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Expression of dendritic cell markers on cultured neutrophils and its modulation by anti-apoptotic and pro-apoptotic compounds

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Abbreviations: DC, dendritic cell; PDTC, pyrroline dithiocarbamate; PE, phycoerythrin; SEB, staphylococcal enterotoxin

Abstract

Neutrophils are also known to acquire the characteristics of dendritic cells (DCs) under the appropriate conditions. In this study, neutrophils were cultivated *in vitro* in the presence or absence of compounds modulating their survival in an attempt to characterize the expression profile of the DC markers. Higher MHC-II, CD80, CD86, CD83, and CD40 expression levels were detected on the surface of the cultured neutrophils for 24 h than on the freshly isolated cells. The annexin V-positive cells showed a higher expression level of the DC markers than the annexin V-negative cells. The population of neutrophils double stained with annexin V and the DC markers increased after being incubated with agonistic anti-Fas Ab. LPS, the anti-apoptotic compound, decreased the CD86 and MHC-II expression levels but 50-60% of the DC marker-positive cells were detected in the annexin V-positive cells. In contrast, CD80, CD86, CD83, and HLA-DR mRNA levels increased in the GM-CSF-treated neutrophils but not in the anti-Fas Ab-treated neutrophils. T cell proliferation was inhibited by co-culturing them with anti-Fas Ab- or LPS-treated neutrophils at a high neutrophil:T cell ratio. However, the superantigen-mediated T cell proliferation was increased by the LPS-treated neutrophils but decreased by the anti-Fas Ab-treated neutrophils. There was a lower level of interferon-γ production in the T cells co-cultured with anti-Fas Ab-treated neutrophils than with the LPS-treated neutrophils. This suggests that apoptotic neutrophils express DC markers on their surface and the differential expression of DC markers might have a detrimental effect on the immune reaction.

Keywords: antigen-presenting cells; antigens, CD95; apoptosis; dendritic cells; neutrophils

Introduction

Neutrophils play an important role in the innate immune response by rapidly migrating into inflamed tissues, releasing proteolytic enzymes, and producing reactive oxygen species (Burg and Pillinger, 2001). Neutrophils have also been implicated in modulating the adaptive immune responses. The release of cytokines from neutrophils modulates the T cell responses, such as chemotaxis and cytokine secretion (Taub *et al*., 1996). Moreover, it has been demonstrated that neutrophils can function as APCs and induce T cell proliferation in a MHC-II restricted manner (Radsak *et al*., 2000). Human peripheral blood and inflammatory neutrophils express functional B7-1-like molecules, and the expression of these molecules is upregulated by the neutrophils of patients with chronic inflammatory disease or Wegener's granulomatosis (Windhagen *et al*., 1999; Iking-Konert *et al*., 2001b, 2002). The *in vitro* generation of dendritic cell (DC)-like cells from immediate precursors of mature neutrophils (Oehler *et al*., 1998) or neutrophils from the peripheral blood using cytokines has also been reported (Yamashiro *et al*., 2000; Iking-Konert *et al*., 2001a). Neutrophils and immature DCs co-localize during pathogenic challenge (van Gisbergen *et al*., 2005). Therefore, it appears that neutrophils are capable of up-regulating molecules to present an antigen against naive T cells or can differentiate into DCs through the appropriate stimuli.

Mature neutrophils are terminally differentiated and short-lived cells, and their apoptosis is known to be an important factor in resolving inflammation (Savill, 1997). The apoptotic rate of neutrophils is dependent on the presence of pro- or anti-inflam-

matory stimuli in the surrounding milieu (Ward *et al*., 1999b). The spontaneous apoptosis of neutrophils can be enhanced by Fas stimulation (Watson *et al*., 1997, 1999). Moreover, the onset of apoptosis by neutrophils *in vitro* is associated with the down-regulation of key pro-inflammatory functions, including cell surface receptor expression (Whyte *et al*., 1993; Dransfield *et al*., 1994). Moreover, several genes encoding proteins involved in antigen presentation are up-regulated during the initial stages of neutrophil apoptosis (Kobayashi *et al*., 2003). It has been shown that MHC-II is synthesized by neutrophils after being stimulated with anti-apoptotic cytokines, such as IFN-γ or GM-CSF (Fanger *et al*., 1997; Radsak *et al*., 2000). In contrast, *Mycobacterium tuberculosis* infection of neutrophils has been shown to induce CD83 expression on the cells at 3 h after infection, the time at which early apoptosis was observed, suggesting that neutrophils differentiate into DC-like cells as they start to undergo apoptosis (Aleman *et al*., 2005). Therefore, the modulation of cell survival can affect the expression of the DC markers on neutrophils. This study investigated whether or not pro- or anti-apoptotic stimuli can modulate the expression of DC markers on neutrophils.

Materials and Methods

Reagents

Histopaque, propidium iodide (PI), LPS, pyrroline dithiocarbamate (PDTC), staphylococcal enterotoxin B (SEB), and brefeldin A were purchased from Sigma (St. Louis, MO). The Giemsa staining solution was purchased from Fluka (Bushes, Switzerland). The dextran was obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). The GM-CSF was acquired from R&D Systems Inc. (Minneapolis, MN). The RPMI-1640 medium was supplied by Gibco-BRL (Rockville, MD). The RT-PCR kit was obtained from Promega (Madison, WI). The FITC or phycoerythrin (PE)-conjugated anti-CD80, CD83, CD86, CD40, MHC-II, HLA-DR, CD16, CD32, CD3 and isotype control Abs (IgG1, IgG2a) were purchased from BD Pharmingen (Flanklin lakes, NJ). The anti-caspase-3 Ab was acquired from Santa Cruz Biotech (Santa Cruz, CA).

