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Human endothelial cells: effect of TNF-α on peripheral blood mononuclear cell adhesion

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SUMMARY

Human umbilical vein endothelial cells (HUVEC) were cultured and treated for varying periods with a range of concentrations of tumour necrosis factor-alpha (TNF-α). After this treatment the proportion of peripheral blood mononuclear cells (PBMC), previously depleted of plastic adherent cells, capable of binding to the endothelial cells was assessed. Few PBMC bound to HUVEC which had not been pretreated with TNF-α but up to 36% bound after pretreatment of the endothelial cells with TNF-α for 10 hr at a concentration of 10 U/ml. Phenotypic characterization of the adherent and non-adherent PBMC subpopulations revealed that natural killer (NK) cells ($CD16^+$) and a proportion of memory helper T cells ($CD4^+$ $CD45RA^-$) bound to TNF-α pretreated HUVEC but that few naive helper T cells ($CD4^+$ $CD45RA^+$) showed similar binding. Cytotoxicity assays for NK activity were used to analyse functionally the adherent and non-adherent PBMC subpopulations. It was found that the cell subpopulation which did not adhere to TNF-α pretreated HUVEC mediated little lysis of K562 target cells. Conversely, the endothelial cell-adherent PBMC subpopulation produced active lysis supporting the phenotypic evidence that NK cells were concentrated within this subpopulation. These results suggest that TNF-α has a rapid and profound up-regulatory effect on the expression of adhesion molecules on the surface of HUVEC. Furthermore, it is apparent that these up-regulated adhesion molecules preferentially bind NK cells and a subset of memory helper T cells from the PBMC population.

INTRODUCTION

The adhesion of leucocytes to vascular endothelium is mediated by interactions between a variety of membrane-associated adhesion molecules.[1] Various cytokines, including tumour necrosis factor-alpha (TNF-α),[2-4] interleukin-1 (IL-1),[4-6] IL-4[7,8] and interferon-gamma (IFN-γ),[8,9] are known to up-regulate the expression of adhesion molecules on cultured vascular endothelial cells and thereby to increase the binding of leucocytes. It has also been reported that injection of TNF-α and IFN-γ up-regulates the expression of adhesion molecules, enhances leucocyte binding within local venules and stimulates cellular extravasation and accumulation at the injection site.[10] These *in vitro* and *in vivo* data suggest that cytokines produced by activated inflammatory cells play an important role in initiating tissue infiltration by leucocytes.

In order to understand how cytokines control the inflammatory process it is necessary to study the specificity of leucocyte adhesion after treatment of endothelial cells with purified cytokine preparations. For example, it has been reported that both TNF-α and IL-1 increase the adhesion of T lymphocytes and polymorphonuclear leucocytes (PMN) to endothelium, whereas IL-4 and IFN-γ increase only the binding of T lymphocytes.[7-11] It has also been reported that memory T lymphocytes bind more avidly than naive T lymphocytes to activated endothelial cells;[12,13] this finding may correlate with up-regulated expression of adhesion molecules on the surface of T cells which have been previously activated.[14]

In the experiments described in this report we examine the effects of TNF-α on cultured human umbilical vein endothelial cells (HUVEC) and investigate the binding of monocyte-depleted peripheral blood mononuclear cell subpopulations both to untreated and to TNF-α-treated endothelial cells. The adherent and non-adherent cell subpopulations were analysed both by phenotypic characterization and by assay of NK-cell function.

Abbreviations: HUVEC, human umbilical vein endothelial cells; ICAM, intercellular adhesion molecule; LFA, lymphocyte function-associated antigen; VCAM, vascular cell adhesion molecule; VLA, very late activation antigen.

