Direct observations of the kinetics of migrating T-cells suggest active retention by endothelial cells with continual bidirectional migration

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Abstract

The kinetics and regulatory mechanisms of T-cell migration through endothelium have not been fully defined. In experimental filter-based assays *in vitro,* transmigration of lymphocytes takes hours, compared to minutes in vivo. We cultured endothelial cell (EC) monolayers on filters, solid substrates or collagen gels, and treated them with tumour necrosis factor-α (TNF), interferon-γ (IFN), or both, prior to analysis of lymphocyte migration in the presence or absence of flow. Peripheral blood lymphocytes (PBL), CD4+ cells or CD8+ cells, took many hours to migrate through EC-filter constructs for all cytokine treatments. However, direct microscopic observations of EC-filters which had been mounted in a flow chamber showed that PBL crossed the endothelial monolayer in minutes and were highly motile in the subendothelial space. Migration through EC was also observed on clear plastic, with or without flow. After brief settling without flow, PBL and isolated CD3+ or CD4+ cells all crossed EC in minutes, but the numbers of migrated cells varied little with time. Close observation revealed that lymphocytes continuously migrated back and forth across endothelium. Under flow, migration kinetics and the proportions migrating back and forth were little altered. On collagen gels, PBL again crossed EC in minutes and migrated back and forth, but showed little penetration of the gel over hours. In contrast, neutrophils migrated efficiently through EC and into gels. These observations suggest a novel model for lymphoid migration, in which endothelial cells support migration but retain lymphocytes (as opposed to neutrophils), and additional signal(s) are required for onward migration.

Introduction

Lymphocytes of all classes must migrate through endothelium in order to home to lymph nodes or to enter inflamed or infected tissue. In the context of inflammation, flowing cells are captured by specialised, fast-acting adhesion receptors (such as vascular cell adhesion molecule-1, VCAM-1, and E- or P-selectin) presented by venular endothelial cells (EC) responding to cytokines such as tumour necrosis factor-α (TNF), interleukin-1β (IL-1) or interferon-γ (IFN) [1]. Initial capture is followed by activation of the lymphocytes by surface-presented chemokine(s). Chemokines induce integrin activation and stabilisation of adhesion, followed by migration over and through the endothelial monolayer. In vitro, flow-based assays have shown that, depending on the stimulus applied to the EC, T-cell capture is possible through VCAM-1, E-selectin or P-selectin (although efficiency for each may depend on the T-cell subset), and that stable adhesion is mediated through binding of activated $\alpha_4\beta_1$-integrin to VCAM-1 and $\alpha_L\beta_2$-integrin to inter-cellular adhesion molecule-1 (ICAM-1) [2-4]. Transendothelial migration of T-cells has been observed within minutes of adhesion in such flow systems, for TNF-treated EC (where blockade of β_2-integrins was inhibitory) [3], for EC stimulated with TNF plus IFN [5] and for EC that had been stimulated with TNF and had stromal-derived factor-1α (SDF, CXCL12) or CCL19 (ELC) added to their surface [6]. In the last study, little migration was seen without an added chemokine (CXCL12 or CCL9), and even then, migration was much more effective in the presence of flow than if flow was stopped.

In contrast, most studies on the regulation of lymphocyte migration through endothelium, have used static assays in which EC have been grown on porous filters. These necessarily tests migration away from the sub-endothelial space as well as through the endothelial cells, and

periods from 2 to 36 hours are required for migration through the construct (e.g [7-9]). In such systems, T-cells spontaneously migrated through unstimulated endothelium/filters over time, [7, 10, 11], although the proportion migrating only reached about 10% of those added. Perhaps surprisingly, treatment of the EC with a range of cytokines (IL-1, TNF, IFN or TNF+IFN) caused modest [12, 13] or negligible [8, 10, 14] increases in T cell transmigration compared to unstimulated EC, again over prolonged periods. Addition of inflammatory chemokines, such as CCL2 (MCP-1), CCL3 (MIP-1α) or CCL5 (RANTES), below the filter slightly increased migration of memory T cells, although CXCL12 (a homeostatic chemokine) caused marked increase in the migration for naïve and memory T-cells [12]. Transendothelial migration studies have also been carried out for EC grown directly on collagen gels, where quite a small proportion of added T cells (~10%) migrated into the gel over 2-4h, and cytokine stimulation of the EC again had little effect [15, 16].

Considering the T-cell phenotype, memory CD4$^+$ T-cells migrated more efficiently than naive cells through resting or cytokine-treated EC (e.g [14, 16]), and in the presence of a chemotactic gradient [12]. Transmigration of T-cells tends to be increased by stimulation prior to assay, for instance, incubation with phorbol dibutyrate (PDB) or IL-2, culture for 2 to 24 hours (which up-regulates expression of $\alpha_L\beta_2$-integrin), or differentiation over weeks [7-10]. Nevertheless, studies using freshly isolated lymphocytes without manipulation reported migration through resting or IL-1β stimulated EC at 24h [11, 14] comparable to that observed in others studies when T cells were cultured overnight (e.g. [7]). It should be noted that in the studies described above, where transendothelial migration occurred in minutes under flow, the T-cells had first been subjected to prolonged incubation in presence [5] or absence of IL-2 [3, 6].

The studies outlined above leave some uncertainties about the rate at which migration occurs, first through the endothelial monolayer and then away from it, and the requirement for flow or for pre-activation of T-cells or endothelial cells for efficient transendothelial migration. Hours are required for T-cells to cross EC/filter constructs or enter collagen gels below EC, as opposed to minutes to cross EC alone, suggesting that assays are strongly influenced by migration through the sub-endothelial matrix and/or filter itself. In vivo, it is likely that naive as well as memory T-cells are recruited to peripheral tissue [17], and peripheral blood lymphocytes (PBL) are evidently recruited without prior activation. While direct studies of the kinetics of lymphocyte recruitment across inflamed endothelium are lacking, studies in the rat have shown that T cells are recruited across the vessel wall in the peripheral lymph nodes and Peyers Patch within 30-40mins of their infusion [18, 19]. To investigate these problems, we studied kinetics of lymphocyte migration through endothelial cells cultured on filters, plastic wells or collagen gels, and treated with different cytokines, and compared results in static and flow-based assays. Previously we used similar approaches to analyse kinetics of neutrophil migration through endothelial monolayers and filters [20-22]. Here we found that lymphocytes could cross endothelial monolayers in minutes, with little evidence of a requirement for flow. The migrated cells were highly motile, and the prolonged periods required to transit filter systems could not be attributed to transendothelial migration itself. However, we made the novel observations that some lymphocytes underwent multiple transits back and forth across endothelial monolayers, and that few motile transmigrated lymphocytes entered collagen gels (e.g., compared to neutrophils). We suggest that EC tend to retain migrating lymphocytes and that a separate signal is required to overcome this and allow lymphocytes to move into the stroma.

