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# Experimental HUVEC expression of adhesion molecules in detecting effects of anti-interleukine agents

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Enhanced leukocyte recruitment is an inflammatory process that occurs during early phases of the vascular dysfunction that characterises atherosclerosis. We evaluated the impact of anti-TNF-α (adalimumab, infliximab and etanercept) and anti-IL-12/23 (ustekinumab) on interactions between human leukocytes and endothelial cells in a flow chamber that reproduced in vivo conditions. Clinical concentrations of anti-TNF-α were evaluated on the leukocyte recruitment induced by a variety of endothelial (TNF-α, interleukin-1β, lymphotoxin-α and angiotensin-II) and leukocyte (PAF, IL-12 and IL-23) stimuli related to inflammation and atherosclerosis. Treatment with anti-TNF-α, even before or after establishing the inflammatory situation induced by TNF-α, diminished leukocyte–endothelial cell in-teractions induced by this stimuli. Our results also implicated adhesion molecules (ICAM-1, VCAM-1 and E-selectin) in the actions of anti-TNF-α in terms of leukocyte adhesion to endothelium. However, anti-TNF-α drugs did not influence the actions of interleukin-1β, but prevented those of lymphotoxin-α and angiotensin-II. However, once established, inflammatory response elicited by the latter three stimuli could not be reversed. Pre-treatment with anti-TNF-α, also prevented leukocyte actions induced by IL-23 on PBMC rolling flux and rolling velocity and by IL-12 on PMN adhesion. Ustekinumab exhibited a more discreet profile, having no effect on leukocyte recruitment induced by any of the endothelial stimuli, while blocking the effects of IL-23 on leukocyte activation and those of IL-12 on PMN adhesion and PAF on PBMC rolling velocity. These findings endorse the idea that biological anti-inflammatory drugs, in particular anti-TNF-α, have the capacity to influence cardiovascular risk accompanying psoriasis and rheumatoid arthritis by ameliorating vascular inflammation.



## 1. Introduction

The increased risk of early cardiovascular disease (CVD) in pa-tients with autoimmune diseases such as psoriasis (PS) and rheumatoid arthritis (RA) has been attributed to an acceleration of atherosclerosis produced by the systemic inflammation that charecterizes abovementioned diseases on the subtle regard (Roubille et al., 2015; Westlake et al., 2011; Spah, 2008; Alexandroff et al., 2009). Manifestations of vascular inflammation involving interaction between adhesion molecules on leukocytes and on the endothelium are one of the early hallmarks of plaque formation and lead to the accumulation of leukocytes in the vessel wall (Ley et al., 2007). Although all immunosuppressive therapies have the potential to interfere with such interactions, there is mounting evidence that anti-in-flammatory biologic drugs used to treat PS and RA can ameliorate vascular systemic inflammation and reduce the risk of CVD (Westlake et al., 2011; Maki-Petaja et al., 2012; Ahlehoff et al., 2013; Tam et al., 2014; Ridker and Luscher, 2014; Nguyen and Wu, 2014; Roubille et al., 2015).

Evidence regarding anti-TNF-α therapies is particularly compelling, probably due to the widespread involvement of said cytokine in wide

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variety of vascular inflammatory responses (Elliott et al., 1994; Westlake et al., 2011; Roubille et al., 2015). However, theories concerning the mechanisms implicated are ra-ther spurious, as these drugs have been related both, with heart failure and a reduction in inflammation and plaque formation. There is also a case for analyzing anti-IL-12/23 agents given the potential implication of both these cytokines in plaque formation and their frequent use in the treatment of PS as alternatives to anti-TNF-α; however, some reports have related them with an increase in adverse cardiovascular events, which calls into ques-tion their validity (Ahlehoff et al., 2013; Alexandroff et al., 2009).

The aim of the present study was to characterize the effects of the most widely used anti-TNF-α-adalimumab (ADA, fully human), infliximab (INF, chimeric origin with human constant and murine variable regions) and etanercept [ETA, human p75 region of tumor necrosis factor-α (TNF-α) receptor linked to the fragment crys-tallisable (Fc) region of human IgG1]-on leukocyte–endothelial cell interactions and endothelial adhesion molecules induced by var-ious stimuli implicated in PS and RA, and to compare the effects with those of the anti-p40 subunit of IL-12/23 ustekinumab (UST) (Nestorov, 2005; Tracey et al., 2008; Koutruba et al., 2010; Price et al., 2007; Singh et al., 2011).

## 2. Materials and methods

We have soughted 250 pubmed database papers regarding diversity of differential effects of anti-TNF-α and anti-IL-12/23 agents on human leukocyte–endothelial cell interactions.

### 2.1. Human umbilical vein endothelial cells (HUVEC) cell culture

The best approach came out as HUVEC were harvested from freshly obtained umbilical cords by collagenase treatment, as previously described (De Pablo et al., 2010). In short, umbilical cord veins were rinsed of blood products with warm phosphate-buffered saline (PBS), after which the vein was filled with collagenase (1 mg/ml) for 17 min at 37 °C. The cords were then gently massaged to ensure detachment of en-dothelial cells from the vessel wall. The digest was collected, centrifuged and pelleted. The pellet was resuspended in en-dothelial cell growth medium (EGM-2) inside T25 culture flasks in which cells were cultured until confluence. After reaching con-fluence, primary cultures were detached with trypsin and trans-ferred to 6-well plate culture dishes. Passage 1 of these primary cultures was subsequently employed. For adhesion studies, HUVEC were cultured on fibronectin (5 μg/ml)-coated 25-mm plastic coverslips until confluent (~48 h).

### 2.2. Leukocyte isolation

Polymorphonuclear (PMN) or peripheral blood mononuclear (PBMC) cells were isolated from whole blood drawn from healthy volunteers and anticoagulated with sodium citrate (De Pablo et al., 2010). Samples were incubated with dextran (3%) for 45 min. PBMC and PMN in the supernatant were separated by gradient density centrifugation (250 g, 25 min) with Ficoll-Paque™ Plus. After red blood cell lysis, leukocytes were washed (HBSS without $Ca^{2+}$ or $Mg^{2+}$) and resuspended in complete RPMI media. The medical ethical committee of the Hospital Clínico Universitario de Valencia approved the study and all participating patients pro-vided written informed consent. All samples were compensated using the appropriate isotype-matched negative control.

### 2.3. Adhesion assay under flow conditions

The parallel plate flow chamber in vitro model has been de-scribed in detail previously (De Pablo et al., 2012; Cai et al., 2006). For adhesion assays, coverslips containing confluent HUVEC monolayers were placed in a circular recess in the bottom plate of the flow chamber (maintained at 37 °C), where a portion (5 mm × 25 mm) of the monolayer was exposed to the flow.