Cell culture

The peripheral blood neutrophils were isolated from healthy young donors using a method involving dextran sedimentation and differential centrifugation through a Ficoll-Hypaque density gradient (Park *et al*., 2002). The donors were confirmed not to have taken any anti-inflammatory drugs for at least three weeks before sampling. Informed consent was obtained from all participants and the local institutional review board at Dong-A University Hospital approved the study. The neutrophils were shown to be 98% pure by microscopy. The contaminating monocytes were depleted using anti-CD14 Ab-coated magnetic beads (Milteny Biotec Inc., Auburn, CA). The detection of $CD14^+$ cells in the separated neutrophils was $< 0.1\%$ in flow cytometry analysis using FITC-conjugated anti-CD14 Ab. The isolated neutrophils (1×10^5/100 μl) were maintained in RPMI 1640 medium supplemented with 5% FBS, 1% glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in 96-well flat bottomed plates at 37°C in a humidified atmosphere containing 5% CO_2.

Morphological assessment of neutrophil apoptosis

The neutrophils incubated in the presence or absence of anti-Fas Ab, or various agents were spun down on a glass slide using a cytospin (Shandon, Pittsburgh, PA). The cells were fixed with methanol and stained with the Giemsa staining solution. Typical apoptotic cells were readily identified by the nuclear condensation and cytoplasmic vacuoles.

Flow cytometric analysis

The level of phosphatidylserine exposure was determined by measuring the extent of annexin V-FITC binding using an apoptosis detection kit (Oncogene Research Product). The cells (1×10^6) were first harvested and washed with PBS. The cells were incubated with IgG of the same isotype at 4°C for 1 h to block nonspecific staining or Fc receptor-mediated binding of Ab. They were then labeled with FITC- or PE-conjugated Abs, incubated on ice for 30 min and washed with an isotonic PBS buffer supplemented with 0.5% BSA. The cells (1×10^4) were subsequently analyzed by flow cytometry (Beckton Dickinson, Franklin Lakes, NJ) (Park *et al*., 2005). Isotype-matched irrelevant Ab was used as a control for nonspecific staining and fluorescence parameters were gated using stained cells with FITC- or PE-conjugated isotype Ab.

RT-PCR analysis

The total RNA from $CD14^-CD66b^+$ cells (1×10^6) was isolated and lysed with TRIZOL reagents (Invitrogen). Each 50 μl PCR reaction mixture contained

1.5 U DNA polymerase, 200 μM dNTP, 50 pmol of the oligonucleotide primers. The reaction was amplified in a DNA thermal cycler for 30 cycles using the following PCR program: 95°C 1 min, 55°C 1 min, 72°C 30 s. Two specific primers were used; β-actin, ATGGATGATGATATCGCCGCG (sense), T-CTCCATGTCGTCCCAGTTG (antisense), (249 bp); CD80, TTGGATTGTCATCAGCCCTGC (sense), AT-TTTCTTCTCCTTTTGCCAGTAG (antisense) (318 bp); CD83, GCCATGTCGCGCGGCCTCCAGCTT (sense), GGACAATCTCCGCTCTGTATTTC (antisense), (440 bp); CD86, AGGACAAGGGCTTG-TATCAA (sense), ATTGCTCGTAACATCAGGGA (antisense), (330 bp); HLA-DR, CGGATCCTTCG-TGTCCCCAC (sense), CTCCCCAACCCCGTAGT-TGTGTCTGCA (antisense), (270 bp).

Western blot analysis

The cell extracts from the freshly isolated or cultured neutrophils were subjected to SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated overnight at 4°C with the primary Abs in a blocking buffer, followed by incubation with the secondary Abs for 1 h at 25°C. The signals were detected using an ECL chemiluminescence kit.

T cell proliferation assay

The T cells were harvested from the mononuclear fraction of the peripheral blood and further purified using anti-CD3 Ab-coated magnetic beads. The purity was determined by staining the cells with FITC-conjugated anti-CD3 Ab and was > 98%. The neutrophils which had been cultured for 24 h with or without LPS (100 ng/ml), GM-CSF (10 ng/ml), or anti-Fas Ab (1 μg/ml) were harvested, washed with the culture medium, and added to 1×10^5 T cells in 96-well culture plates. The mixture was cultured for 24 h at 37°C in 5% CO_2 in the presence of SEB (100 ng/ml) and pulsed for 18 h with 1 μCi [^{3}H] thymidine (NEN-DuPont, Boston, MA). The T cells were cultured without neutrophils as a reference. The cells were harvested and the levels of radioactivity incorporated were determined using a scintillation counter.

Cytokine assay

The IFN-γ levels in the culture supernatant were quantified using a ELISA kit (R&D Systems). The analyses were carried out according to the manufacturer's instructions.

Statistical analysis

The results are presented as a mean ± SD. A Student's *t*-test was used to compare the means of the unpaired samples. A P value < 0.05 was considered significant.

Results

Detection of DC markers on neutrophils in culture condition

The expression levels of the co-stimulatory molecules (CD80 and CD86), CD40, CD83, and MHC-II by the cultured neutrophils were analyzed using immunofluorescent flow cytometry. As shown in Figure 1A, the number of cells stained positively to each marker increased when the neutrophils were cultured for 24 h in a simple medium containing 5% FBS. The percentages of CD80$^+$, CD86$^+$, CD83$^+$, MHC-II$^+$, and CD40$^+$ expressing neutrophils after 24 h culture were 20 ± 4%, 62 ± 8%, 12 ± 6%, 60 ± 5%, and 6 ± 3%, respectively (Figure 1A and 1B). Time course analysis showed that the expression of each molecule on the neutrophil surface increased in a time dependent manner (Figure 1B). The percentage of CD86$^+$ cells increased significantly after 12 h. There was no further increase in the surface expression of these molecules after culturing for an additional 24 h (data not shown). Moreover, the percentage of CD83-positive cells, which might be a specific surface marker of DC maturation, was higher in the cultured neutrophils than in the freshly isolated cells. Next, double staining was performed using FITC-conjugated anti-CD66b Ab and PE-conjugated anti-CD80 Ab in order to exclude contamination by CD14$^+$ monocytes or other APCs because CD66b is a marker for the neutrophils. As shown in Figure 1C, more than 98% of the freshly isolated cells were CD66b positive, and CD66b expression level did not change in the cultured cells. More than 15% of the cultured cells tested were positive for both CD66b and CD80.