Methods

Isolation of human peripheral blood lymphocytes (PBL), T cells and neutrophils

Venous blood from healthy individuals was collected in EDTA tubes (Sarstedt, Leicester, UK).

Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation of blood on

histopaque 1077, and PBL were prepared by panning of PBMC on culture plastic to remove

monocytes [23]. In some experiments, to obtain activated T-cells, PBMC were cultured for 7

days in the presence of 10μg/ml phytohaemagglutinin (PHA; Sigma). PHA initially induced

clumping and proliferation of lymphocytes, but by day 7 cells had dispersed and were spherical

and smaller than freshly isolated PBL (judged by Coulter Counter volume distribution). In other

experiments, T-cells ($CD3^+$) or $CD4^+$ T-cells were purified from PBMC by negative selection

using magnetic Dynabeads (Dynal, Wirral, U.K.) and a cocktail of monoclonal antibodies to

remove cells bearing CD19, CD11b (HIB19 and ICRF44 respectively, both from BD

Pharmingen, UK), CD14, CD16 (RM052 and 3G8 respectively, Beckman Coutler, UK) and, in

the case of $CD4^+$ T cell selection, CD8 (OKT8, eBioscience, UK) [24]. Isolated cells were

washed, counted, and adjusted to a final concentration of $2x10^6$/ml in phosphate buffered saline

containing Ca^{2+} and Mg^{2+} or Medium 199 (Gibco Invitrogen Compounds, Paisley, Scotland)

supplemented with 0.15% bovine serum albumin (Sigma-Aldrich, Poole, UK) (PBSA or

M199BSA respectively). On a few occasions, neutrophils were isolated using a two-step density

gradient as described [20-22] and suspended at $2x10^6$/ml in M199BSA.

Isolation and culture of endothelial cells

HUVEC were isolated from umbilical cords as previously described [25] and cultured in

M199 supplemented with 20% fetal calf serum (FCS), 10ng/ml epidermal growth factor, 35μg/ml

gentamycin, 1µg/ml hydrocortisone (all from Sigma) and 2.5µg/ml amphotericin B (Gibco Invitrogen Compounds). Primary HUVEC were dissociated using trypsin/EDTA (Sigma) and seeded on either six-well tissue culture plates (Falcon; Becton Dickinson Labware, NJ, USA), uncoated low-density 3.0µm pore polycarbonate Transwell filters (which were placed in matching plates; BD Pharmingen, Oxford, UK), glass chamber slides (Lab-tek, Nalge Nunc International, Naperville, IL) or collagen gels (see below). Seeding density was chosen to yield confluent monolayers within 24h. Tumour necrosis factor-alpha (TNF; 100U/ml; Sigma) and/or interferon gamma (IFN; 10ng/ml; Peprotech Inc., London, UK) were added to confluent monolayers for 4 or 24h before the assay with neutrophils or lymphocytes respectively.

To form collagen gels, type 1 collagen dissolved in 0.6% acetic acid (2.15mg/ml; First Link Ltd, West Midlands, UK) was mixed with 10xM199 and FCS (1.66ml, 0.1ml and 0.34ml respectively). The pH was neutralised by addition of 0.15ml 1N NaOH, and 1ml was dispensed into a 6-well plate and allowed to gel at 37°C. The gel was then equilibrated with HUVEC culture medium for 48h before seeding and culture with HUVEC as above. In some experiments, CXCL10 (IP-10) or CXCL12 (SDF1-α) (80 or 800ng/ml; Peprotech) was added to the collagen after neutralisation with NaOH. The gel was polymerised as above and equilibrated with HUVEC culture medium containing chemokine at the same concentration.

Analysis of lymphocyte migration

1. Migration through endothelial cells on Transwell filters under static conditions

Lymphocyte migration was assessed using 24-well format Transwell filters. HUVEC were washed to remove residual cytokines, fresh M199+BSA was placed in the lower chamber

and PBL were added to the upper chamber. The lymphocytes were allowed to settle, adhere and migrate through HUVEC at 37°C in a CO_2 incubator for the desired period. Migration was stopped at the chosen time by transferring the filter into a fresh well, leaving the transmigrated cells in the original lower chamber. The lymphocytes suspended in the upper chamber were removed, and pooled with cells obtained when the filter was washed twice. These cells were taken to represent non-adherent lymphocytes. The non-adherent and transmigrated cells were counted using a Coulter Multisizer II (Coulter Electronics Ltd, Essex, UK). From the known number of added lymphocytes, the percentage of lymphocytes that adhered, and the percentage of lymphocytes that transmigrated were calculated.

In some experiments, the surface phenotypes of adherent and transmigrated lymphocytes were assessed by flow cytometry. Freshly isolated, non-adherent (upper chamber) or transmigrated (lower chamber) lymphocytes were labelled with anti-CD4-PE or anti-CD8-FITC (Becton Dickinson, Oxford, UK) for 30min on ice. Fixed volume counts for positively labelled cells were made using a Coulter XL flow cytometer and analysed using WinMDI. In this way, we calculated the percentage adhesion and transmigration for CD4$^+$ and CD8$^+$ T-cell subsets.

2. Microscopic observation of migration through endothelial cells under static conditions

Adhesion and transmigration were assessed by direct microscopic observation as previously described [26]. HUVEC in 6-well plates were washed with PBSA to remove residual cytokines and purified PBL, CD3$^+$ or CD4$^+$ T-cells were added for 5min. Non-adherent cells were removed from the HUVEC by gentle washing with PBSA (which took 2min) and video recordings of the endothelial surface were made using phase contrast videomicroscopy as follows: (i) 5 fields were briefly recorded immediately after washing to analyse number of

adherent cells and their position above or below the monolayer (see below); (ii) a single field was recorded for 15 minutes to follow lymphocyte behaviour; (iii) a further 5 fields were briefly recorded to analyse the position of lymphocytes above or below the monolayer. Manipulations and microscopy were carried out inside a Perspex box held at 37°C.

The video recordings were digitised and analysed offline using Image-Pro Plus software (DataCell Ltd, Finchampstead, UK). The numbers of adherent cells were counted in the video fields, averaged and then converted to cells per mm^2 using the calibrated microscope field dimensions, and multiplied by the known surface area of the HUVEC to calculate the total number adherent. This number was divided by the known total number of lymphocytes added, to obtain the percentage of the lymphocytes that had adhered. Each lymphocyte was classified as either: (i) phase bright, with round or distorted shape, and adherent to the surface of the HUVEC; (ii) phase dark and spread, and migrating below the HUVEC. The percentage of adherent lymphocytes that had transmigrated was calculated at each time, with time zero taken as the end of the settling period. The migration velocities of phase-dark lymphocytes underneath the HUVEC were measured by digitising a sequence of images 1min apart for 6min. In each digitised image, cells were outlined and the position of their centroid determined. Migration velocity (µm/min) was the average distance moved by the centroid per minute.