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MATERIALS AND METHODS

Preparation and culture of HUVEC

Human umbilical cords were collected immediately after parturition and endothelial cells were prepared and cultured by use of a

modification of the method described by Jaffe *et al.*[15] Briefly, the umbilical vein was cannulated and the vessel was perfused with heparinized Medium 199 (Northumbria Biologicals, Cramlington, Northumberland, U.K.). The distal end of the cord was cross-clamped, the vein was filled with 0·25% (w/v) collagenase (Sigma, Poole, Dorset, U.K.) in Medium 199 and the proximal end was clamped. After incubation at room temperature for 30 min the clamps were removed and the contents of the vein were recovered by perfusion with Medium 199 supplemented with 10% (v/v) foetal calf serum. The cells were collected by centrifugation at 400 ***g*** for 10 min and the pellet was suspended in 10 ml of endothelial cell (EC) medium composed of Medium 199 supplemented with 20% foetal calf serum (Northumbria Biologicals), endothelial cell growth factor (25 μg/ml; Sigma), heparin (100 μg/ml; Sigma) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin, Northumbria Biologicals). The cells were cultured at 37° in an atmosphere of 5% CO_2 in 25 cm^2 culture flasks (Falcon; Becton-Dickinson, Cowley, Oxfordshire, U.K.) which had previously been treated with phosphate-buffered saline (PBS) containing 1% (w/v) gelatin (Sigma). For subculture the adherent cells were harvested using a solution of 0·05% Trypsin and 0·02% EDTA in PBS (Northumbria Biologicals). In some cases the recovered HUVEC were suspended in EC medium containing 10% DMSO prior to freezing and storage in liquid nitrogen. A small aliquot of cells propagated from each umbilical cord was characterized by culture on plastic microscope slides before ethanol fixation and immunofluoresence staining with FITC-conjugated anti-factor VIII antibody (ICN Flow, High Wycombe, Bucks, U.K.). HUVEC were used between the 3rd and the 7th passage for all the experiments described in this report.

Human peripheral blood mononuclear cells (PBMC)

PBMC were isolated from healthy donors by Ficoll–metrizoate (Lymphoprep; Nycomed, Birmingham, W. Midlands, U.K.) density gradient centrifugation[16] of 10 ml of heparinized blood. The PBMC were then incubated in PBMC medium composed of RPMI-1640 (Northumbria Biologicals) supplemented with 10% FCS, antibiotics and 10 mM HEPES buffer (Northumbria Biologicals) in horizontal 25-cm^2 tissue culture flasks at 37° in 5% CO_2. After 1 hr the cells which were non-adherent to plastic were collected; these cells were used throughout this study and are referred to as PBMC.

^{51}Cr-labelling of cells

PBMC or K562 cells (human erythroleukaemia line; ECACC, Porton Down, Sussex, U.K.) were suspended in 0·2 ml of PBMC medium and 200 μCi of $Na_2{}^{51}CrO_4$ (Amersham, Amersham Bucks, U.K.) were added. After incubation for 90 min the labelled cells were washed four times by centrifugation in PBMC medium.

PBMC-HUVEC adhesion assay

The PBMC-HUVEC adhesion assay was performed in accordance with the procedure described by Cavender *et al.*[5] Briefly, HUVEC monolayers were propagated on gelatin pretreated flat-bottomed 96-well plates (Falcon) and incubated for varying periods with EC culture medium supplemented with titres of TNF-α (Asahi Chemical Industry Co. Ltd, Tokyo, Japan; 2×10^6 U/mg protein determined by cytotoxicity assay on murine LM cells in the absence of actinomycin-D) varying between 0 and 10^6U/ml. After this treatment the HUVEC monolayers were washed three times with warm PBMC medium, 2×10^5 Cr-labelled PBMC were added in 0·2 ml of PBMC culture medium and the plates were incubated for 60 min at 37°. The wells were then washed gently three times with warm PBMC medium to remove non-adherent PBMC and the remaining adherent PBMC were lysed by addition of 0·2 ml of 1% (v/v) Triton X-100 (Sigma). The amount of released ^{51}Cr present in the lysate was measured by gamma-spectrometry (LKB-Wallac Clinnigamma 1272; Pharmacia-LKB, Milton Keynes, Beds, U.K.). The percentage of adherent PBMC was calculated by application of the following formula:

$$\% \text{ adhesion} = \frac{\text{c.p.m. in 0·2 ml of lysate}}{\text{c.p.m. in 0·2 ml of original cell suspension}} \times 100.$$