The expression level of DC markers on apoptotic neutrophils

The markers characteristic of DCs were up-regulated in the neutrophils cultured for 24 h *in vitro* but the cultured neutrophils contained a similar number of apoptotic and aged cells. Therefore, this study investigated which cells up-regulated the DC markers on their surface after culturing for 24 h. The level of cell survival was assessed by the morphological changes and flow cytometric analysis

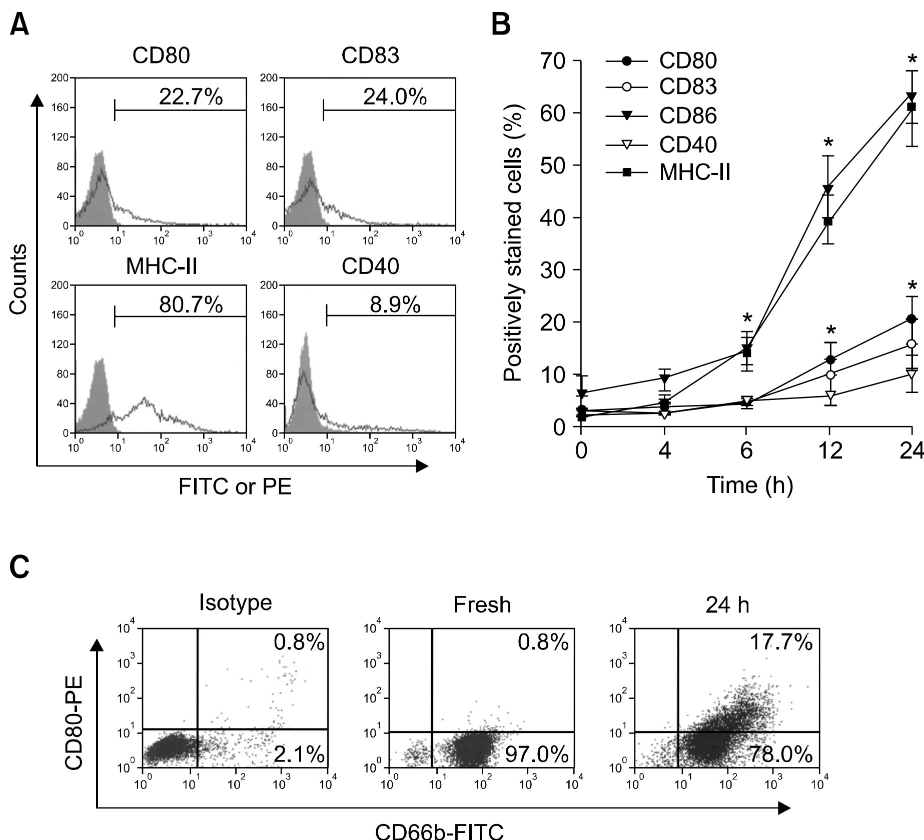

Figure 1. The expression of CD80, CD86, CD83, MHC-II, and CD40 on *in vitro* cultured neutrophils. (A) Neutrophils (1×10^6) were isolated, cultured for 24 h, harvested, and preincubated with isotype-matched control Abs. FITC-conjugated Abs were then used to detect CD80, CD83, MHC-II, and CD40, as described in "Materials and Methods". Fluorescence parameter was gated using FITC-conjugated isotype control Ab and the gray areas in the plots indicate the isotype controls. The results are representative of three independent experiments. (B) Neutrophils were incubated for the indicated times and harvested. The cells were preincubated with isotype-matched IgG for 1 h and then stained with FITC-conjugated Abs. Their expression levels were analyzed using flow cytometry. The results are shown as a mean $\pm$ SD of positively stained cells from three independent experiments. (C) Freshly isolated or 24 h-cultured neutrophils were stained with anti-CD66b Ab-FITC and anti-CD80 Ab-PE as described in "Materials and Methods". The results are representative of three independent experiments. The numbers indicate the percentage of positively stained cells. *$P < 0.05$ vs. freshly isolated cells.

of PI staining and annexin V binding. The neutrophils rapidly underwent spontaneous apoptosis in the *in vitro* culture. More than 50-60% of the cells were annexin V-positive and had a typical apoptotic cell morphology after 24 h of culture (Lee *et al*., 2004). The cells were then double stained for the PE-conjugated DC markers and annexin V-FITC. As shown in Figure 2A, the majority of the DC marker-positive cells also were positive to annexin V-FITC. Treating the neutrophils with agonistic anti-Fas Ab for 24 h significantly increased both the rate of apoptosis and the percentage of DC makers-positive cells, although the population of $CD83^+$ and annexin V^+ did not significantly increase. This suggests that apoptotic neutrophils express DC markers on their cell surface under the culture conditions examined.

The relationship between the expression level of Fcγ receptor (FcγR) and DC markers was next investigated. The CD16 (FcγRIII) expression level decreased in neutrophils cultured for 24 h (Figure 2B) (Dransfield *et al*., 1994). GM-CSF restored the CD16 expression level but Fas stimulation decreased its level. However, the CD32 (FcγRII) expression level was not changed under any of the culture conditions examined. The CD80-positive cells were mainly detected in the CD16-negative cells and CD32-positive cells, and Fas stimulation increased the population of $CD16^-CD80^+$ cells or $CD32^+CD80^-$ cells. Moreover, a 24 h treatment with GM-CSF decreased the CD80 expression level on neutrophils compared with the cells cultured with the media alone, even though GM-CSF-treated neutrophils expressed a higher level of CD80 than