3. Microscopic observation of migration through endothelial cells into collagen gels under static conditions

HUVEC on collagen gels were washed with M199+BSA to remove residual cytokines, and purified PBL, PHA stimulated PBL or neutrophils were added for 10min. Non-adherent cells were removed from the HUVEC by gentle washing with PBSA, and phase-contrast video-

microscope recording were made 0.25h, 1h, 3h and 24h after the original addition of leukocytes. Five video-fields were recorded. In each field, images were first recorded at the endothelial surface, and then recordings were made as the microscope was focussed gradually down in 50μm steps. Cells visible with the endothelial monolayer were counted and divided into those which were phase bright (above EC) and those which were phase dark (just below EC). Cells within each 50μm step were counted as they came into focus; these cells were typically irregular in shape and phase bright. The focal depth of the gels was approximately 300μm. After averaging counts in the 5 fields, data were expressed as the percentage of the adherent cells in each vertical region. On some occasions, a single field at the endothelial surface was recorded for 5 minutes to analyse migratory behaviour of the leukocytes. All manipulations of gels and microscopy were carried out at 37°C.

4. Microscopic observation of migration through endothelial cells under conditions of flow

Filters coated with HUVEC were cut from the Transwell holders and placed on a 75x25mm coverslip. Alternatively, the bases of chamber slides coated with HUVEC were freed from the fluid reservoirs attached to their surface. The coverslips or slides were incorporated into a parallel-plate flow chamber and attached to a perfusion system mounted on the stage of a phase-contrast videomicroscope enclosed in a Perspex chamber at 37°C, as described [21, 26]. The flow channel dimensions were 20 x 4 x 0.13mm (length x width x depth) for the filters, and 50 x 10 x 0.25 mm for the chamber slides. At one end, they were connected to a Harvard withdrawal syringe pump which delivered flow at a rate equivalent to a wall shear stress of 0.1Pa. At the other end, they were connected to an electronic switching valve (Lee Products, Gerards Cross, UK) which selected flow from two reservoirs, containing PBL in PBSA or cell-free PBSA. A four-minute bolus of PBL was perfused over the HUVEC followed by cell-free wash buffer.

Video recordings were made of a series of microscope fields along the centreline of the flow channel after 2 and 11min of washout, and between these times a single field was recorded.

Video recordings were analysed essentially as above, except that lymphocytes adherent to the surface of HUVEC could be classified as either rolling adherent (spherical cells moving over the surface much slower than free-flowing cells) or stationary adherent (typically with distorted shape and actually migrating slowly on the surface). Phase-dark transmigrated cells could be counted for either substrate, but when observing filters in the flow chamber, recordings were also made of the underside of the filter where a few cells might be found. This was achieved by focussing the microscope stage up and down 10μm (i.e., the filter thickness). The sum of the numbers adherent in all categories was divided by the number perfused during the bolus to obtain total PBL adhesion as % of cells perfused.

Statistical analysis

Effects of multiple treatments were tested using analysis of variance (ANOVA), followed by comparison to control by Dunnett test. Single treatments were compared to controls by paired t-test.

Results

Kinetics of lymphocyte migration through endothelial cells and 3μm-pore filters

Settling of lymphocytes onto endothelial cell/filter constructs and quantification of the number collected from the back has been widely used to assess 'transendothelial' migration. In initial experiments with unstimulated or TNF-treated HUVEC, we found that few PBL migrated

through the filter after 2 or 4 hours (e.g. 2.4 ± 1.0% of added cells migrated through TNF-stimulated HUVEC at 4h; mean SEM, n=3). The proportion increased by 24h (e.g. 11.0 ± 3.2% of added cells which migrated through TNF-stimulated HUVEC; mean SEM, n=4) and so we made comparisons between variously-treated HUVEC at this time. Figure 1 shows that endothelial cells treated with cytokines (TNF or INF alone, or together) tended to support greater lymphocyte transmigration compared to unstimulated HUVEC, although there was no consistent difference between the cytokine treatments. This trend was also evident when CD4$^+$ or CD8$^+$ T cells were analysed separately (Figure 1), and the two types of T-cell behaved similarly to each other. The proportion of lymphocytes that were adherent was high after 24 hours (~50%) and not significantly affected by cytokine treatments (data not shown). Such long contact times are not physiological, and presumably increase non-specific background adhesion. We thus reduced the initial contact time by washing off non-adherent lymphocytes after 10min, whilst maintaining the 24h migration endpoint. This decreased lymphocyte adhesion, and there was now a tendency toward greater adhesion for endothelial cells stimulated with TNF+IFN compared to untreated cells (21.8 ± 3.7% vs. 14.8 ± 6.0% of added PBL adherent respectively; mean ± SEM from 6 experiments). However, transmigration was also much lower (3.4 ± 0.6% vs. 1.6 ± 0.5% of added PBL transmigrated respectively).

These findings showed that PBL and the major T-cell subclasses took hours to migrate through endothelial/filter constructs, a distance of only ~10μm, and that cytokine stimulation of the endothelial cells only increased recruitment about two-fold. Lymphocyte recruitment across the wall of inflamed vessels is expected to be more rapid and stimulus-specific. Thus we observed lymphocyte interactions with HUVEC treated with TNF plus IFN (the treatment inducing most efficient transmigration) on filters under flow conditions. Few flowing PBL

adhered to unstimulated HUVEC cultured on filters, but many more were adherent when the endothelial cells had been stimulated with TNF plus IFN ($0.6 \pm 0.3\%$ vs. $5.8 \pm 2.0\%$ of PBL perfused, respectively; mean $\pm$ SEM, n=4). The cells adherent to the cytokine-treated EC were firmly attached with only a small percentage rolling, and within 11 minutes about 30% had migrated through the endothelial monolayer (Figure 2). At this time, very few lymphocytes had migrated through the filter itself (Figure 2). The migration velocities of the phase-dark cells under the HUVEC averaged about 5µm/min (4.9 ± 0.6 µm/min; mean $\pm$ SEM of means from 3 experiments).

Thus, lymphocytes adhered and migrated quickly through cytokine-stimulated monolayers in the presence of flow. They then migrated freely under the monolayer but did not appear below the filter within minutes. Indeed, it took hours to negotiate the filter in the static assay. Since direct observation of kinetics of migration through the endothelium or the filter was not possible in the 'standard' static filter assay, it is not possible to conclude at this stage the exact step at which the hold-up occurred. It is possible that transendothelial migration was slower in the absence, compared to the presence, of flow [27], or that lymphocytes quickly crossed the endothelial monolayer but were held up by the filter in either case. To clarify this point, we compared microscopic observations of migration kinetics through HUVEC under static or flow conditions, using clear, solid substrates.