A circular glass window in the top plate of the chamber allowed real-time microscopic examination of the monolayer exposed to the flow. Images in a single field of view were recorded over a 5-min period during which leukocyte parameters were determined. Leukocyte rolling was calculated by counting the number of leukocytes passing a reference point in the monolayer during a period of 1 min. The velocities of 20 con-secutive leukocytes in the field of focus were determined by measuring the time required to travel a distance of 100 μm. Leukocyte adhesion was determined by counting the number of leukocytes that maintained stable contact with the monolayer for 30 s.

Two series of experiments were carried out in order to perform a comprehensive evaluation of the mechanisms and cell populations implicated and the pharmacological features of the drugs under assay. Initially, we focused on the role of the endothelium when activated by stimuli at doses that have been correlated with the onset of the inflammatory process in atherosclerosis (Libby, 2012; Alvarez et al., 2004): TNF-α (25 ng/ml, 4 h), IL-1β (inter-leukin-1β, 80 IU/ml, 4 h), LT-α (lymphotoxin-α, 3 ng/ml, 4 h) and Ang-II (angiotensin-II, 10 nM, 4 h) (Nakada et al., 1998; Yamagata et al., 2012; Suna et al., 2008; Mateo et al., 2006). When necessary, HUVEC were pre-treated (0.5 h before) or post-treated (0.5, 4 or 24 h after activation of the endothelium) with clinically relevant concentrations of ADA (3–11 mg/ml), INF (200 mg/ml), ETA (5 mg/ ml) or UST (3–15 mg/ml) (Rigby, 2007; Nestorov, 2005; Furst et al., 2006; Gottlieb et al., 2007).

The second group of experiments was designed to explore the role of leukocytes. Hence, PMN or PBMC were stimulated with the general leukocyte activator Platelet-activating factor (PAF; 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine, 10 mM, 1 h) (Mon-trucchio et al., 2000) or with stimuli specifically known to provoke leukocyte activation in psoriatic arthritis (Koutruba et al., 2010; Suzuki et al., 2014; Lowes et al., 2013): IL-12 (interleukin-12, 5 ng/ ml, 1 h) and IL-23 (interleukin-23, 50 ng/ml, 1 h) (Allavena et al., 1994). When necessary, PMN or PBMC were pre-treated (0.5 h before activation of leukocytes) with clinically relevant con-centrations of ADA and UST. Post-activation treatment could not be performed because of limits in the leukocytes' integrity.

### 2.4. Expression of adhesion molecules in HUVEC

Endothelial adhesion molecules [ICAM (intercellular adhesion molecule)-1, VCAM (vascular adhesion molecule)-1 and E-selectin] were analyzed as described previously (Alvarez et al., 2004; Ibiza et al., 2009). HUVEC were grown to confluence in 6-well plates and thereafter stimulated with TNF-α (25 ng/ml) for 4 h at 37 °C. Some cells were treated with ADA (0.01–11 mg/ml) 0.5 h before or 24 h after activation of the endothelium. Cells were detached with trypsin, placed in suspension, incubated with the corresponding antibody (20 min, on ice, in darkness), fixed (in formaline 10%) and analyzed for protein expression according to forward- and side-scatter characteristics in a FACS Calibur cytometer (BD, Franklin Lakes, NJ, USA). In each case, 10,000 cells were analyzed and the mean of the specific fluorescence intensity was employed as a marker of the expression of the epitope.

### 2.5. Materials

Dulbecco's PBS, with ($DPBS^{+}$) or without (DPBS ) $Ca^{2+}$ and $Mg^{2+}$, EGM-2 culture media, HBSS and fetal bovine serum were acquired from LONZA (Verviers, Belgium). Recombinant TNF-α, human serum albumine (HSA, Albuminate 25%), RPMI1640 sup-plemented with 20 mM HEPES, fibronectin, formaline, dextran, IL-1β, IL-12, PAF and Ang-II were supplied by Sigma Chemical Co (St. Louis, MO, USA). LT-α and IL-23 were acquired from Prospecbio. Ficoll-Paque TM Plus was supplied by GE Healthcare Life Sciences (Amersham, UK). Plastic coverslips with a diameter of 25 mm were obtained from Nunc, supplied by Thermo Fisher Scientific. PBS, collagenase, and trypsin were acquired from Gibco Invitrogen, Life Technologies (Carlsbad, CA, USA). Fluorescein isothyocianate (FITC) and phycoerithrin (PE) conjugated control antibodies and anti-bodies against E-selectin, ICAM-1 or VCAM-1 were purchased from BD Bioscience. Adalimumab (Hu-mira$^{S}$), infliximab (Remicade$^{S}$), etanercept (Enbrel$^{S}$) and uste-kinumab (Stelara$^{S}$) were used in the form of their clinically available preparations.

### *2.6. Data analysis and statistics*

Data are mean7S.E.M of 3–6 experiments. Statistical sig-nificance was considered to be o0.05 by one-way ANOVA analysis of variance