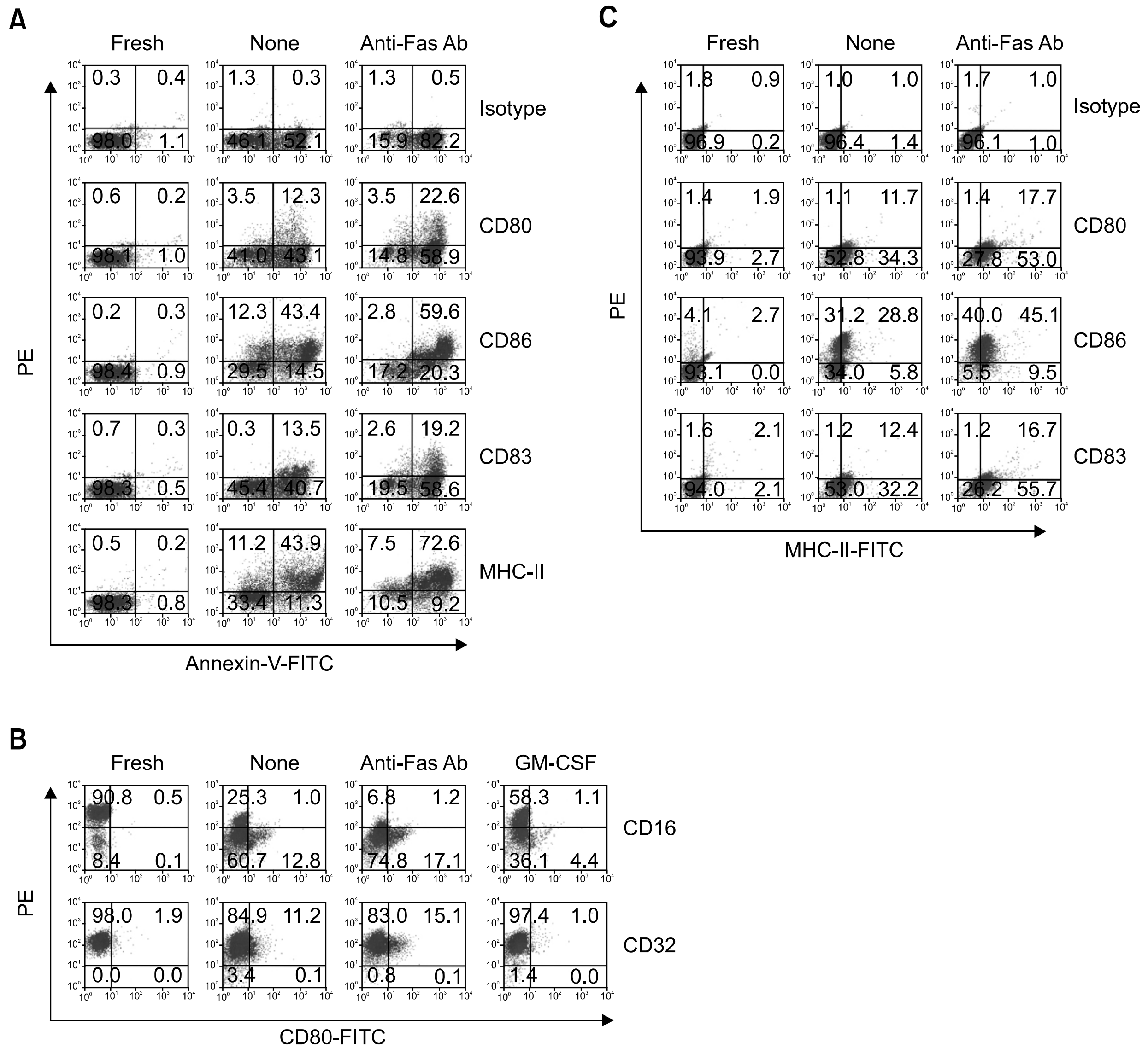

Figure 2. The expression of DC markers on apoptotic neutrophils. (A) Neutrophils were incubated with or without 1 μg/ml anti-Fas Ab for 24 h. The cells were harvested, preincubated with control IgG for 1 h, and double-stained with PE-conjugated Abs and annexin V-FITC. (B) Neutrophils were incubated with or without anti-Fas Ab or 10 ng/ml GM-CSF for 24 h. The cells were harvested, preincubated with control IgG for 1 h, and double-stained with PE-conjugated anti-CD16 or anti-CD32 Ab and FITC-conjugated anti-CD80 Ab. (C) Cells were treated as in panel A and doubly stained with FITC-conjugated anti-MHC-II Ab and PE-conjugated anti-CD80, anti-CD83, or anti-CD86 Ab. Fluorescence parameter was gated using FITC- and PE-conjugated isotype control Abs (Isotype). The results are representative of three independent experiments.

the freshly isolated neutrophils. The population of $CD16^-CD80^+$ cells was also decreased by the GM-CSF treatment. Markers of costimulatory molecules appeared in variable percentages of the apoptotic cells together with high levels of expression of MHC-II. Thus, a double immuno-staining was performed next by using FITC-conjugated anti-MHC-II Abs and PE-conjugated anti-CD80, anti-CD86, or anti-CD83 Ab to possibly identify whether there was a correlation between expression levels of MHC-II and expression levels of costimulatory molecules on apoptotic neutrophils. As shown in Figure 2C, the percentage of populations of $CD80^-MHC\text{-}II^+$ was higher than those of $CD80^+MHC\text{-}II^+$ (53.3 $\pm$ 8.5% vs 18.9 $\pm$ 4.5%, $P < 0.001$). However, CD86 expression was mainly found to be in the MHC-II^+-population. The CD83-positive cells were mainly detected in the MHC-II-positive cells (17.5 $\pm$ 2.7% vs 1.2 $\pm$ 0.5%, $P < 0.01$). These findings indicate that DC markers are differentially expressed on the apoptotic neutrophils.