Separation of adherent and non-adherent PBMC

HUVEC monolayers were cultured on gelatin pretreated 6-well plates (Falcon) for 4 hr in EC medium either with or without 10 U/ml of TNF-α and were washed three times with warm PBMC medium. 3×10^6 PBMC in 2 ml of PBMC-medium were then added both to wells containing HUVEC cells and to wells without HUVEC (control PBMC) and the plates were incubated for 1 hr at 37°. Non-adherent PBMC were collected from both the HUVEC-containing and the control wells by aspiration and the remaining adherent PBMC and HUVEC were washed and treated with 0·2% EDTA-PBS for 30 min at 4° before aspiration. Before NK cell assay (below) the adherent PBMC were separated from HUVEC by flow cytometric cell sorting on the basis of size (forward light scatter) and granularity (side scatter) using a FACS 420 cell sorter (Becton Dickinson). Control experiments demonstrated that the cell-sorting procedure did not modify the cytolytic potential of NK cells within PBMC preparations.

Immunofluorescence staining and analysis of PBMC

Each of the PBMC samples was divided into aliquots which were washed by centrifugation with PBS containing 0·5% BSA (Sigma) and 0·1% NaN_3 and the resulting cell pellets were stained by addition of the antibodies listed in Table 1. Each staining step was performed on ice for 20 min and was followed by three washes by centrifugation in PBS–BSA–NaN_3. No

Table 1. Monoclonal antibodies used for phenotypic analysis

CD No.	Specificity	Conjugate	Source
CD3	T cells	FITC	DAKO*
CD4	Helper/inducer T cells	PE	B.D.†
CD8	Suppressor/cytotoxic T cells	FITC	DAKO
CD11a	LFA-1α; ICAM-1 receptor	—	DAKO
CD16	Fc Receptor on NK cells	PE	B.D.
CD18	β chain to CD11a, b. c	—	DAKO
CD29	VLA β chain	—	Coulter‡
CD45RA	Naive T cells	FITC	B.D.
CDw49d	VLA4α; VCAM-1 receptor	—	Immunotech§

* Dakopatts, High Wycombe, Bucks, U.K.
† Becton-Dickinson, Cowley, Oxon, U.K.
‡ Coulter Immunology, Luton, Beds, U.K.
§ Immunotech, Marseille, France.

further staining steps were necessary after labelling with fluorochrome-conjugated primary antibodies. However, in the case of non-conjugated primary antibodies the cells were additionally incubated with PE-conjugated sheep anti-mouse Ig (Sigma). Flow cytometric acquisition and analysis of between 10,000 and 20,000 cells was carried out using a FACScan flow cytometer and FACScan software (Becton-Dickinson). Lymphocytes were gated on the basis of their characteristic volume (forward-angle light scatter) and granularity (side-scatter); in all cases large, granular HUVEC, which had become detached from the monolayer, were excluded by this gating.