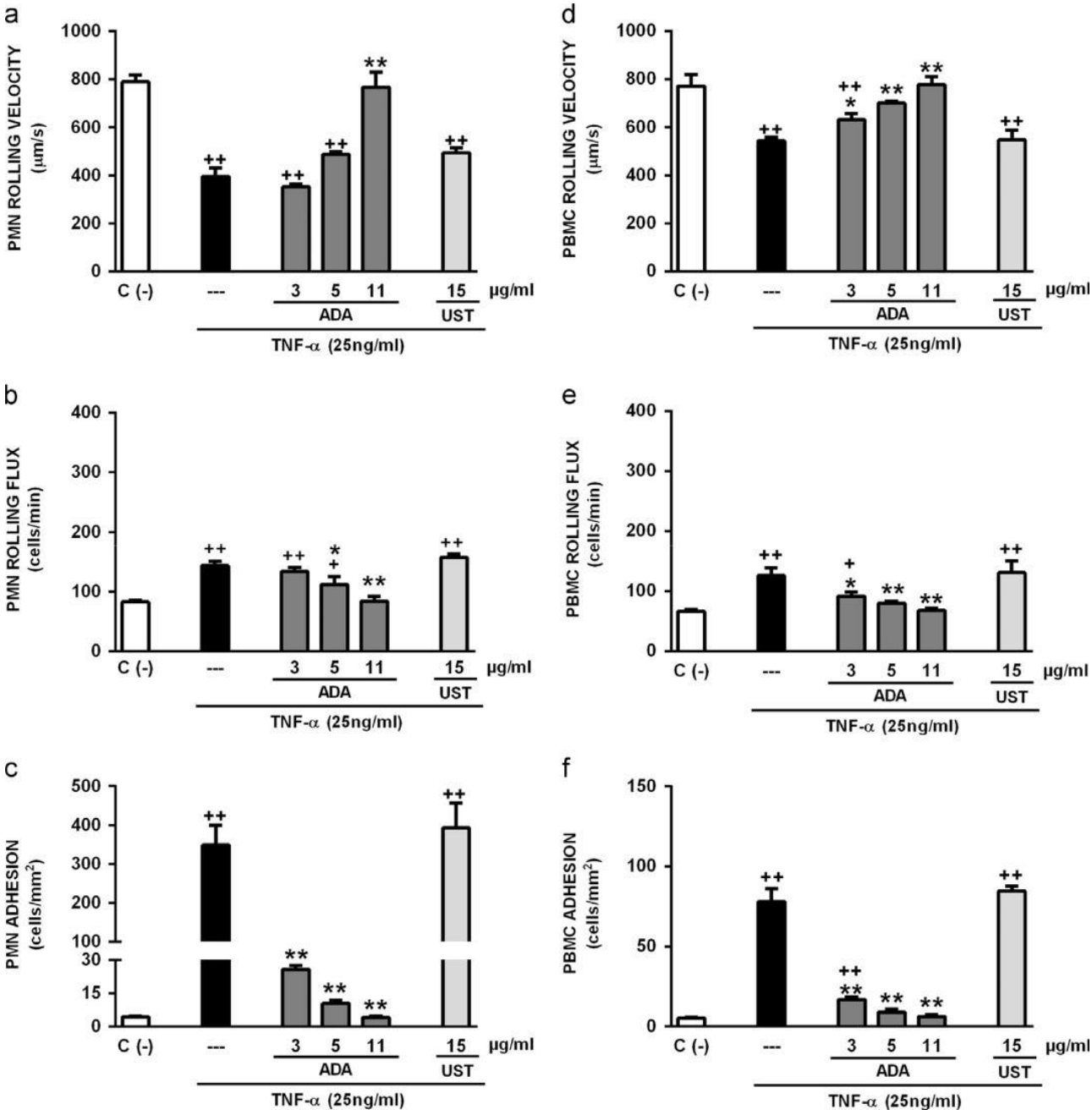


**Fig. 1. Effects of pre-treatment with adalimumab or ustekinumab on PMN- or PBMC-endothelial cell interactions induced by TNF-α.** HUVEC were pre-treated (0.5 h) with adalimumab (ADA, 3–11 mg/ml) or ustekinumab (UST, 15 mg/ml) before stimulation (4 h) with tumor necrosis factor-α (TNF-α, 25 ng/ml) or medium (control). Poly-morphonuclear (PMN) cells rolling velocity (a), rolling flux (b) and adhesion (c) and peripheral blood mononuclear cells (PBMC) rolling velocity (d), rolling flux (e) and adhesion (f) were quantified after assembling the flow chamber. Results are mean S.E.M., n>4. $^{+}$P<0.05 or $^{++}$P<0.01 vs. corresponding value in control group and *Po0.05 or **P <0.01 vs. corresponding value in TNF-α-treated group (ANOVA followed by Newman–Keuls test).

of variance, with Newman–Keuls post-test correction to compare multiple variances.

## 3. Results

### *3.1. Effects on cell interactions induced by endothelial stimuli*

As expected, only a small number of PMN or PBMC cells ad-hered to unstimulated HUVEC (Figs. 1, 2, 4, 5 and 6), thus re-producing the conditions of a normal non-inflamed vessel (Supplementary material S1). In order to mimic the typical scenario of the development of the athero-sclerotic plaque, HUVEC were sti-mulated with TNF-α, IL-1β, LT-α or Ang-II.

TNF-α (Supplementary material S2) induced a significant de-crease in the rolling velocity and an increase in the rolling flux and adhesion of both PMN and PBMC (Figs. 1 and 2). ADA prevented leukocyte (PMN and PBMC)-endothelial cell interactions induced by TNF-α in a dose-dependent manner (Supplementary movies S3, S4 and S5). The actions of the anti-TNF-α on adhesion were more pronounced than those on rolling, and this pattern was particu-larly obvious in PMN, in which the lowest concentration employed (3 mg/ml) significantly reduced adhesion while having no effect on rolling velocity or flux (Fig. 1). Of note, 24 h treatment with concentrations of ADA lower than 0.1 mg/ml specifically reversed