mRNA and protein expression levels of DC markers in cultured neutrophils

The mRNA expression levels of the various DC markers were measured after 2, 4, 6, 12, and 24 h culture with or without GM-CSF or anti-Fas Ab (Figure 3A). The HLA-DR mRNA expression level of cultured neutrophils with media alone increased after 2 h culture and decreased thereafter. However, the presence of GM-CSF caused the HLA-DR mRNA expression level to increase for up to 24 h, whereas anti-Fas Ab failed to increase the HLA-DR mRNA level. The CD80 and CD86 mRNA levels were not dramatically changed at the early time points but were increased in the presence of GM-CSF after 24 h. CD83 mRNA was up-regulated after 2 h culture with the media alone and was decreased after prolonged incubation. The reduction of CD83 mRNA level was attenuated by GM-CSF but not by anti-Fas Ab. However, there was no correlation between the alterations in the CD80, CD86, CD83, and HLA-DR mRNA levels and the changes in cell surface expression detected by flow cytometry. Therefore, surface expression appears to be the result of translational or post-translational events.

The intracellular protein levels of DC markers in cultured neutrophils were measured by Western blotting using commercially available Abs. As shown in Figure 3B, the intracellular proteins of CD80 and CD86 in the freshly isolated neutrophils were not detected but their levels increased after culturing for 24 h. The total HLA-DR protein level in the neutrophils cultured for 24 h was similar to that in the freshly isolated neutrophils. GM-CSF increased the HLA-DR protein level in the neutrophils cultured for 24 h but did not affect the CD80 and CD86 protein levels. When the neutrophils were stimulated with anti-Fas Ab, there was a slight increase in the level of HLA-DR proteins but the CD80 and CD86 protein levels were relatively unchanged.

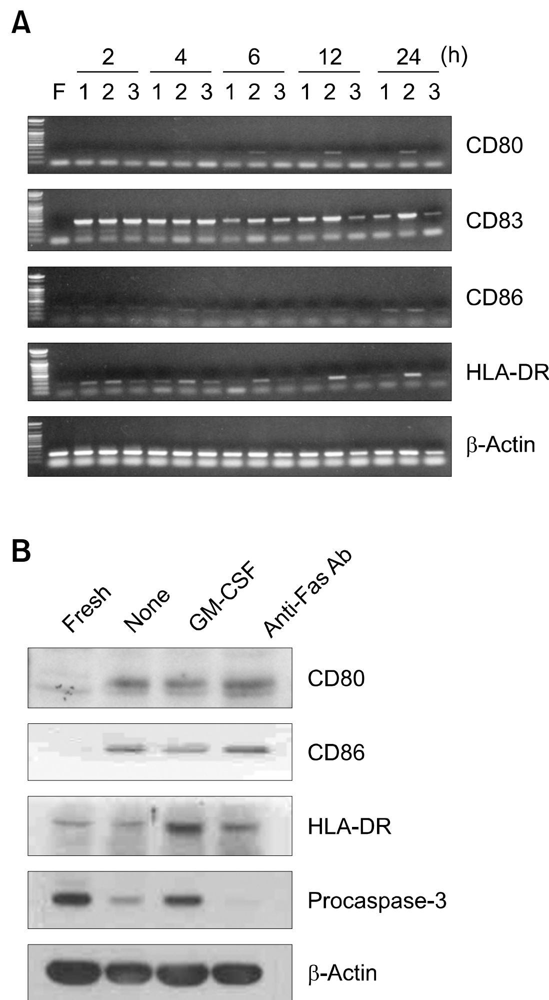

Figure 3. The expression levels of mRNA and proteins of DC markers in cultured neutrophils. (A) $CD14^{-}CD66b^{+}$ cells (1×10^6) were cultured for the indicated times with or without GM-CSF (lane 2) or anti-Fas Ab (lane 3). Lane 1 represents the neutrophils cultured with media alone. mRNA purification and RT-PCR were performed as described in “Materials and Methods”. mRNA expression of β-actin was used as a control. (B) The cell lysates were prepared and subjected to SDS-PAGE and immunoblotting using anti-CD80, CD86, HLA-DR, procaspase-3, and β-actin Abs. As a positive control, the amount of procaspase-3 proteins in the lysates of cultured neutrophils was measured. A representative of three separate experiments from three donors is shown.

Effect of pro-apoptotic compounds on the expression of DC markers

It has been shown that brefeldin A, an inhibitor of the vesicle transport event, and PDTC, an NF-κB inhibitor, induce the apoptosis of neutrophils (Ward *et al*., 1999a; Lee *et al*., 2003). These pro-apoptotic compounds increased the rate of apoptosis by up to 90% after 24 h culture (Lee *et al*., 2004). The CD80, CD83, and CD86 levels were higher in the neutrophils treated with brefeldin A or PDTC than in the neutrophils cultured with media alone (Figure 4). Moreover, CD80, CD83, and CD86 were expressed exclusively on the annexin V-positive cells. However, the MHC-II level increased slightly in the presence of pro-apoptotic compounds after culturing for 24 h compared with the media alone and most of the MHC-II-positive cells were detected in the annexin V-positive population. The neutrophils incubated with LPS, another anti-apoptotic agent for neutrophils, showed a significant delay in the rate of spontaneous apoptosis. After 24 h in the presence of LPS, only 20-30% of cells were apoptotic. The CD80-, CD83-, CD86-, or MHC-II-positive cells made up $<$ 10% of the total number of neutrophils but 50-60% of the DC marker-positive cells in the