NK assay

^{51}Cr-labelled K562 cells (target cells) were added at 2×10^3 cells/well to the wells of round-bottomed 96-well plates in a volume of 0·1 ml of PBMC medium. These targets were then mixed with 0·1 ml of either adherent, non-adherent or control PBMC (effector cells) in effector : target cell ratios of 100 : 1, 50 : 1, 25 : 1, 12·5 : 1, 6·25 : 1 and 3·13 : 1. These cell mixtures were incubated for 4 hr at 37°, the plates were centrifuged and 0·1 ml of supernatant from each well was collected for gamma-counting. Spontaneous release was determined by incubating target cells without effector cells. Maximum release was measured after the lysis of target cells by incubation with 1·0% (v/v) Triton X-100. Cytotoxicity was calculated using the following formula:[17]

$$\% \text{ specific lysis} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100.$$

RESULTS

Primary HUVEC cultures formed a 'cobblestone' monolayer that was routinely assessed for purity by immunofluorescence staining; in all cases more than 99% of the cells examined showed positive staining of Factor VIII. After the establishment of initial confluency the cells were split in the ratio of 1 : 2 on every third or fourth day. Before performing cell-binding assays PBMC preparations were routinely depleted of plastic-adherent mononuclear cells. This treatment reduced the proportion of $CD14^+$ (Leu-M3; Becton-Dickinson) monocytes in the cell preparations to less than 3%.

The results presented in Fig. 1a show how increasing the concentration of TNF-α used for a 4 hr preincubation of HUVEC increased the proportion of PBMC which binds to the endothelial cell monolayer. The TNF-α titration was performed in the range between 1×10^{-3} and 1×10^6 U/ml and it was found that PBMC binding increased from a background 5% to a value of 25% within a TNF-α concentration range of 0·01–1 U/ml but that binding then remained relatively constant; a TNF-α concentration of 10 U/ml was used in all subsequent experiments.

The effect on PBMC binding of increasing the period of preincubation of HUVEC with 10 U/ml of TNF-α from 0 to 72 hr is shown in Fig. 1b. These results show that TNF-α rapidly enhanced the ability of HUVEC to bind PBMC, with significant binding occurring after treatment for 30 min and half maximal binding occurring within 2 hr. Maximal binding occurred after treatment for 10 hr when 36% of the PBMC population were adherent to the HUVEC; after treatment for periods in excess of this time the proportion of bound PBMC decreased. A preincubation period of 4 hr was used in all subsequent assays.

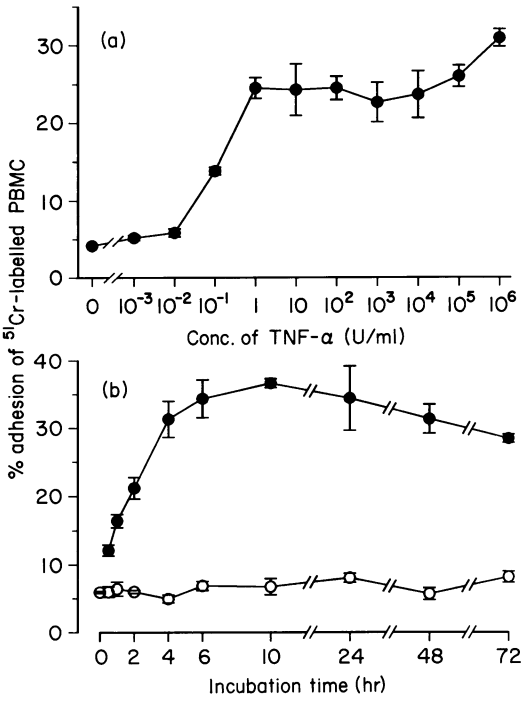

Figure 1. Effect on PBMC adhesion of pretreating HUVEC with TNF-α. (a) Relationship between dose of TNF-α used to pretreat HUVEC for 4 hr and the proportion of PBMC subsequently bound in a 1 hr assay. The error bars represent standard deviation on triplicate samples. (b) Relationship between time of incubation of HUVEC either with TNF-α at 10 U/ml (●) or with medium (○) and subsequent binding of PBMC in a 1 hr asay. The error bars represent standard deviation of triplicate samples.