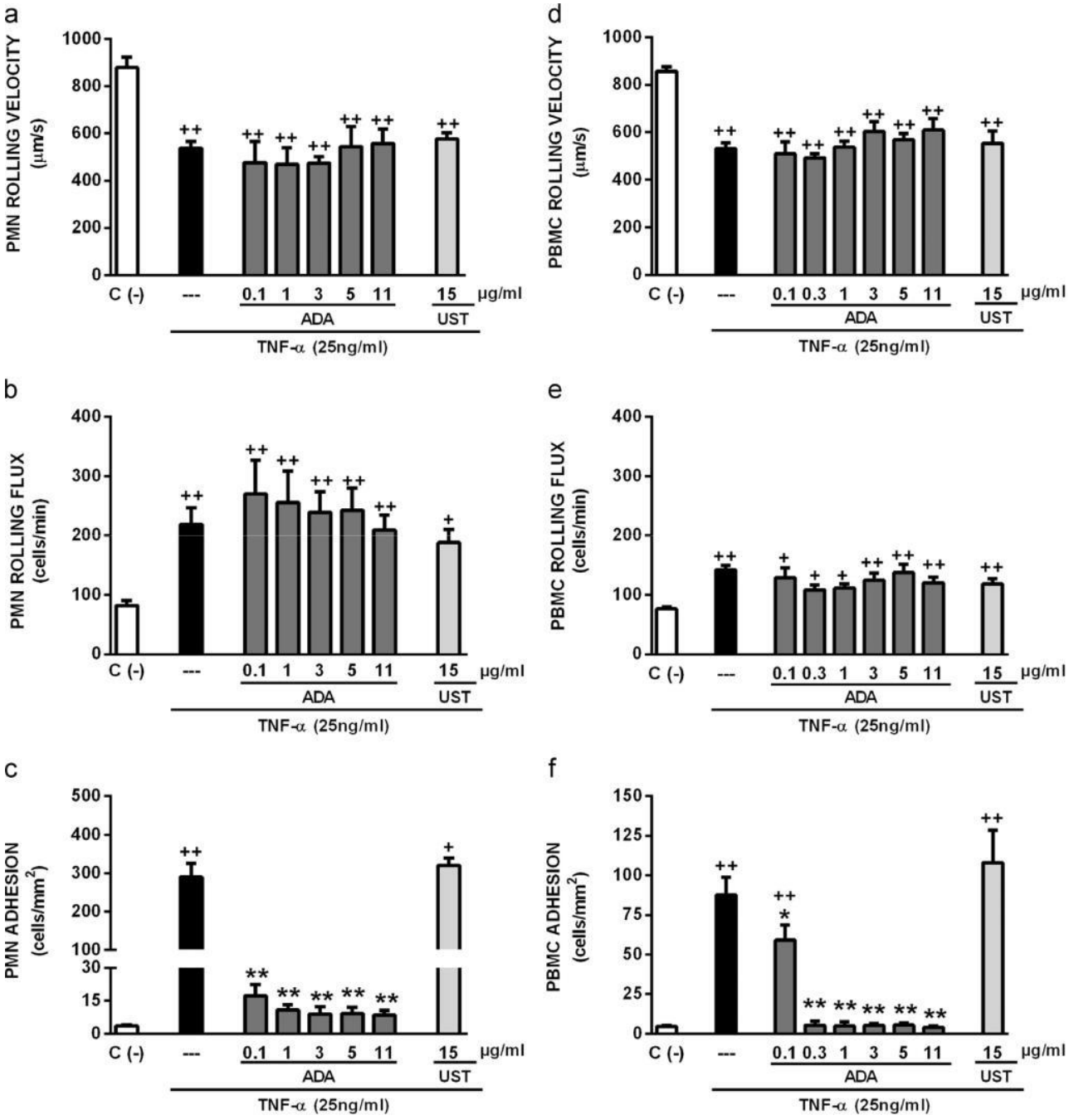


**Fig. 2. Capacity of adalimumab and ustekinumab to reverse PMN- or PBMC-endothelial cell interactions induced by TNF-α.** HUVEC were treated (4 h) with tumor necrosis factor-α (TNF-α, 25 ng/ml) or medium (control) before incubation with adalimumab (ADA, 0.1–11 mg/ml) or ustekinumab (UST, 15 mg/ml) for a further 24 h period. Polymorphonuclear (PMN) cells rolling velocity (a), rolling flux (b) and adhesion (c) and peripheral blood mononuclear cells (PBMC) rolling velocity (d), rolling flux (e) and adhesion (f) were quantified after assembling the flow chamber.

**Table 1**
Capacity of adalimumab, infliximab and etanercept to reverse PMN- or PBMC-endothelial cell interactions induced by TNF-
HUVEC were treated (4 h) with tumor necrosis factor-α (TNF-α, 25 ng/ml) or medium (control) before incubation with adalimumab (ADA, 11 mg/ml), infliximab (INF, 200 mg/ ml) or etanercept

| | | Vehicle | TNF-α | TNF-α+ADA | TNF-α+INF | TNF-α+ETA |
|---|---|---|---|---|---|---|
| **PMN** | **Rolling velocity (μm/seg)** | 898,81+26,7 | 546,33±24,8[aa] | 557,87±61,9[aa] | 563,25±70,6[aa] | 651,33+75,7[aa] |
| | **Rollingflux (cells/min)** | 65,04±5,7 | 218,75±28,3[aa] | 209,25±25,3[aa] | 176,57±14,5[aa] | 166,08±23,1[aa] |
| | **Adhesion (cells/mm²)** | 2,62±0,3 | 290,32±35,1[aa] | 8,46±2,2[bb] | 11,25±7,6[bb] | 10,73±6,1[bb] |
| **PBMC** | **Rolling velocity (lm/seg)** | 875,72±22,8 | 531,38±25,1[aa] | 609,33±49,8[aa] | 667,10±28,9[aa] | 659,23±28,9[aa] |
| | **Rollingflux (cells/min)** | 65,31±5,9 | 142,33±7,5[aa] | 120,5±9,3[aa] | 106,67±8,8[aa] | 108,67±14,5[aa] |
| | **Adhesion (cells/mm²)** | 3,22±0,4 | 87,72±11,1[aa] | 3,93±1,2[bb] | 1,95±0,7[bb] | 4,94±0,8[bb] |

(ETA, 5 mg/ml) for a further 24 h period. Polymorphonuclear (PMN) cells rolling velocity, rolling flux and adhesion and peripheral blood mononuclear cells (PBMC) rolling velocity, rolling flux and adhesion were quantified after assembling the flow chamber. Results are mean7S.E.M., n Z3.

[aa] Po0.01 vs. corresponding value in control group.

[bb] Po0.01 vs. corresponding value in TNF-α-treated group (ANOVA followed by Newman–Keuls test).

the adhesion of leukocytes following endothelium activation (4 h) by TNF-α without affecting earlier steps of the vascular in-flammatory process, such as leukocyte rolling velocity and flux (Fig. 2). Shorter periods of incubation with ADA (i.e., 0.5 h or 4 h) had no effect on the changes in leukocyte parameters induced by 4 h incubation with TNF-α (data not shown). 24 h incubation with INF and ETA produced a similar profile to ADA in terms of rever-sing leukocyte recruitment induced by 4 h TNF-α; i.e., leukocyte adhesion was reversed without disturbing rolling velocity or flux (Table 1). UST, whether used pre- or post-TNF-α, had no effect on any of the leukocyte–endothelial cell interactions induced by this inflammatory cytokine.

Supplementary material related to this article can be found online at http://dx.doi.org/10.1016/j.ejphar.2015.08.054.

TNF-α (25 ng/ml) increased the expression of VCAM-1, ICAM-1 and E-selectin (Fig. 3, Supplementary Fig. S1) in HUVEC. ADA both prevented and reversed the induction of these adhesion mole-cules, even at very low concentrations (0.3 mg/ml).

IL-1β and LT-α also induced a significant decrease in the rolling velocity and an increase in the rolling flux and adhesion of both PMN and PBMC (Figs. 4 and 5). In the case of Ang-II, there was a similar and significant decrease of rolling velocity and an increase in rolling flux both in PMN and PBMC, while only PMN adhesion was increased by this peptide (Figs. 4 and 5). TNF-α evidently had no bearing on the effects of IL-1β on leukocyte recruitment, since pre-treatment with ADA did not alter the response of PMN or PBMC to this stimulus. On the contrary, some TNF-α-activated receptors seemed to be implicated in the inflammatory actions of LT-α, as all parameters of PMN- and PBMC endothelial interactions elicited by this cytokine were significantly prevented by the highest dose of ADA employed (11 mg/ml). Once again, ADA had a greater impact on adhesion (a complete return to control levels) than on rolling velocity and flux, which were only partially pre-vented (Fig. 4). ADA's effect on the actions of Ang-II on PMN were similar to those on LT-α, while Ang-II has no bearing on PBMC (Fig. 4). Finally, pre-incubation with UST had no effect on leuko-cyte–endothelial cell interactions induced by IL-1β, LT-α or Ang-II (Fig. 4). In a scenario where the endothelium had previously been ac-tivated by IL-1β, LT-α or Ang-II (4 h), 24 h post-treatment with either ADA or UST did not reverse the leukocyte–endothelial cell interactions elicited by all three stimuli (Fig. 5).