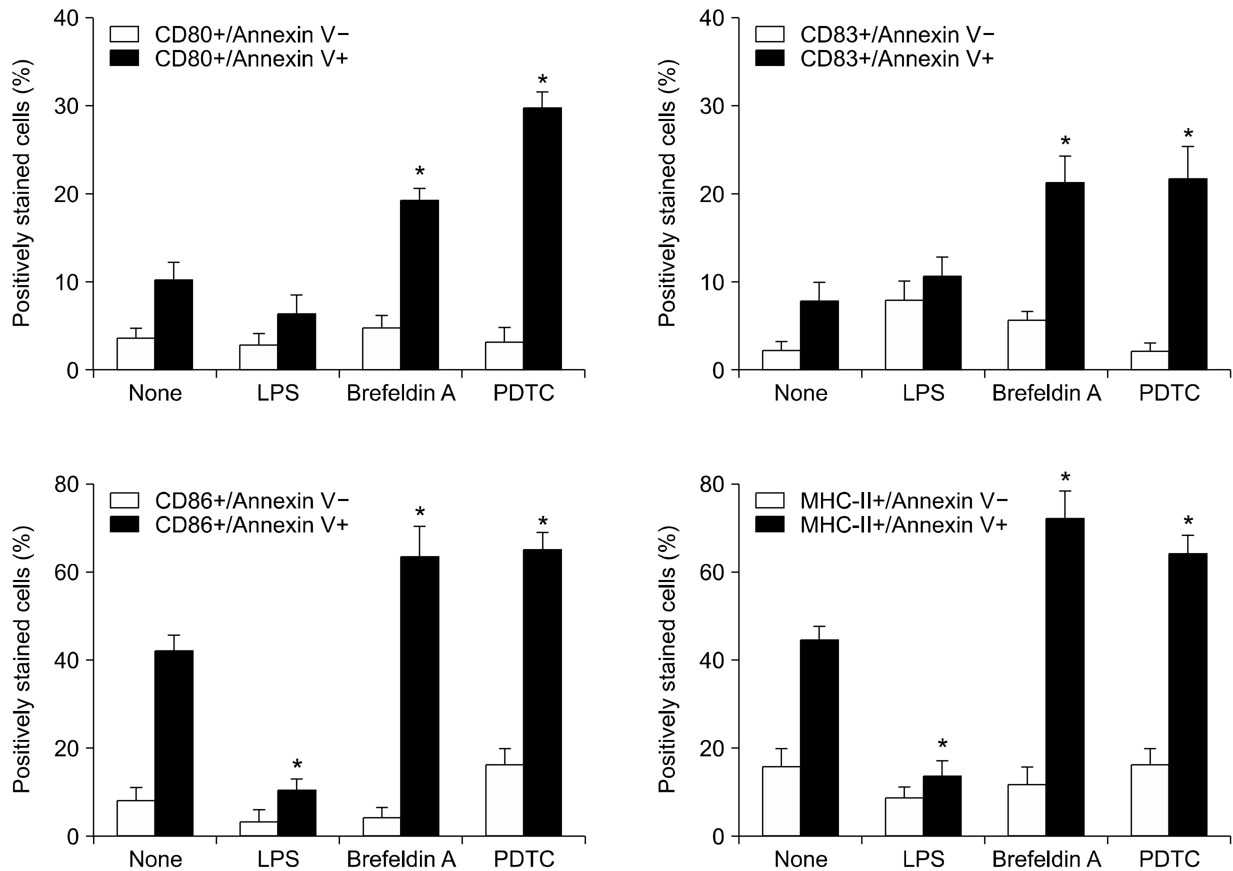

Figure 4. Increased expression of DC markers on neutrophils by pro-apoptotic compounds. Neutrophils were cultured in the presence of LPS (100 ng/ml), brefeldin A (10 μM), or PDTC (10 μM) for 24 h. The cells were harvested, preincubated with control IgG for 1 h, and stained with annexin V-FITC and PE-conjugated anti-CD80, CD83, CD86, or MHC-II Abs, as described in Figure 2. The mean ± SD of annexin V-positive or -negative cells among DC markers-stained cells is presented from three independent experiments. $^*P < 0.05$ vs. None.

LPS-treated neutrophils were annexin V-positive.

Effect of cultured neutrophils on T cell activation

The ability to stimulate T cells is one of the key functions of DCs. Therefore, this study examined the amount of IFN-γ secreted from the mixed culture of T cells and neutrophils which had been cultured with the medium alone, LPS, GM-CSF, or anti-Fas Ab. When the anti-Fas Ab or LPS-pretreated neutrophils were harvested, washed, and co-cultured with T cells for 24 h, the levels of IFN-γ secreted in the culture media containing Fas-stimulated neutrophils was lower than containing the LPS-treated neutrophils. However, the level of IFN-γ secretion under both conditions increased significantly at a 10:1 ratio of neutrophils (Figure 5A). It has been shown that MHC-II-expressing neutrophils induce superantigen-mediated T cell activation (Fanger *et al.*, 1997). When neutrophils, which had been treated with or without GM-CSF, LPS, or anti-Fas Ab, were co-cultured with the T cells for 24 h in the presence of SEB, the magnitude of the response of T cells to SEB was proportional to the number of cultured neutrophils. However, more IFN-γ secretion was observed with the GM-CSF-treated neutrophils at a ratio of approximately 1:10 than that observed with the Fas-stimulated neutrophils (Figure 5B). Next, we examined the T cell proliferation in the co-cultured condition with neutrophils for 24 h in the presence of SEB because 50-60% of the neutrophils were already apoptotic at 24 h of culture. As shown in Figure 6, SEB alone stimulated the proliferation of T cells but the addition of LPS- or anti-Fas Ab-treated neutrophils did not enhance the level of T cell proliferation at low neutrophil:T cell ratios. On the other hand, T cell proliferation was further stimulated by LPS-treated neutrophils at a neutrophil:T cell ratio of 10:1 in the presence of SEB but inhibited by anti-Fas Ab-treated neutrophils. This suggests that apoptotic neutrophils expre-