Lymphocyte migration through endothelial cells on clear substrates in absence of flow

When PBL were allowed to settle for 5min, few (~5%) adhered to unstimulated HUVEC cultured in multi-well plates, but cytokine-stimulated endothelial cells supported much higher levels of attachment (Figure 3A). We were surprised to find that 2 minutes after washing, a

significant proportion of the adherent cells had transmigrated, and that after a further 15 minutes, this proportion remained essentially the same (Figure 3B). Transmigration was higher for cytokine-treated monolayers, particularly in the presence of IFN. Although some adherent cells did transmigrate through unstimulated HUVEC in minutes, the absolute number observed was small due to the low level of adhesion. Examining migration through cytokine-treated EC in more detail, we recorded individual fields and repeatedly assessed transmigration. While there were minor fluctuations in the proportion of PBL transmigrated, there were no significant upward trends (Figure 3C). We also noted the velocity of migrated cells, which averaged about 8µm/min and tended to be faster in the presence of IFN (Table 1A). Thus, in a static assay with short initial contact times, we detected cytokine-specific induction of lymphocyte adhesion and could observe transendothelial migration within minutes.

The detailed analysis of individual fields revealed another unexpected phenomenon. PBL were seen to continue transmigrating (going from phase-bright to phase-dark) throughout the entire 15min period, but other cells were migrating in the opposite (basal to apical) direction. The continual forward and reverse migration explained the nearly constant level of transmigration observed at any time. Some cells made several transits back and forth within the observation period, and some stayed in the same compartment throughout. To quantify this behaviour, we followed individual cells second-by-second over a period of 6 minutes, and recorded if and when they moved between the basal and apical surfaces of the HUVEC. A sequence of pictures of a multiply-migrating cell is shown in Figure 4A, and some typical behaviours are illustrated schematically in Figure 4B. For cytokine-treated HUVEC, about 20% of PBL made at least one transit during the observation period and about a third of these made multiple transits (Table 1A). By measuring the number of transits during the 6-minute period, we

calculated the average interval between transits for those cells that moved between compartments (mean ~4 min) (Table 1A). The values varied little between the cytokine treatments (TNF, IFN or TNF+IFN). Transits were seen with unstimulated HUVEC (data not shown), but again, the number of cells observed was small. On average, half of the transits were forward and half in the reverse direction, which was consistent with the observation noted above, that overall levels of transmigration were constant over the total observation period.

We wondered whether specific lymphocyte sub-populations might be more efficient in migration or prone to reverse migration, and so carried out a series of experiments comparing PBL to purified $CD3^+$ and $CD4^+$ T cells from the same donors. We observed no significant difference in the proportion of these lymphocyte populations adhering or transmigrating through endothelial cells stimulated with TNF+IFN (Figure 5). In addition, the multiple-transit behaviour of purified T cell subpopulations, and the velocities of migrated cells were not significantly different from PBL (Table 1B).

Lymphocyte migration through endothelial cells on clear substrates under flow

Unstimulated endothelial cells cultured in chamber slides consistently failed to recruit flowing lymphocytes (Figure 6A). However, after cytokine stimulation, the endothelial cells efficiently captured much greater numbers of flowing lymphocytes, with greater adhesion observed on endothelial cells stimulated with TNF or TNF+IFN compared to IFN alone (Figure 6A). As with HUVEC cultured on filters, few captured lymphocytes rolled, and most adhered firmly. Nearly a half of the adherent PBL transmigrated through the cytokine-stimulated monolayers within 11 minutes, with IFN being the most effective inducer of migration (Figure 4B). Velocity of migrated cells averaged about 8μm/min, which is similar to that observed in

the static assay, and again, velocity tended to be faster in the presence of IFN (Table 1C).

We checked whether multiple transits were observed under flow as well as in the static assay. In fact, the proportions of cells undergoing at least one transit, or undergoing multiple transits in a six-minute period were similar, if a little higher, in the presence of flow (Table 1C). Overall, the average intervals between transits were nearly identical to the values obtained in the static assay (Table 1). Again, values for these variables were similar for the different cytokine treatments.

Lymphocyte migration through endothelial cells and into collagen gels

The above findings suggested that under static or flow conditions, lymphocytes quickly crossed endothelial monolayers, but were reluctant to move on from the subendothelial space, and that this might have been linked to repeated migration back and forth. However, even the filters represent a solid barrier to migration over most of their surface, and so we decided to observe migration out of the subendothelium into collagen gels over prolonged periods. First, we analysed neutrophil migration through TNF-treated HUVEC into gels, since we had characterised neutrophils previously in all the other models used here [e.g., 21, 22, 26]. Figure 7A shows changes in the distribution of neutrophils above and just below the EC, and in the gel over time. After 15 minutes, a high proportion of adherent neutrophils had penetrated the monolayer and were visible, phase-dark just below it. By 1 hour, a few more cells had migrated under the EC, but nearly all of the transmigrated cells were now found in the gel. The cells moved further into the gel by 3 hours, and by 24 hours, the neutrophils were essentially evenly distributed throughout the depth of the gel. The lymphocytes were much less efficient in entering the gels (Figure 7B). A significant proportion of adherent cells had migrated through the endothelial

monolayer within 15 minutes, as in the other models. This proportion increased to about 50% after 1 hour, but <5% were found in the gel at this time. By 3 hours, about 10% were in the gel, but these had not penetrated far compared to the neutrophils. Even at 24 hours only 10% were in gel, and most of these were still in the first 100µm.

We also recorded the behaviour of the lymphocytes at the endothelial surface after 15min, to allow comparison to the observations on solid substrate. Again, we observed cells undergoing forward and backward migration, with some making multiple transits through the endothelial monolayer, at frequencies comparable to those seen on the clear plastic (Table 1D). The phase-dark cells were highly motile and had velocity averaging 5-6µm/min (Table 1D).

Effects of lymphocyte activation or the presence of a subendothelial chemokine

We considered whether activated T-cells would migrate more efficiently into gels. However, while PHA activation significantly increased lymphocyte migration through the endothelial monolayer (64.6 ± 3.5% vs. 45.2 ± 6.4% of adherent cells migrated at 3h following PHA treatment vs. freshly isolated PBL respectively; mean ± SEM, n=3; p<0.05 by paired t-test), migration into the gel was not significantly altered (9.1 ± 3.9% vs. 5.1 ± 4.9% migrated at 24h with or without PHA respectively; mean ± SEM, n=3). Furthermore, we still observed multiple transits back and forth across EC by PHA-activated lymphocytes. In fact, the proportions of cells undergoing at least one transit (~14%), or undergoing multiple transits (~5%) in a six-minute period were similar to freshly isolated lymphocytes. Thus, activation was insufficient to induce migration away from the subendothelial space, suggesting the need for a second signal presumably from within the tissue.