### *3.2. Effects on cell interactions induced by leukocyte stimuli*

Leukocytes were stimulated with three mediators that have been correlated with inflammation in atherosclerosis or PS. PAF, a broad and unspecific leukocyte activator, induced a significant decrease in rolling velocity and an increase in rolling flux and adhesion of both PMN and PBMC (Fig. 6). ADA had no effect on these actions, and, while UST also lacked an influence on any of the effects of PAF on PMN, it specifically reduced PBMC rolling flux. IL-23 induced a reduction in PMN and PBMC rolling velocity and increased rolling flux. While ADA reversed the effects of IL-23 on PBMC only, UST blocked these effects on both leukocyte popula-tions. IL-12 had no effect at physiological doses and affected only PMN at very high doses (50-fold), reducing their rolling velocity and increasing their rolling flux and adhesion. Both ADA and UST reversed the effects of IL-12 on adhesion, but not on rolling velo-city or rolling flux (Fig. 6).

## 4. Discussion

The exacerbation of leukocyte–endothelial interactions is an inflammatory process associated with the early phases of the vascular dysfunction that characterises important cardiovascular diseases (Krieglstein and Granger, 2001). The aim of the present study was to compare the actions of two of the most potent anti-inflammatory treatments for PS – anti-TNF-α and anti-IL-12/23 agents – on a variety of endothelial (TNF-α, IL-1β, LT-α and Ang-II) and leukocyte (PAF, IL-12 and IL-23) stimuli implicated in en-dothelial dysfunction and/or PS pathogenesis (Tracey et al., 2008; Koutruba et al., 2010; Price et al., 2007; Singh et al., 2011). We employed a dynamic in vitro model that is widely used to analyze the multistep recruitment of leukocytes and the vascular anti-in-flammatory actions of drugs (Victor et al., 2011; Luu et al., 2007). In this system, human leukocytes (PMN and PBMC) flow over a monolayer of human endothelial cells in a way that closely re-sembles processes (rolling and adhesion) that are critical for he-mostasis and vascular cell integrity and which precede the for-mation of an atherosclerotic plaque in vivo (Goetz et al., 1999; De Pablo et al., 2013). Our results demonstrate that clinically relevant concentrations of the three anti-TNF-α drugs tested, but not of UST, significantly undermine leukocyte recruitment when the endothelium is activated. However, when leukocytes are activated, UST reduces some, but not all, of these interactions, while, sur-prisingly, ADA also exerts a significant effect. Pre-incubation with concentrations of ADA (3–11 μg/ml) that mimicked those present in patients (Rigby, 2007; Nestorov, 2005; Furst et al., 2006) prevented all the effects induced by subsequent administration of TNF-α, one of the most potent stimuli of leu-kocyte–endothelial interplay (Griffin et al., 2012).

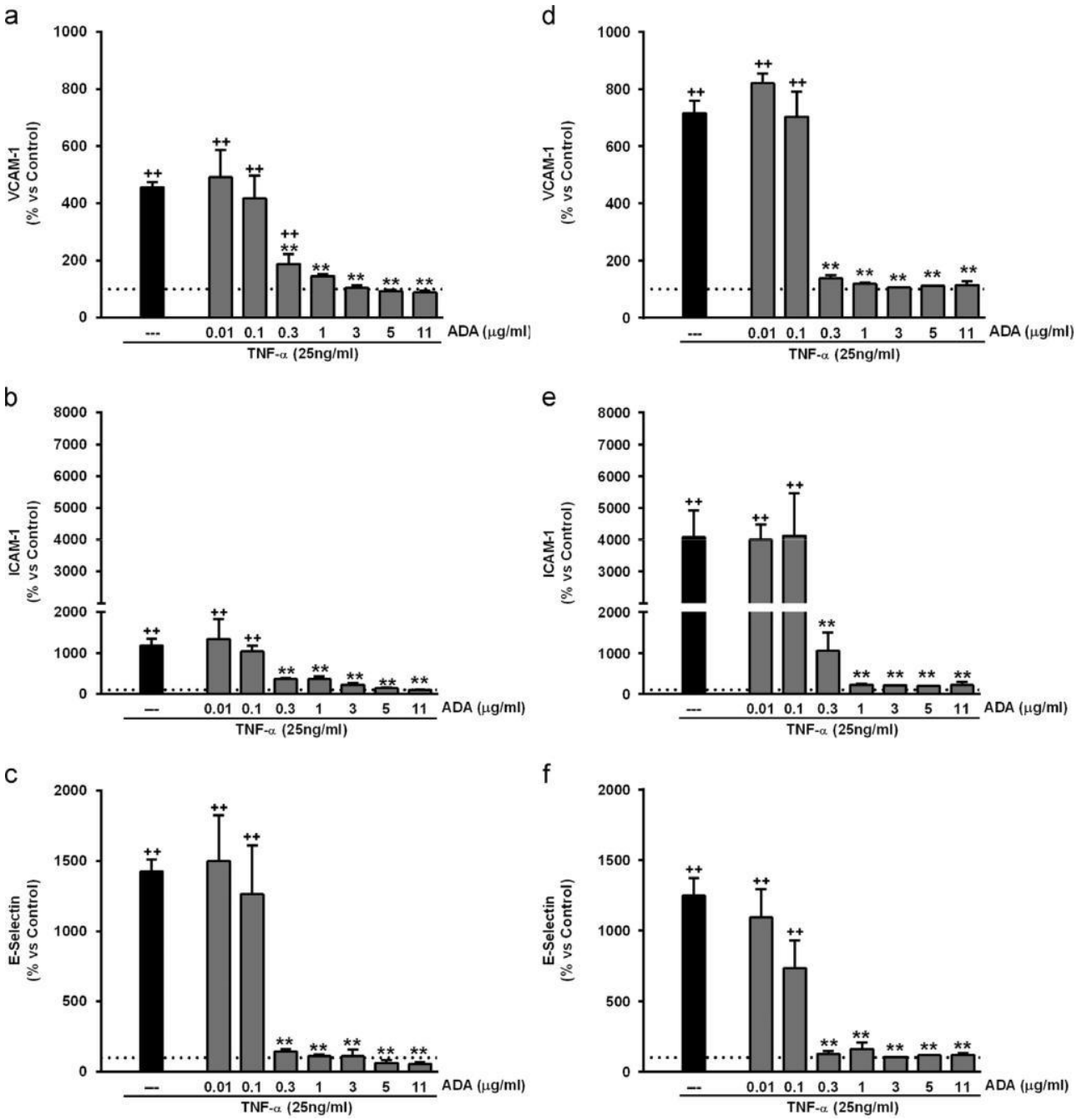


**Fig. 3. Effects of adalimumab on the expression of endothelial adhesion molecules induced by TNF-α.** HUVEC were treated with adalimumab (ADA, 0.01–11 mg/ml) 0.5 h before (a–c) or 24 h after (d-f) stimulation (4 h) with tumor necrosis factor-α (TNF-α, 25 ng/ml) or medium (control). Expression of vascular cell adhesion molecule-1 (VCAM-1) (a and d), intracellular adhesion molecule-1 (ICAM-1) (b and e) and E-selectin (c and f) was analyzed by flow cytometry. Fluorescein isothyocianate (FITC) or phycoerithrin (PE)-fluorescence values are expressed as a percentage of the mean fluorescence intensities of control cells (100%). Results are mean7S.E.M., nZ3. $^{+}P<.05$ or $^{++}P<0.01$ vs. corresponding values in control group and $^{**}P>0.01$ vs. corresponding value in TNF-α-treated group (ANOVA followed by Newman–Keuls test).