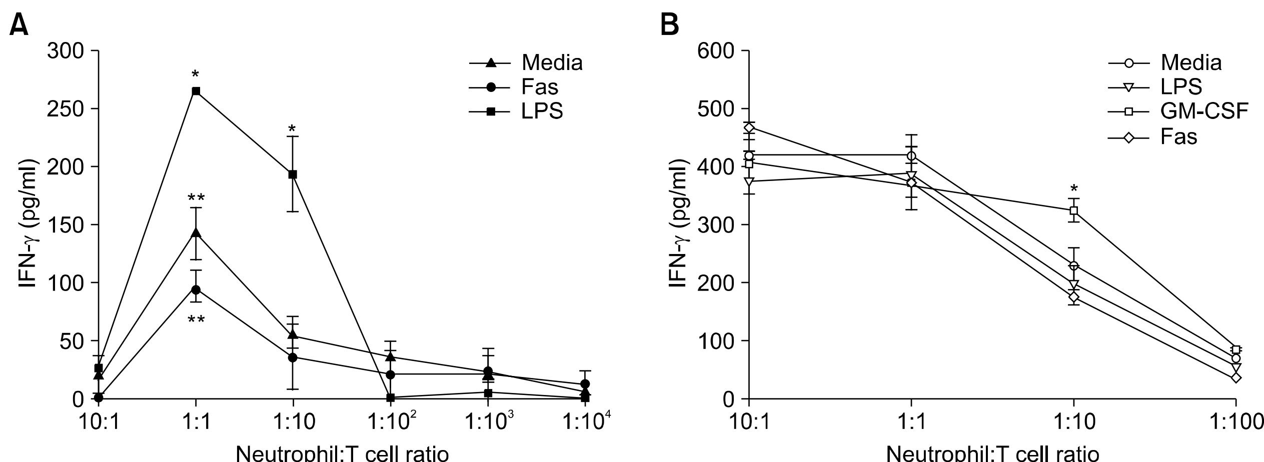

Figure 5. IFN-γ production by T cells co-cultured with anti-Fas Ab-, GM-CSF-, or LPS-treated neutrophils. $CD14^{-}CD66b^{+}$ cells (1×10^6/ml) were cultured with or without GM-CSF, LPS, or anti-Fas Ab for 24 h, harvested, and washed with medium twice. The cells were then mixed with T cells (1×10^5) in the indicated ratios and cultured for 24 h in the absence (A) or presence (B) of SEB (100 ng/ml). The culture media were withdrawn and the IFN-γ concentrations were measured by ELISA. The results are presented as the mean ± SD of a triplicate assay from two separate cultures. *$P < 0.001$ vs. anti-Fas Ab-treated neutrophils, **$P < 0.05$ vs. T cell alone.

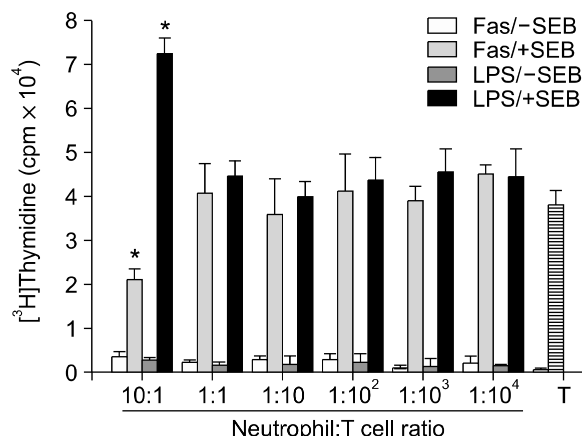

Figure 6. T cell proliferation in co-cultured condition with anti-Fas Ab- or LPS-treated neutrophils. $CD14^{-}CD66b^{+}$ cells were separated and treated as shown in Figure 5. The cells were then harvested and washed twice with the media. These cells were mixed with the indicated ratio of T cells for 24 h in the presence of SEB (100 ng/ml). The mixed cells were further cultured for 18 h after adding 1 μCi [^{3}H]thymidine. T cells stimulated with SEB were used as the positive control, and T cells cultured alone were used as the negative control. The incorporation of [^{3}H]thymidine was determined using a scintillation counter. The results are presented as a mean ± SD of triplicate cultures. *$P < 0.05$ vs. T cell alone.

ssing DC markers on their surfaces have no stimulatory activity on T cells.

Discussion

Recent experimental data suggests that neutrophils have the potential to trans-differentiate into DCs (Oehler *et al*., 1998; Yamashiro *et al*., 2000; Iking-Konert *et al*., 2001a). Therefore, neutrophils appear to have different phenotypic characteristics depending on a variety of factors, including the survival duration. This study showed that the number of neutrophils expressing MHC-II, co-stimulatory molecules, and CD83 was increased by culturing neutrophils for 24 h without any cytokines. This suggests that these neutrophils acquired the DC phenotype. Almost all annexin V-positive cells expressed MHC-II, whereas only a portion of annexin V-positive cells expressed other DC markers. Thus, the expression of all DC markers was not regulated in a similar fashion. It is unclear whether cells expressing DC markers go into apoptosis or cells that become apoptotic express DC markers. It has been shown that the increased apoptotic signals delivered by recombinant FasL induce CD80 expression (de Carvalho Bittencourt *et al*., 2002) and that the sodium butyrate-induced CD86 expression is related to the apoptosis of HL-60 cells (Suzuki *et al*., 2003). Another group has also reported that monocytes differentiate into DCs without the addition of exogenous cytokines in an *in vitro* model of trans-endothelial migration (Randolph *et al*., 1998). The possibility that the increased detection of DC markers on the surface of neutrophils may be due to contaminating monocytes and lymphocytes can be ruled out for the following reasons: (1) the freshly isolated neutrophils tested were negative for the CD14 marker, (2) the proportion of DC maker-positive cells was too high to be derived from monocytes or lymphocytes after purification, (3) all the DC markers were

detected on the $CD14^-$ and $CD66b^+$ cells. Furthermore, the possibility of the nonspecific binding of Abs to the cell surfaces can be also ruled out by the differential expression of the DC markers on the apoptotic or aged cells. In addition, our data indicate that the CD16 expression level decreased in neutrophils cultured for 24 h and the CD80-positive cells were mainly detected in the CD16-negative cells. Moreover, the apparent DC marker detection could be the consequence of the poor fluorescence compensation or, alternatively, of an analysis that included cell debris or necrotic cells which are known to non-specifically bind antibodies. In this regard, we included the related plot showing isotype controls after fluorescence compensation.

