M. (1997) Interleukin-10 promotes activation-induced cell death of SLE lymphocytes mediated by Fas ligand. *Journal of Clinical Investigation* **100**, 2622–2633.

Gerschenson, L. E. & Rotello, R. J. (1992) Apoptosis: a different type of cell death. *FASEB Journal* **6**, 2450–2455.

Green, D. & Reed, J. (1998) Mitochondria and apoptosis. *Science* **281**, 1309–1312.

Grigg, J. M., Savill, J. S., Sarraf, C., Haslett, C. & Silverman, M. (1991) Neutrophil apoptosis and clearance from neonatal lungs. *Lancet* **338**, 720–722.

Haslett, C. (1992) Resolution of acute inflammation and the role of apoptosis in the tissue fate of granulocytes. *Clinical Science* **83**, 639–648.

Havemose-Poulsen, A., Holmstrup, P., Stoltze, K. & Birkedal-Hansen, H. (1998) Dissolution of type I collagen fibrils by gingival fibroblasts isolated from patients of various periodontitis categories. *Journal of Periodontal Research* **33**, 280–291.

Ikuta, K., Kameoka, M. & Luftig, R. B. (1997) AIDS pathogenesis: the role of accessory gene mutations, leading to formation of long-lived persistently infected cells and/or apoptosis-inducing HIV-1 particles. *Virus Research* **52**, 145–156.

Iwai, K., Miyawaki, T., Takizawa, T., Konno, A., Ohta, K., Yachie, A., Seki, H. & Taniguchi, N. (1994) Differential expression

of bcl-2 and susceptibility to anti-Fas-mediated cell death in peripheral blood lymphocytes, monocytes, and neutrophils. *Blood* **84**, 1201–1208.

Knudson, C. M., Tung, K. S., Tourtellotte, W. G., Brown, G. A. & Korsmeyer, S. J. (1995) Bax-deficient mice with lymphoid hyperplasia and male germ cell death. *Science* **270**, 96–99.

Koulouri, O., Lappin, D. F., Radvar, M. & Kinane, D. F. (1999) Cell division, synthetic capacity and apoptosis in periodontal lesions analysed by in situ hybridisation and immunohistochemistry. *Journal of Clinical Periodontology* **26**, 552–559.

Kroemer, G. (1997) The proto-oncogene Bcl-2 and its role in regulating apoptosis. *Nature Medicine* **3**, 614–620.

Lee, A., Whyte, M. & Haslett, C. (1993) Inhibition of apoptosis and prolongation of neutrophil longevity by inflammatory mediators. *Journal of Leukocyte Biology* **54**, 283–288.

Liles, W. C., Kiener, P. A., Ledbetter, J. A., Aruffo, A. & Klebanoff, S. J. (1996) Differential expression of Fas (CD95) and Fas ligand on normal human phagocytes: implications for the regulation of apoptosis in neutrophils. *Journal of Experimental Medicine* **184**, 429–440.

Marzo, I., Brenner, C., Zamzami, N., Jurgensmeier, J., Susin, S. A., Vieira, H. L., Prevost, M. C., Xie, Z., Matsuyama, S., Reed, J. C. & Kroemer, G. (1998) Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis. *Science* **281**, 2027–2031.

Miyasaki, K. T. (1991) The neutrophil: mechanism of controlling periodontal bacteria. *Journal of Periodontology* **62**, 761–774.

Murray, J., Barbara, J. A., Dunkley, S. A., Lopez, A. F., Van Ostade, X., Condliffe, A. M., Dransfield, I., Haslett, C. & Chilvers, E. (1997) Regulation of neutrophil apoptosis by tumor necrosis factor-alpha: requirement for TNFR55 and TNFR75 for induction of apoptosis in vitro. *Blood* **90**, 2772–2783.

Nagase, H. (1997) Activation mechanisms of matrix metalloproteinases. *Biological Chemistry* **378**, 151–160.

Ochiai, K., Kagami, M., Matsumura, R. & Tomioka, H. (1997) IL-5 but not interferon-gamma (IFN-gamma) inhibits eosinophil apoptosis by up-regulation of Bcl-2 expression. *Clinical and Experimental Immunology* **107**, 198–204.

Oltvai, Z. N., Millman, C. L. & Korsmeyer, S. J. (1993) Bcl-2 heterodimerizes in vivo with a conserved homolog Bax, that accelerates programmed cell death. *Cell* **74**, 609–619.

Page, R. C., Offenbacher, S., Schroeder, H. E., Seymour, G. J. & Kornman, K. S. (1997) Advances in the pathogenesis of periodontitis: summary of developments, clinical implications and future directions. *Periodontology 2000* **14**, 216–248.