Furthermore, the effects arising from deferred blockade of TNF receptors were not noticeable after short periods (0.5 h or 4 h) of anti-TNF-α incubation and reached significance only after 24 h, which is evidence of the multiple inflammatory pathways trig-gered following administration of the cytokine and its reduced role in subsequent responses. In addition, ADA had no influence on the actions of IL-1β, but prevented the actions of LT-α and Ang-II when pre-administered. However, ADA failed to reverse the inflammatory response elicited by the latter two stimuli once it had been established. The effects described with ADA, which binds to TNF-α and avoids the interaction of this cytokine with its two receptors (TNF receptor 1 and TNF receptor 2) (Van et al., 2011), are generally in line with published evidence implicating TNF-α in the actions of the stimuli we have evaluated in this study. Thus, whereas IL-1β exerts its action through receptors unrelated to TNF-α (Dinarello, 2011), LT-α exhibits certain similarity with the tertiary and quaternary structure of TNF-α (Buhrmann et al., 2013), which probably explains its susceptibility to TNF-α blockade. In-deed, its actions are a result of its binding to a variety of receptors, including TNF receptor 1 and TNF receptor 2 and the more specific LT-β receptor (Etemadi et al., 2013). Lastly, Ang-II binds to angio-tensin-II receptor type I, which activates signal pathways shown to upregulate TNF-α production (Abadir et al., 2011).

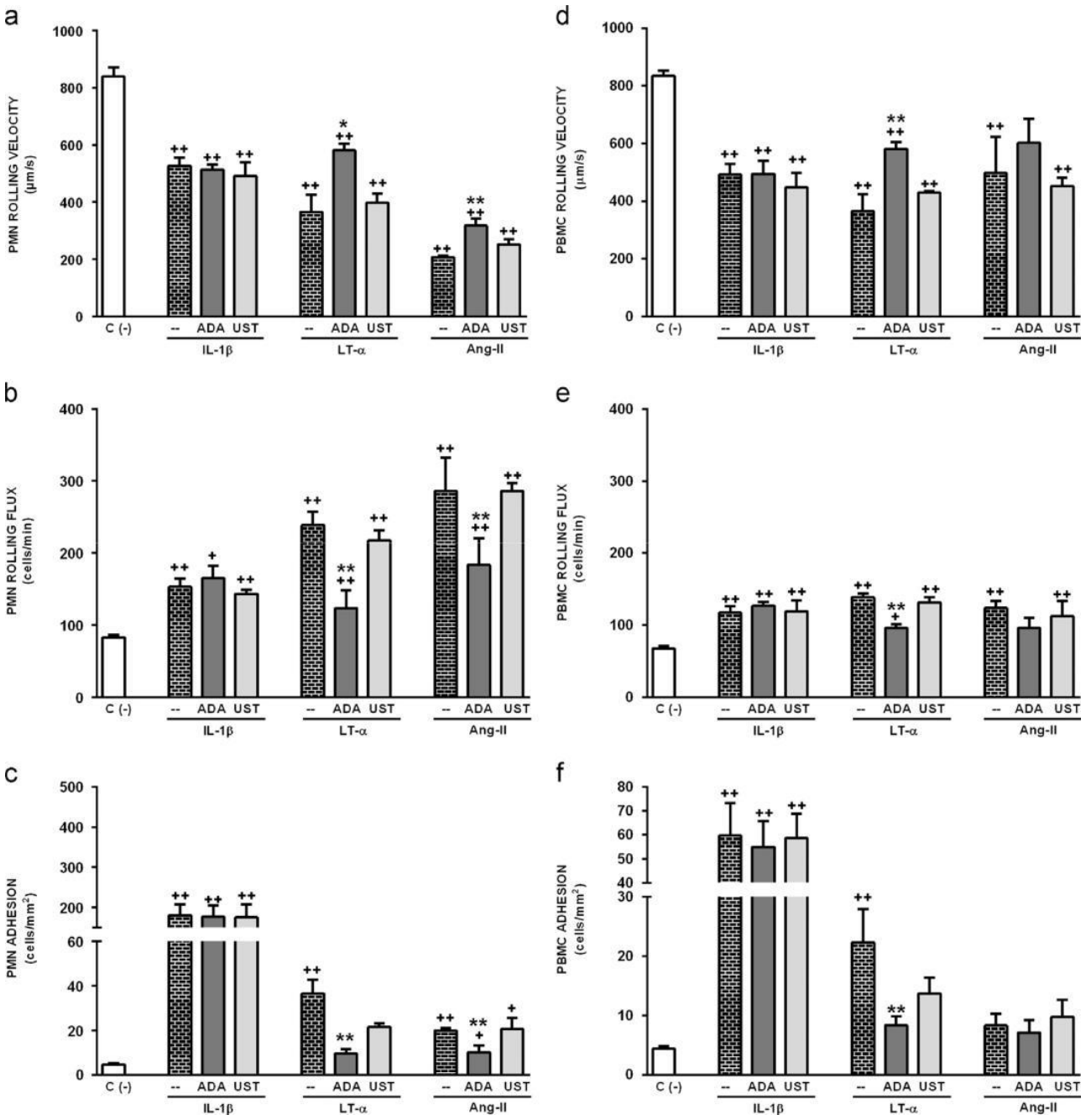


**Fig. 4. Effects of pre-treatment with adalimumab or ustekinumab on PMN- or PBMC-endothelial cell interactions induced by IL-1β, LT-α or Ang-II.** HUVEC were pre-treated (0.5 h) with adalimumab (ADA, 11 mg/ml) or ustekinumab (UST, 15 mg/ml) before stimulation (4 h) with interleukin-1β (IL-1β, 80 IU), lymphotoxin-α (LT-α, 3 ng/ml), angiotensin II (Ang-II, 10 nM) or medium (control). Polymorphonuclear (PMN) cells rolling velocity (a), rolling flux (b) and adhesion (c) and peripheral blood mononuclear cells (PBMC) rolling velocity (d), rolling flux (e) and adhesion (f) were quantified after assembling the flow chamber. Results are mean7S.E.M., nZ3. $^{+}P<0.05$ or $^{++}P<0.01$ vs. corresponding value in control group and $^{*}P<0.05$ or $^{**}P<0.01$ vs. corresponding value in IL-1β, LT-α or Ang-II-treated group (ANOVA followed by Newman– Keuls test).