Chemokines CXCL10 and CXCL12 were added to gels at a concentration (80ng/ml) which would be expected to increase chemotaxis through filters (unpublished observations). However, in 2 experiments with each, PBL migration through cytokine-treated endothelium or into the gel were not increased, and the tendency to migrate back and forth across the endothelial monolayer remained unaltered (data not shown). The chemokines most likely diffused across the EC and into the surrounding medium during endothelial culture, washing and the adhesion assay itself, diluting any gradient. We thus increased the concentration of CXCL10 added to the gel 10-fold, and observed a small but consistent increase in the migration of lymphocytes into the gel when compared to untreated gels (Figure 7C). Of note, the total level of transmigration across the endothelial monolayer was not increased (averaging $51.2 \pm 9.8\%$ or $54.3 \pm 5.6\%$ for gels with or without chemokine respectively; mean $\pm$ SEM from 3 experiments m,easured at 24h), but the presence of CXCL10 tended to reduce lymphocyte migration back and forth across the endothelial monolayer. The proportion of adherent cells undergoing one or more transit over 6 minutes was reduced from $14.2 \pm 4.8\%$ to $6.9 \pm 2.2\%$, and the proportion undergoing more than one transit was reduced from $6.9 \pm 2.3\%$ to $3.9 \pm 1.8\%$ (mean $\pm$ SEM from 3 experiments) although these trends did not reach statistical significance. Thus the presence of a chemokine, such as CXCL10, could promote lymphocyte migration away from the endothelium into the underlying matrix.

Discussion

Using direct microscopic observation of endothelial monolayers treated with different cytokines, we found that freshly-isolated peripheral blood lymphocytes could migrate across endothelial monolayers in minutes in the presence or absence of flow. While the lymphocytes migrated at about 5-10µm/min underneath the endothelial cells, they did not move quickly

through 10μm-thick porous filters or into collagen gels. Interestingly, a significant proportion of PBL could be seen to migrate back and forth across the endothelial monolayer, sometimes repeatedly. In consequence, the proportion under the endothelium did not vary much over about 15 minutes. The above phenomena could be observed for CD3+ T-cells (which would be expected to make up the great majority of PBL) and the CD4+ T-cell subset, as well as PHA-activated cells. In non-visual, static, filter-based assays, little transmigration was detected within hours, and 24 hours were needed to obtain a proportion of lymphocytes under the filter comparable to that seen in minutes during direct observations of transendothelial migration. After 24 hours, penetration of gels by lymphocytes was inefficient (e.g., compared to neutrophils). Given the speed at which lymphocytes were seen to migrate under EC, it seems that EC tended to retain lymphocytes in their vicinity, and that a signal to migrate through stroma and/or across the filters was lacking. Studies in which exogenous chemokine was added to collagen gels, and penetration of the matrix was increased, supported this concept.

Efficient migration of T-cells through EC treated with TNF and IFN (reaching ~40% of those adherent) has been described previously under flow conditions similar to those used here [5]. In studies using TNF alone, Luscinskas et al., observed that migration occurred in minutes but did not quantify the proportion of cells migrating [3]. Cinamon et al., found that only ~5% of adherent T-cells migrated through TNF-treated EC under flow, but this proportion increased greatly when SDF was added to the endothelial surface [6]. Most striking was their observation that if flow was stopped, there was negligible transmigration. In each of these flow-based studies, isolated T-cells were pre-incubated for prolonged periods, presumably to induce activation and greater migration, although this was only explicitly stated by Piali et al. [5].

We did not observe marked differences in migration behaviour in the presence or absence of flow. Flow is required to model the capture processes operating in the vasculature, and here, as expected, cytokine-treated EC supported much greater adhesion than unstimulated EC in presence of flow. However, we found that the proportion of adherent cells transmigrating, and the frequency with which cells migrated in and out of the monolayer, were similar with or without flow, for EC treated with TNF, IFN or both. Interferon tended to induce the highest level of migration and to induce lymphocytes to migrate more rapidly, but the trends were similar with or without flow. Throughout the study, treatment of EC with cytokines increased the efficiency of transmigration, as well as capture in flow-based assays, but migration did not require addition of an exogenous chemokine. Previously, presence of flow and binding of SDF to the surface of TNF-treated HUVEC were found necessary to obtain efficient trans-endothelial migration of T-cells [6]. However, the definition of transmigration was different from that used here (only cells which were observed to change once from phase-bright to phase-dark were counted) and the T-cells had been cultured for 15-18h before analysis.

Lymphocytes were not purposefully activated in most of our studies, on the basis that PBL are recruited directly from the circulation during inflammation in vivo. While this recruitment may be more efficient for memory cells, naive cells are also recruited to non-lymphoid tissue [17]. Here, migration behaviour was broadly similar for PBL, T-cells, and CD4$^+$ and CD8$^+$ subsets. We have found preferential migration of CD45RA-negative (i.e., memory phenotype), CD4$^+$ or CD8$^+$ cells through Transwell filters (assessed using flow cytometry; unpublished observations) in line with reports by others [15, 16, 28]. Thus, it is likely that PBL migrating through endothelial monolayers were enriched in memory cells, but pre-separation of naive and memory populations would be required to define their relative migration efficiencies e.g., in flow

models. In comparing results here to previous work with activated T-cells, it may be worth noting that there is considerable inter-donor variation in migration of T-cells (e.g., compared to neutrophils) in our experience. Gathering data from several studies in our laboratory, we have observed transendothelial migration through EC treated with TNF and IFN in direct microscopic flow assays between 2 and 50% of adherent PBL (mean =17%; n=26). When we did study activated lymphocytes, they migrated more efficiently through cytokine-stimulated endothelial monolayers than resting lymphocytes, in agreement with previous reports [7-10]. Despite this, the activated lymphocytes did not migrate efficiently into an underlying collagen gel, indicating that activation per se was insufficient to generate the necessary signals for migration away from the subendothelial space. Nevertheless, the possibility that antigen-presentation by EC specifically facilitates migration of cognate T-cells cannot be discounted [29].

The data presented here reveal some problems relating to interpretation of widely-used, filter-based migration assays. Using prolonged incubations under static conditions, there is a high level of lymphocyte adhesion and significant transmigration through filters, even without cytokine stimulation of EC. With cytokine treatment, lymphocytes still take far longer to cross filters than to cross endothelial monolayers, and far longer than is required to enter tissue in vivo. When we reduced the initial contact time between PBL and EC before wash-off of non-adherent cells, fewer PBL adhered, but the proportion of the adherent cells that migrated through the filter was still small after many hours. Practically, this suggests that mechanisms found to support or regulate migration in such assays, might not be specific to the transendothelial (as opposed to the trans-filter) stage.