The CD80 and CD86 proteins were not expressed in the freshly isolated neutrophils but their intracellular protein levels were higher in the cultured neutrophils for 24 h. The CD83 and HLA-DR mRNA expression levels were increased in the neutrophils after 2 h culture with media alone. The GM-CSF treatment increased the mRNA expression level of the DC markers, including CD80 and CD86. However, there was increased expression of the surface DC markers on the neutrophils cultured with anti-Fas Ab but their mRNA expression levels were not changed by anti-Fas Ab. Other group has also shown that HLA-DR surface expression on neutrophils is induced by the cell surface interactions of neutrophils with the cell lines of the lymphoid phenotype and is not altered by the cycloheximide treatment (Vella *et al*., 2002). This indicates that the phenomenon is independent of the *de novo* gene activation and protein synthesis (Vella *et al*., 2002). NF-κB might be the main transcriptional activator involved in inducing CD80 and CD86 gene expression (Zhao *et al*., 1996; Yoshimura *et al*., 2001), and it is also known to be an anti-apoptotic activator (Ward *et al*., 1999a). However, NF-κB inhibitor did not attenuate the expression of CD80 and CD86, rather their expression level on the apoptotic neutrophils was increased. Overall, the surface expression of CD80, CD83, CD86, and MHC-II might occur via the translocation of the molecules from the cytosolic fraction or post-transcriptional events. It is possible that the surface DC markers on the apoptotic neutrophils may be transient but not inducible like anionic phosphatidylserine, which is relocated to the outer surface of the plasma membrane during apoptosis.

It has been suggested that the temporally regulated expression of the co-stimulatory molecule might initiate T cell-dependent immunity (Sethna *et al*., 1994). Among the many co-stimulatory signals, members of the B7 family on the APCs interact with CD28 and the cytotoxic T lymphocyte antigen-4 on T cells. CD80 and CD86 are involved in the Th1 and Th2 responses, respectively (Kuchroo *et al*., 1995). It was reported that neutrophils promote the Th1 responses in experimental models of infection with *Legionella pneumophillia* or *Toxoplasma gondii* (Bliss *et al*., 2000; Tateda *et al*., 2001). However, it is still unclear if T cells can interact with neutrophils that express MHC and co-stimulatory molecules. Mencacci *et al*. (2002) reported that Gr-1^+ neutrophils inhibit the activation of IFN-γ-producing $CD4^+$ T cells and induce apoptosis through a CD80/CD28-dependent mechanism. They suggested that alternatively activated neutrophils expressing CD80 might adversely affect the Th1-dependent resistance in fungal infections. This study showed that CD80 and CD86 were also detected on the cultured neutrophils and further elevated in the apoptotic cell population. This suggests that apoptotic neutrophils might have more ability to interact with T cell receptors than aged cells. However, there was no correlation between the production of cytokines or T cell activation and the expression level of DC markers on the cultured neutrophils. On the contrary, LPS-treated neutrophils expressed a lower level of MHC-II but induced higher T cell activation and proliferation as compared with apoptotic neutrophils. Apoptotic cells become positive for PI staining in the later phase and such late apoptotic cells are presumed to exhibit distinct biological functions compared with early apoptotic cells, annexin V-positive but PI-negative cells. The immature DCs express little or no co-stimulatory molecules on their surface. Once activated, the DCs undergo a process of maturation, which is reflected by the increased expression of MHC, co-stimulatory molecules, and CD83 (Kim *et al*., 2006). Moreover, the capacity of antigen uptake and processing is lost in matured DCs but the ability of antigen presentation is increased. Apoptotic neutrophils express co-stimulatory molecules and CD83, which indicates that these cells are similar to the mature DC phenotype. However, it was reported that neutrophil functions such as phagocytic activity are down-regulated in apoptotic cells (Whyte *et al*., 1993). We can speculate that the absence of costimulatory function in apoptotic neutrophils may be due to the variable expression of DC markers on these cells and the phenotypically different subset of apoptotic neutrophils display different cytokine profile for T cell activation. Thus, it seems that apoptotic neutrophils expressing DC markers do not satisfy all criteria sufficiently to be regarded as APCs. Moreover, apoptotic neutrophils showing

immature DC characteristics might function to suppress immune reactions because repetitive stimulation of naïve T cells with immature DCs induces tolerance. More study will be needed to determine whether or not apoptotic neutrophils affect the T cell functions if phagocytosis of apoptotic neutrophils does not occur.

Apoptosis is a prerequisite for the clearance of neutrophils by macrophages and for resolving inflammation (Savill, 1997). The innate immunity may be terminated after the phagocytosis of apoptotic neutrophils but apoptotic neutrophils might also contribute the development of adaptive immunity because bacteria and bacterial products in the inflammatory loci may be cross-presented by macrophages or DCs phagocytosing apoptotic neutrophils (van Gisbergen *et al*., 2005). It was reported that the uptake of apoptotic neutrophils by DCs leads to the enhanced expression of CD83 and MHC-II on the DCs but a reduced capacity of DCs to stimulate mixed leukocyte reaction (Clayton *et al*., 2003). Positive staining of CD83 by an immunohistochemical assay in the inflammatory sites might involve both aged and apoptotic neutrophils (Iking-Konert *et al*., 2002). There was no evidence that neutrophils differentiate into DC-like cells in inflammation tissue until now. However, it is possible that apoptotic neutrophils in inflammatory loci may stain positively for the DC markers. Recently, it was reported that increased apoptosis accompanies the expression of DC markers in neutrophils in a pleural effusion from *Mycobacterium tuberculosis* patients (Aleman *et al*., 2005). Thus, it is possible that neutrophil apoptosis can affect the development of adaptive immunity by influencing the expression of various antigen-presenting molecules on their surface.

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