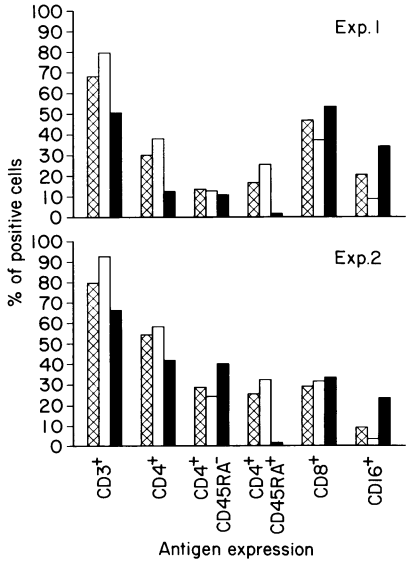

Figure 2. Results of two representative experiments in which the phenotype of PBMC subpopulations was examined. The subpopulations investigated were: PBMC (▩), PBMC non-adherent to TNF-α-treated HUVEC (□) and PBMC adherent to TNF-α treated HUVEC (■).

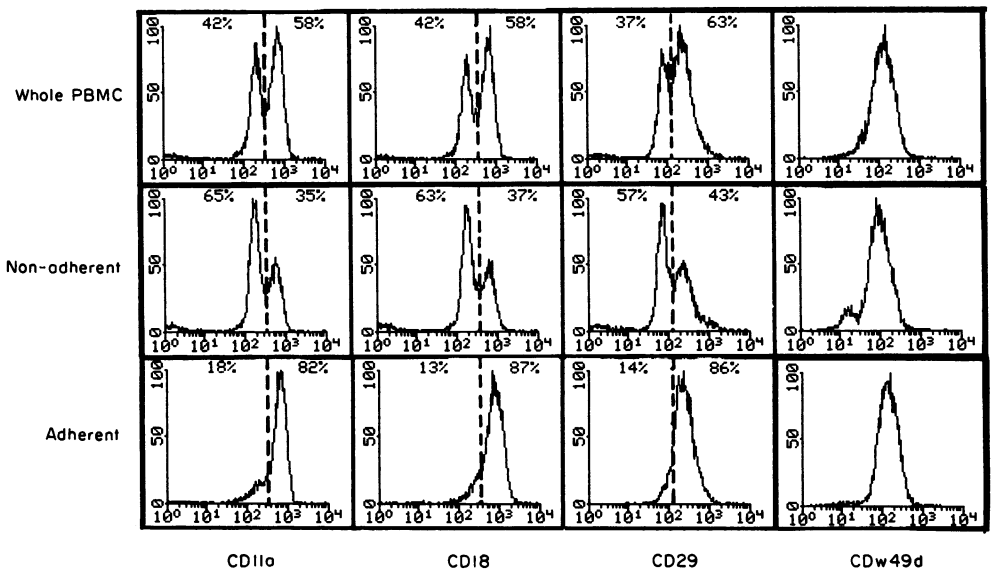

Figure 3. Flow cytometric fluorescence profiles of PBMC and PBMC divided into TNF-α pretreated HUVEC adherent and non-adherent subpopulations after labelling with antibodies specific for CD11a, CD18, CD29 and CDw249d and appropriate counterstaining. The data were analysed using LYSYS software (Becton-Dickinson).