The interaction of leukocytes with endothelial cells is controlled by the interplay of adhesion molecules present in both cell groups (Ley et al., 2007; Muller, 2009). However, until now, there has been very little evidence of the implication of these molecules in the protective cardiovascular actions of anti-TNF-α, and the few existing reports have assessed non-functional soluble forms of adhesion molecules in plasma (Gonzalez-Gay et al., 2006; den Broeder et al., 2002; Klimiuk et al., 2004). In addition, most studies have been performed with INF and ETA, and so very little data has been obtained regarding newer, "non-chimeric" anti-TNF-α. Our results clearly endorse a role for adhesion molecules in the actions of anti-TNF-α, particularly as we have measured the expression of functional mole-cules – not just soluble ones – in the very same endothelial cell monolayer in which the flow chamber studies were performed. We can confirm previous evidence that TNF-α induces the expression of ICAM-1, VCAM-1 and E-selectin (Zhang et al., 2002), and we demonstrate that this enhancement is prevented and reversed by concentrations of ADA well below clinical levels. This profile of the actions of anti-TNF-α is of special relevance, since adhesion represents a stable interaction of white cells with the endothelium and occurs immediately in retrospective ground.

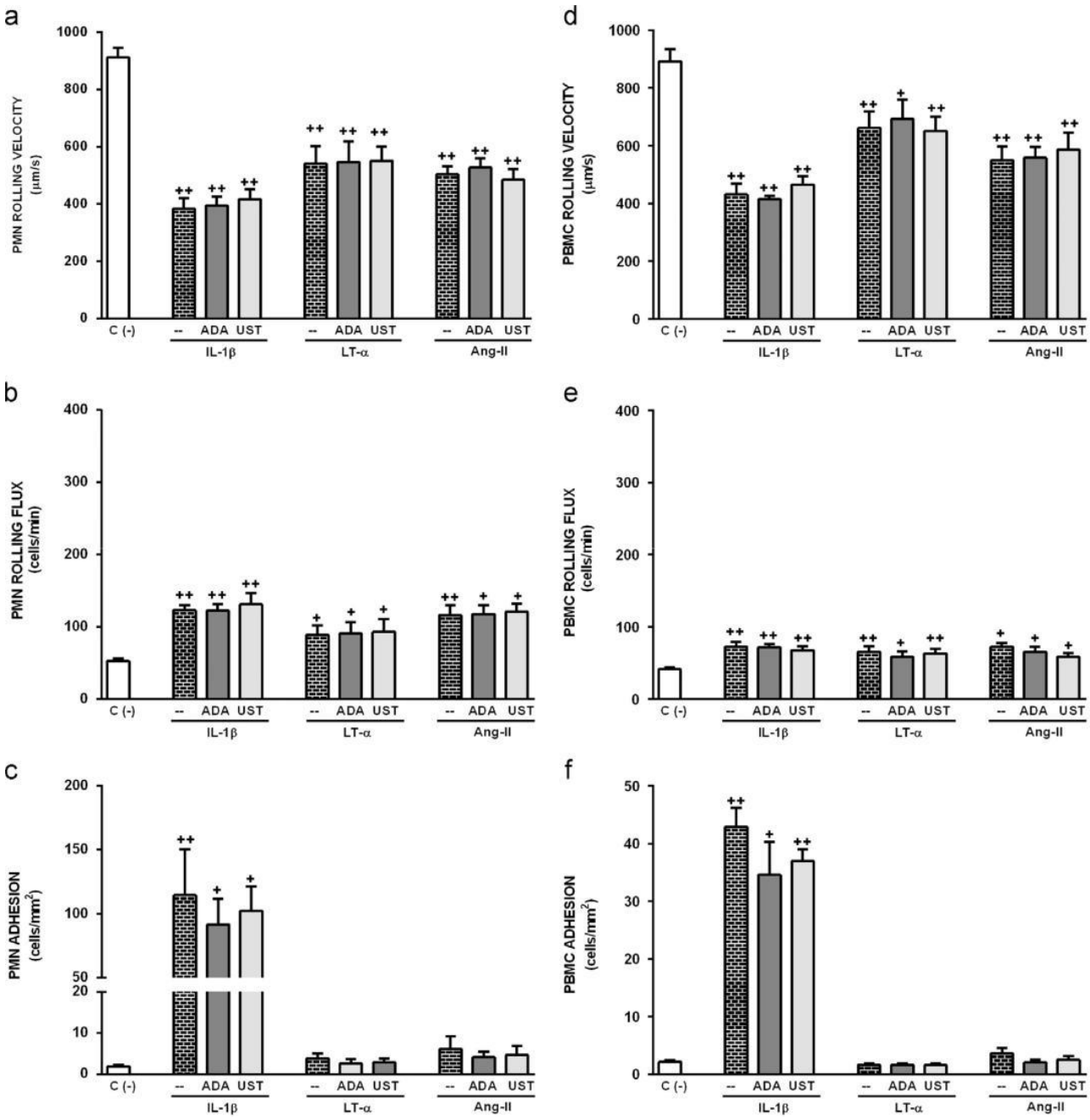


**Fig. 5. Capacity of adalimumab and ustekinumab to reverse PMN- or PBMC-endothelial cell interactions induced by IL-1β, LT-α or Ang-II.** HUVEC were treated (4 h) with interleukin-1β (IL-1β, 80 IU), lymphotoxin-α (LT-α, 3 ng/ml), angiotensin II (Ang-II, 10 nM) or medium (control) before incubation with adalimumab (ADA, 11 mg/ml) or ustekinumab (UST, 15 mg/ml) for a further 24 h period. Polymorphonuclear (PMN) cells rolling velocity (a), rolling flux (b) and adhesion (c) and peripheral blood mononuclear cells (PBMC) rolling velocity (d), rolling flux (e) and adhesion (f) were quantified after assembling the flow chamber. Results are mean7S.E.M., n Z4. $^{+}$Po0.05 or $^{++}$P<0.01 vs. corresponding value in control group (ANOVA followed by Newman–Keuls test).

relevance, since adhesion represents a stable interaction of white cells with the endothelium and occurs immediately prior to dya-pedesis/emigration, the “point of no return” in leukocyte in-flammatory vascular enrollment (Muller, 2009). Of note, this response is in keeping with that of drugs such as natalizumab (anti-$\alpha_4$ integrin antibody) and roquinimix, which also abolish leuko-cyte adhesion, but not rolling, under TNF-α-stimulating conditions (Coisne et al., 2009; Zhang et al., 2000). UST had no effect on the leukocyte recruitment induced by any of the four stimuli used to activate the endothelium, as was ex-pected given the limited evidence of the implication of IL-12 and IL-23, whose p40 subunit is blocked by UST, in the inflammatory responses of HUVEC. However, UST prevented the effects of IL-23 on leukocyte activation and the PMN adhesion induced by IL-12. UST would seem to block both cytokines, thus avoiding their binding to the IL-12 β1 receptor chain of both IL-12 (IL-12 β1/β2) and IL-23 (IL-12β1/IL-23) receptor complexes (Suzuki et al., 2014; Lowes et al., 2013; Benson et al., 2011). IL-12 exerted a differential profile on both leukocyte subpopulations in terms of leukocyte–endothelial cells interactions by inducing them only on PMN but not on PBMC. Similar results were observed by Allavena et al. (1994) when evaluated the chemotactic response of PMN and PBMC to the cytokine IL-12. However, the effects of IL-12 on PMN rolling were not blocked by UST, but this result needs to be in-terpreted with caution given the clinical concentration of IL-12 on psoriatic patients (36.6717.93 pg/ml) Arican et al., 2005).