In our experience, neutrophils migrate through endothelial monolayers and migrate

underneath the monolayers at similar rates to the lymphocytes observed here, but a larger proportion can be directly observed to migrate through filters in minutes in a flow assay [21]. In static trans-filter assays, neutrophils can be collected as early as 15-30 minutes after addition to the upper surface. Here, we found that neutrophils that had migrated through EC quickly moved into collagen gels, and became uniformly dispersed with time. This suggests random diffusion into the gel. Lymphocytes did not 'diffuse' in this way, and indeed it seems that most were actually retained in or near the endothelial cell layer. The inefficiency of lymphocyte migration away from the sub-endothelial space was linked with the tendency of many migrated cells to move back through the monolayer to the luminal surface in minutes. We have not observed such behaviour with neutrophils. Taken together, these observations suggest that lymphocytes continually interacted with EC and that a critical signal was lacking in the in vitro models, which was required to drive migration of lymphocytes away from endothelium into tissue. Here, the addition of CXCL10 to the collagen under EC moderately enhanced lymphocyte migration into the gel, and reduced the tendency to move back and forth across the endothelial monolayer. In general, stromal chemoattractants may increase efficiency of migration into tissue for all leukocytes, but it seems that neutrophils, as opposed to lymphocytes, do not need such a signal to free themselves from endothelium. Given the heterogeneity in the chemokine receptors expressed by different lymphocytes, more than one subendothelial chemokine might be required to induce efficient migration of the entire population. Conversely, stromal production of a restricted chemokine subset could provide a further 'subendothelial' level of control within the recruitment cascade.

Reverse migration has been described for monocytes, over hours after migration though HUVEC cultured on amniotic tissue [30] or over tens of minutes under flow after migration

through TNF-treated HUVEC cultured in glass capillaries [31]. We described reverse migration of neutrophils over hours in a similar flow model [32]. Reverse migration of neutrophils has since been observed directly in a inflamed microvessels of the zebra fish [33]. Here, some PBL and T-cells shuttled back and forth between these compartments over minutes. Repeat migration of lymphocytes between the lumen and sub-endothelial spaces has not been described previously. Nearly 10% of all adherent cells made more than one transit in a 6-minute period, and of cells that started underneath the endothelial monolayer, over 40% underwent reverse migration in the same time. Whether comparable behaviour occurs in vivo is unknown, and we are not aware of any published real-time observations of migration of lymphocyte (as opposed to neutrophils) in inflamed vessels.

In conclusion, lymphocytes (and specifically T-cells) migrated across endothelial cells in minutes, were highly motile thereafter, and some at least migrated back and forth across the endothelium repeatedly. The presence of flow improved the specificity of the assays (in the sense that binding to unstimulated EC was negligible and effects of cytokines more clear-cut than in static assays), but did not influence strongly the initial migration, or the back and forth movement in the models used here. It appears that lymphocytes may be actively retained by endothelial cells and require a signal from another source to induce their migration onward from the sub-endothelial space. Consequently, the process of lymphocyte recruitment appears to involve an additional regulated stage, in all probability to minimise non-specific sub-set recruitment.

Acknowledgements

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Table 1: Characteristics of migration of lymphocytes migrating through endothelial cell monolayers on clear substrates

Treatment/Cell	Cells undergoing 1 or more transits (%)	Cells undergoing more than 1 transit (%)	Transit interval (min)	Migration velocity (µm/min)
A. Migration of PBL through EC exposed to different cytokines - **Static assay (n=4)**				
TNF	18.3 ± 8.6	6.6 ± 3.8	4.4 ± 0.9	6.4 ± 0.2
IFN	25.9 ± 5.6	7.5 ± 3.9	4.4 ± 0.5	10.5 ± 1.4
TNF+IFN	21.5 ± 4.3	9.6 ± 4.2	3.9 ± 0.2	8.9 ± 1.0
B. Different lymphocyte subsets migrating through EC treated with TNF+INF - **Static assay (n=3)**				
PBL	17.5 ± 5.6	7.1 ± 3.4	3.6 ± 0.5	8.8 ± 2.4
CD3+	15.9 ± 6.0	5.2 ± 3.4	4.6 ± 0.8	6.1 ± 0.3
CD4+	19.2 ± 2.8	5.6 ± 2.9	3.8 ± 0.8	7.1 ± 1.6
C. Migration of PBL through EC exposed to different cytokines - **Flow-based assay (n=4)**				
TNF	23.9 ± 1.8	11.1 ± 3.8	3.7 ± 0.5	5.8 ± 0.5*
IFN	31.5 ± 4.0	13.7 ± 4.1	3.9 ± 0.5	9.6 ± 1.5
TNF+IFN	32.5 ± 5.2	13.6 ± 7.9	4.4 ± 1.0	
D. Migration of PBL through EC on collagen gels - **Static assay (n=3)**				
TNF+IFN	21.0 ± 2.5	7.8 ± 4.1	4.2 ± 0.4	5.4 ± 0.8

Individual lymphocytes were tracked over the 6min period and their location above or beneath the endothelium analysed every 10s. The percentages of lymphocytes migrating through the monolayer once (1 transit) or migrating back and forth (>1 transit) were determined. The average interval between transits for those cells that did move between compartments was also determined. The velocity of migration under the monolayer was measured over a 5-minute period. Data are the mean ± SEM from n independent experiments. * ANOVA showed significant effect of treatment on migration velocity, with TNF significantly different from IFN by Tukey test (both $p<0.05$).

Figure Legends

Figure 1: Effects of different cytokine treatments on migration of PBL and T-cell subsets through endothelial cells and their supporting Transwell filters. HUVEC were stimulated with 100U/ml TNF alone, 10ng/ml IFN alone or with both for 24h. Lymphocytes were allowed to adhere and migrate for 24h after which the number of lymphocytes transmigrating was counted and expressed as a percentage of the added cells. Data are mean ± SEM from 2-8 independent experiments.

Figure 2: Behaviour of lymphocytes recruited to cytokine-stimulated endothelial cells in a flow-based assay. A 4 min bolus of lymphocytes was perfused over HUVEC that had been cultured in Transwell inserts and treated with TNF+IFN for 24h. The behaviour of the adherent lymphocytes (rolling, stationary adherent, migrated through the endothelial monolayer or migrated through the filter) was evaluated 11min after bolus perfusion. Data are mean ± SEM from 4 independent experiments.