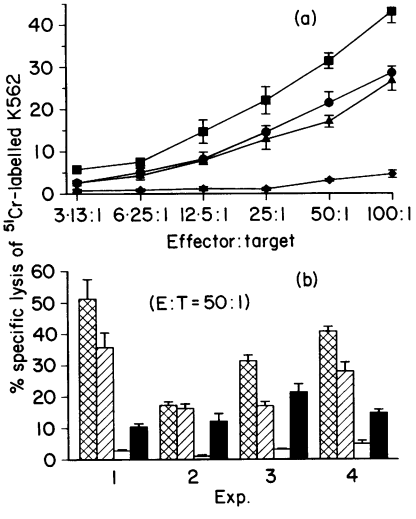

Figure 4. Cytotoxicity of PBMC and PBMC subpopulations separated by adherence to HUVEC for K562 cells. (a) Cytotoxicity of four PBMC subpopulations at varying effector to target cell ratios. The effector cells used were: PBMC (■), PBMC adherent to TNF-α-treated HUVEC (●), PBMC non-adherent to TNF-α-treated HUVEC (◆) and PBMC non-adherent to untreated HUVEC (▲). The error bars represent standard deviation of triplicate samples. (b) Four representative cytotoxicity experiments performed at a constant effector to target cell ratio of 50:1. The PBMC subpopulations were: PBMC (▩), PBMC non-adherent to untreated HUVEC (▨), PBMC non-adherent to TNF-α treated HUVEC (□) and PBMC adherent to TNF-α treated HUVEC (■). The adherent PBMC were separated from HUVEC by cell sorting in experiments 2, 3 and 4 but not in experiment 1. The error bars represent standard deviation of triplicate samples.

Figure 2 shows the results of two representative phenotypic analyses of the mononuclear cell subpopulations which were either adherent or non-adherent to TNF-α pretreated HUVEC. It was found that NK cells ($CD16^+$) were concentrated in the adherent subpopulation, indicating avid binding of these cells; NK cells showed little binding to untreated HUVEC (data not shown). Naive $CD4^+$ T cells ($CD45RA^+$)[14,18] were almost entirely contained in the non-adherent cell population, whereas memory $CD4^+$ T cells ($CD45RA^-$) showed a variable distribution between the non-adherent and adherent groups, indicating that amongst $CD4^+$ T cells only a portion of the memory subpopulation bind to TNF-α pretreated HUVEC. Representative fluorescence histograms recorded after immunofluorescence staining for the CD11a/CD18 (LFA-1) and CDw49d/CD29 (VLA-4) adhesion molecules on PBMC are shown in Fig. 3. The histograms for CD11a, CD18 and CD29 showed a bimodal distribution in the level of expression of these markers. The ratio between the number of cells expressing high and low levels of these molecules was approximately 60:40 for the initial PBMC population. However, this ratio was reversed to approximately 40:60 in cells which were non-adherent to TNF-α pretreated HUVEC, whilst more than 80% of HUVEC adherent cells expressed high levels of these adhesion molecules. The level of expression of the CDw49d antigen was similar in each of the PBMC subpopulations.

The results of a representative NK-cell mediated cytotoxicity experiment are shown in Fig. 4a. In this experiment the relative abilities of various PBMC subpopulations to mediate lysis of K562 targets were compared at a range of effector to target cell ratios. The four subpopulations used were PBMC, PBMC which had been separated into adherent and non-adherent subpopulations on the basis of binding to HUVEC which had been pre-treated with TNF-α and cells which were not adherent to untreated HUVEC. Results obtained from four representative cytotoxicity experiments conducted using a fixed effector to target cell ratio of 50:1 are shown in Fig. 4b; in the

first of these experiments detached HUVEC were not separated from adherent PBMC by flow cytometric sorting before the NK assay. In each set of data the cytolysis mediated by PBMC which were non-adherent to TNF-α pretreated HUVEC was significantly less than the lysis mediated by the adherent cell subpopulation. The presence of contaminating HUVEC in the adherent PBMC effector population used in the first experiment may account for the low lytic potential of these cells relative to that of PBMC.