Preshaw, P. M., Schifferle, R. E. & Walters, J. D. (1999) Porphyromonas gingivalis lipopolysaccharide delays human polymorphonuclear leukocyte apoptosis in vitro. *Journal of Periodontal Research* **34**, 197–202.

Quesniaux, V. F. & Jones, T. C. (1998) Granulocyte–macrophage colony stimulating factor. In: *The cytokine handbook*, ed. Thomson, A., 3rd edition, pp. 637–670. London: Academic Press.

Rasko, J. E. J. & Gough, N. M. (1994) Granulocyte–Macrophage colony stimulating factor. In: *The cytokine handbook*, ed. Thomson, A., 2nd edition, pp. 343–369. London: Academic Press.

Reynolds, J. J. & Meikle, M. C. (1997) Mechanisms of connective tissue matrix destruction in periodontitis. *Periodontology 2000* **14**, 144–157.

Romanelli, R., Mancini, S., Laschinger, C., Overall, C. M., Sodek, J. & McCulloch, C. A. (1999) Activation of neutrophil collagenase in periodontitis. *Infection and Immunity* **67**, 2319–2326.

Rossomando, E., Kennedy, J. E. & Hadjimichael, J. (1990) Tumor necrosis factor alpha in gingival crevicular fluid as a possible indicator of periodontal disease in humans. *Archives of Oral Biology* **35**, 431–434.

Savill, J. S., Wyllie, A. H., Henson, J. E., Wolport, M. J., Henson, P. M. & Haslett, C. (1989) Macrophage phagocytosis of aging neutrophils in inflammation: programmed cell death in the neutrophil leads to its recognition by macrophages. *Journal of Clinical Investigation* **83**, 865–867.

Sawa, T., Nishimura, F., Ohyama, H., Takahashi, K., Takashiba, S. & Murayama, Y. (1999) In vitro induction of activation-induced cell death in lymphocytes from chronic periodontal lesions by exogenous Fas ligand. *Infection and Immunity* **67**, 1450–1454.

Seymour, G. J. (1991) Importance of the host response in the periodontium. *Journal of Clinical Periodontology* **18**, 421–426.

Seymour, G. J., Gemmell, E., Reinhardt, R. A., Eastcott, J. & Taubman, M. A. (1993) Immunopathogenesis of chronic inflammatory periodontal disease: cellular and molecular mechanisms. *Journal of Periodontal Research* **28**, 478–486.

Simon, H. U. (1999) Apoptosis in inflammatory diseases. *International Archives of Allergy and Immunology* **118**, 261–262.

Simon, H., Yousefi, S., Schranz, C., Schapowal, A., Bachert, C. & Blaser, K. (1997) Direct demostration of delayed eosinophil apoptosis as a mechanism causing tissue eosinophilia. *Journal of Immunology* **158**, 3902–3908.

Stashenko, P., Fukiyoshi, P., Obernesser, M. S., Prostak, L., Haffajje, A. D. & Socrenasky, S. S. (1991) Levels of interleukin-1b in tissue from sites of active periodontal disease. *Journal of Clinical Periodontology* **18**, 548–554.

Steinbeck, M. & Roth, J. (1989) Neutrophil activation by recombinant cytokines. *Reviews of Infections Diseases* **11**, 549–568.

Tonetti, M., Imboden, M., Gerber, L. & Lang, N. (1995) Compartmentalization of inflammatory cell phenotypes in normal gingiva and peri-implants keratinized mucosa. *Journal of Clinical Periodontology* **22**, 735–742.

Tonetti, M. S., Imboden, M. A., Gerber, L., Lang, N. P., Laissue, J. & Mueller, C. (1994) Localized expression of mRNA for phagocyte-specific chemotactic cytokines in human periodontal infections. *Infection and Immunity* **62**, 4005–4014.

Tsujimoto, Y. & Shimizu, S. (2000) Bcl-2 family: life-or-death switch. *FEBS Letters* **466**, 6–10.

Weinmann, P., Gaehtgens, P. & Walzog, B. (1999) Bcl-Xl- and Bax-alpha-mediated regulation of apoptosis of human neutrophils via caspase-3. *Blood* **93**, 3106–3115.

Zhang, J., Bardos, T., Mikecz, K., Finnegan, A. & Glant, T. T. (2001) Impaired Fas signaling pathway is involved in defective T cell apoptosis in autoimmune murine arthritis. *Journal of Immunology* **166**, 4981–4986.

Zychlinsky, A. & Sansonetti, P. (1997) Apoptosis as a proinflammatory event: what can we learn from bacteria-induced cell death? *Trends in Microbiology* **5**, 201–204.

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