Figure 3: Effects of different cytokine treatments on lymphocyte recruitment to endothelial cells cultured in multi-well plates. HUVEC were stimulated with 100U/ml TNF alone, 10ng/ml IFN alone or with both for 24h. Lymphocytes were allowed to settle for 5min, non-adherent cells were washed off and lymphocyte adhesion and transmigration were analysed by phase contrast microscopy. **(A)** Effect of cytokines on lymphocyte adhesion measured at 2min after wash-off. **(B)** Effect of cytokines on lymphocyte transmigration through HUVEC at 2min and 17min after wash-off. **(C)** Time courses of lymphocyte transendothelial migration for different cytokines. Data are mean ± SEM from 4 independent experiments. In A and B, ANOVA showed a significant effect of cytokine treatment on lymphocyte adhesion and transmigration; $p<0.01$. * = $p<0.05$, ** = $p<0.01$ compared to untreated by Dunnett test.

Figure 4: The migration behaviour of individual lymphocytes over a 6min period for HUVEC treated with TNF+IFN. **(A)** Video-micrographs of a lymphocyte migrating from above the endothelial monolayer (phase-bright) to underneath (phase dark), back to the top and then under again. **(B)** Schematic representation of typical lymphocyte migratory behaviours. Individual cells were tracked over a 6min period. Positions above (a) or below (b) the HUVEC monolayer are plotted for 5 cells: Cell 1 stayed above the monolayer and cell 5 stayed below. The others made one or more transits between the compartments. Similar behaviours were seen for HUVEC treated with TNF or IFN separately.

Figure 5: Comparison of recruitment of different lymphocyte sub-sets to endothelial cells cultured in multi-well plates. HUVEC were stimulated with TNF+IFN for 24h. PBL, or purified $CD3^+$ or $CD4^+$ T cells were allowed to settle for 5min, non-adherent cells were washed off and adhesion and transmigration were analysed by phase contrast microscopy. **(A)** Effects of cytokines on lymphocyte adhesion measured at 2min and 17min after wash-off. **(B)** Lymphocyte transmigration through HUVEC at 2min and 17min after wash-off. Data are mean ± SEM from 4 independent experiments.

Figure 6: Effects of different cytokine treatments on recruitment of lymphocytes to endothelial cells in a flow-based assay. A 4 min bolus of lymphocytes was perfused over HUVEC that had been cultured on chamber slides and treated with TNF alone, IFN alone or with both for 24h. **(A)** Effects of cytokines on lymphocyte adhesion measured at 2min after wash-off. **(B)** The behaviour of the adherent lymphocytes (rolling, stationary adherent or migrated through the endothelial monolayer) measured 11min after wash-off. Data are mean ± SEM from 3-4 independent experiments. In (A), ANOVA showed a significant effect of treatment ($p < 0.01$). * = $p < 0.05$ and ** = $p < 0.01$ compared to untreated by Dunnett test.