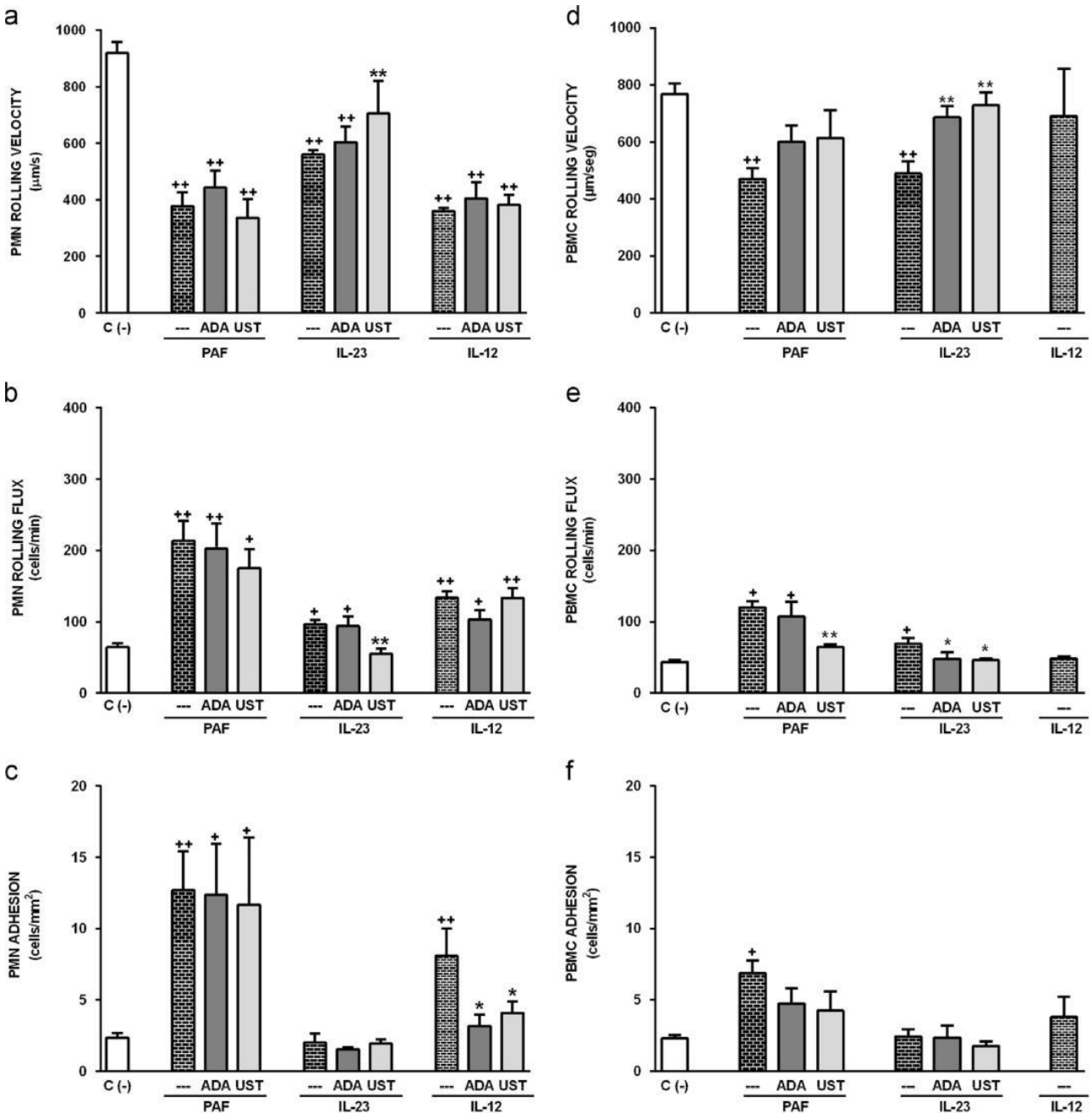


**Fig. 6. Effects of pre-treatment with adalimumab or ustekinumab on PMN- or PBMC-endothelial cell interactions induced by PAF, IL-23 or IL-12.** PMN and PBMC were pre-treated with adalimumab (ADA, 11 mg/ml) or ustekinumab (UST, 15 mg/ml) for 0.5 h before stimulation (1 h) with platelet-activating factor (PAF, 10 mM), interleukin-23 (IL-23, 50 pg/ml), interleukin-12 (IL-12, 5 ng/ml) or medium (control). Polymorphonuclear (PMN) cells rolling velocity (a), rolling flux (b) and adhesion (c) and peripheral blood mononuclear cells (PBMC) rolling velocity (d), rolling flux (e) and adhesion (f) were quantified after assembling the chamber. Results are mean7S.E.M., nZ4. $^{þ}$Po0.05 or $^{þ\,þ}$Po0.01 vs. corresponding value in control and *Po0.05 or **P o0.01 vs. corresponding value in PAF or IL-23 or IL-12-treated group (ANOVA followed by Newman–Keuls test).

In summary, our results demonstrate that clinically relevant concentrations of three distinct and widely used anti-TNF-α drugs inhibit the leukocyte recruitment induced by a wide variety of stimuli that act on both the endothelium and leukocytes. UST exhibits a more discreet profile, blocking the actions of IL-23 and IL-12 on leukocyte activation without modifying those arising from endothelial stimuli activation. These findings endorse the idea that treatment with currently prescribed biological anti-in-flammatory drugs can have a positive impact on CVD risk by reducing the vascular systemic inflammation associated with PS and RA. However, the degree of such effects varies considerably; in light of our in vitro results obtained in human cells, the wider profile of actions of anti-TNF-α – owing to the ubiquity of the mediator in the inflammatory cascade – seems to promise a more potent, and thus more clinically relevant effect. Importantly, this potential CV effect is in addition to these drugs' ability to coun-teract the inflammatory conditions that characterize the dermatologic and rheumatic diseases.

*Conflict of interest*

None of the other authors report any potential conflicts.

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