DISCUSSION

It has been reported that certain cytokines enhance the adhesive interactions which occur between endothelial cells and leucocytes; this binding is thought to be the first step in leucocyte extravasation. Both TNF-α and IL-1 enhance the binding of T cells[2,5] and PMN[3,6] with different kinetics; binding of PMN reaches a maximum earlier and decreases more rapidly than does T-cell binding.[11] This difference correlates with the different kinetics for up-regulation of endothelial cell-associated adhesion molecules such as ELAM-1,[4,19] which is specific for PMN and both ICAM-1[4,19] and VCAM-1,[20,21] which have specificity for T cells. It is also known that IFN-γ and IL-4 induce T-cell binding but not PMN binding to endothelial cells;[7,11] IFN-γ and IL-4 do not upregulate ELAM-1 expression and have an antagonistic effect on ICAM-1 expression with IFN-γ up-regulating and IL-4 down-regulating expression.[8] It has also been reported that amongst the T-cell subpopulations the memory cells bind the most avidly to TNF-α or IL-1-stimulated endothelial cells.[12,13] This finding correlates with the up-regulation of adhesion molecules, including CD2, LFA-1, LFA-3 (receptor for CD2) and CD29 (Table 1) on the surface of memory T cells.[14]

The ability of various cytokines to induce different adhesion molecules on endothelial cells with varying up-regulation kinetics may be of profound importance in the initiation and development of inflammatory reactions. Different leucocyte subpopulations show both qualitative and quantitative differences in adhesion molecule expression. Consequently the composition of the leucocyte population bound to vascular endothelial cells is likely to be highly variable and dependent on the presence of cytokines and hence on the local immune status.

In this study it has been demonstrated that pretreatment of HUVEC with TNF-α enhanced the adhesion of PBMC to HUVEC in a dose and time-dependent manner (Fig. 1a, b). In order to investigate the relative binding of different PBMC subpopulations to TNF-α pre-treated and untreated endothelial cells, PBMC were separated into HUVEC adherent and non-adherent subpopulations which were each examined by phenotypic analysis using a panel of monoclonal antibodies (Table 1). It was found that NK cells ($CD16^+$) were concentrated in the cell subpopulation which bound avidly to TNF-α pretreated HUVEC (Fig. 2) but that few of these cells bound to untreated HUVEC. This suggests that TNF-α up-regulates expression of adhesion molecules on the surface of HUVEC which are capable of binding NK cells. Amongst the $CD4^+$ T cells a variable proportion of $CD45RA^-$ memory cells bound to TNF-α pretreated HUVEC. Naive $CD4^+$ T cells expressing CD45RA showed little affinity for any of the HUVEC preparations.

It has been reported that memory $CD4^+$ T cells express higher levels of the LFA-1 and CD29 adhesion molecules than are expressed by naive $CD4^+$ T cells.[14,22] The results in Fig. 3 show that the subpopulation of PBMC which is adherent to TNF-α pretreated HUVEC also expreses high levels of these antigens. Hence the differential binding observed for memory and naive $CD4^+$ T cells may be explained by the former group of cells expressing higher levels of HUVEC binding adhesion molecules than the latter group of cells.

As may be predicted from the phenotypic data (Fig. 2), the cytolytic activity due to NK cells in the PBMC subpopulation which was non-adherent to TNF-α pretreated HUVEC was low (Fig. 4a, b). Intriguingly, NK activity in the PBMC subpopulation which bound to TNF-α pretreated HUVEC was not as great as may be expected from the high percentage of $CD16^+$ cells present in this subpopulation; indeed the lytic capacity of these cells never exceeded that of the initial PBMC preparation. The reason underlying this reduced lysis is not clear.

TNF-α is known to enhance NK-cell cytolytic activity *in vitro*.[23,24] However, Kist *et al.*[25] reported that a 24-hr intravenous infusion of TNF-α caused a large decrease in the percentage of circulating CD16-positive cells with a concomitant decrease in the ability of PBMC to lyse K562 target cells. The results presented in this report suggest that this inconsistency may reflect the ability of TNF-α to stimulate the binding of NK cells to endothelial cells, thereby removing NK cells from the circulation.

In conclusion, it has been demonstrated that NK cells and some $CD4^+$ memory T cells show avid binding to TNF-α stimulated HUVEC. These data suggest that TNF-α may play an important role in initiating local vascular adhesion of NK and memory cells which may facilitate subsequent infiltration of inflammatory tissues.

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