Figure 7: Migration of leukocytes through EC and into collagen gels over time for A. Neutrophils adherent to HUVEC treated with TNF for 4h; B. Lymphocytes adherent to HUVEC treated with TNF+IFN for 24h. C. Lymphocytes adherent to HUVEC treated with TNF+IFN for 24h, with or without addition of CXCL10 to the gel. Leukocytes were allowed to settle on HUVEC for 10 min, non-adherent cells were washed off.   In A and B, cells were counted that were above the EC (phase bright), just below the EC (phase dark and spread) or present within different regions as the microscope was focussed down into the gel. Data were collected 0.25 (☐), 1 (▨), 3 (▨) and 24h (▨) after initial addition of leukocytes, and calculated as the percentage of the adherent cells.   In C, the percentage of adherent lymphocytes which had entered the gel was assessed with (■) or without (☐) CXCL10 added. Data are mean ± SEM from 3 experiments. In C, ** = p<0.01 compared to untreated gel by paired t-test.
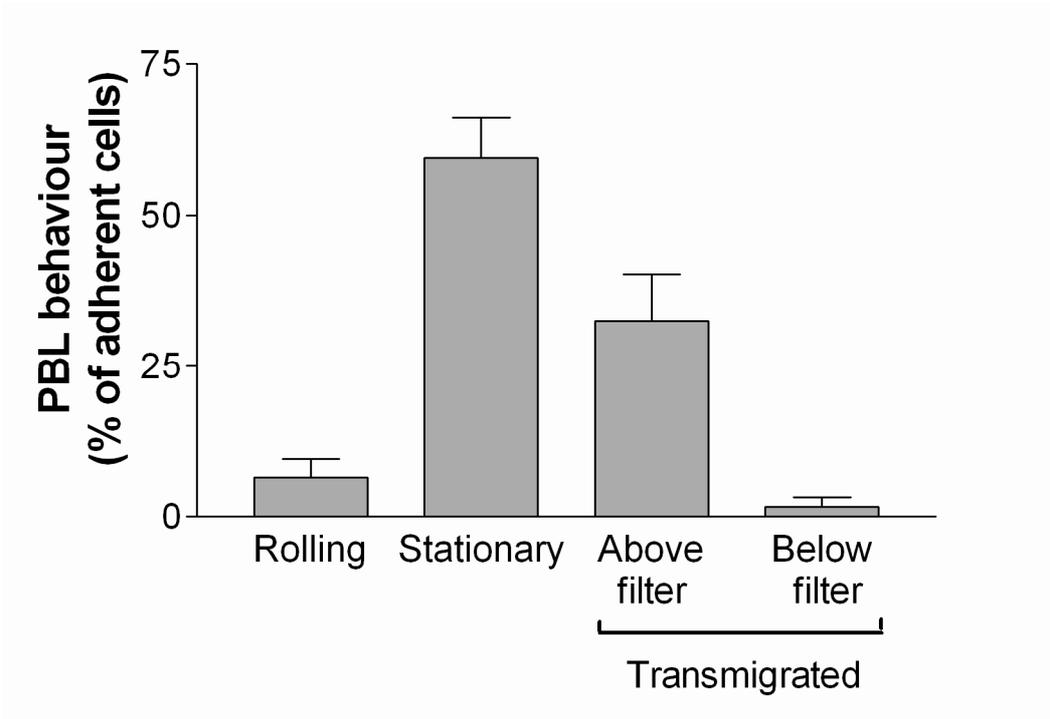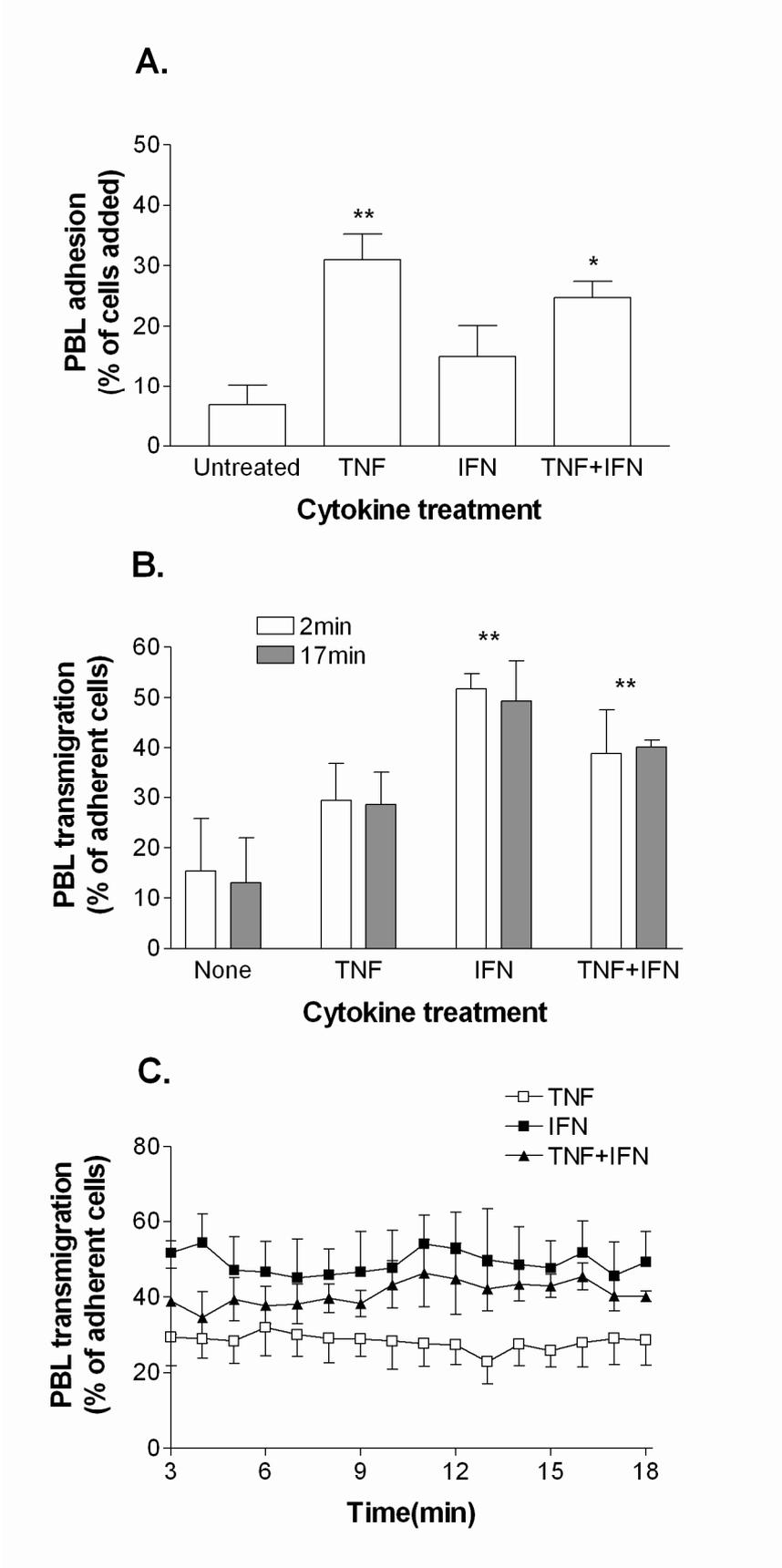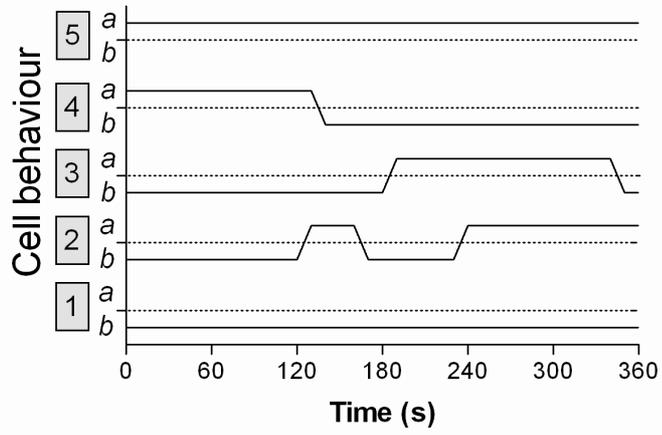
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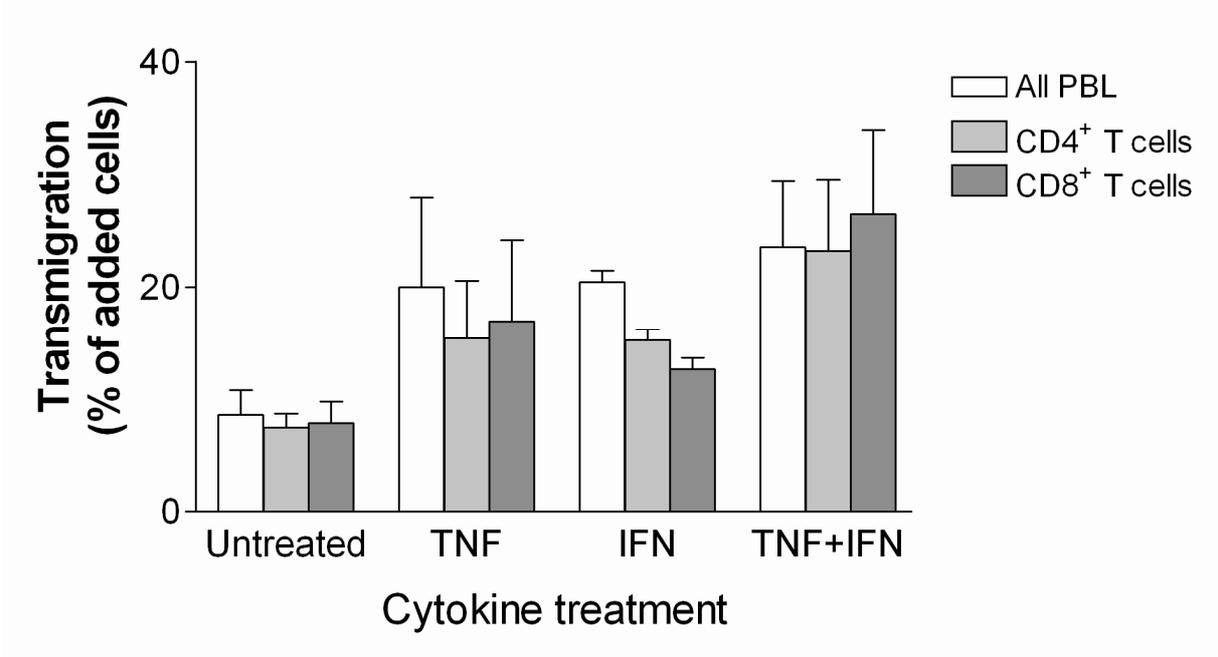
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