Functional role of gap junctions in cytokine-induced leukocyte adhesion to endothelium in vivo

Loreto P. Véliz,1 Francisco G. González,1 Brian R. Duling,3 Juan C. Sáez,1,2 and Mauricio P. Boric1

1Departamento de Ciencias Fisiológicas, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, and 2Núcleo Milenio Inmunología e Inmunoterapia, Santiago, Chile; and 3Cardiovascular Research Center, University of Virginia School of Medicine, Charlottesville, Virginia

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Véliz LP, González FG, Duling BR, Sáez JC, Boric MP. Functional role of gap junctions in cytokine-induced leukocyte adhesion to endothelium in vivo. Am J Physiol Heart Circ Physiol 295: H1056–H1066, 2008. —To assess the hypothesis that gap junctions (GJs) participate in leukocyte-endothelium interactions in the inflammatory response, we compared leukocyte adhesion and transmigration elicited by cytokine stimulation in the presence or absence of GJ blockers in the hamster cheek pouch and also in the cremaster muscle of wild-type (WT) and GJ blocker-treated animals. We also studied the hypothesis that gap junctions (GJs) participate in inflammation; tumor necrosis factor-α (TNF-α); endothelial-connexin 43 knock-out mice; gap junction blockers

THE INFLAMMATORY RESPONSE is mediated by cells of the innate immune system and occurs in tissues affected by a traumatic injury, or after invasion by a pathogenic agent. Inflammation involves numerous vascular events, including arteriolar vasodilatation and blood flow increase, enhanced vascular permeability to macromolecules, and accumulation and infiltration of leukocytes (2, 32, 35). The secretion of several proinflammatory molecules, such as cytokines, chemokines, and neurotransmitters, orchestrates these events (2). In the damaged region, leukocytes are directed by the action of chemotactic agents and cytokines to attach to the vessel wall and to leave the circulation (35, 38). Leukocyte adhesion to the endothelium and subsequent transmigration depend on the expression of specific adhesion molecules on the surface of each cell type, following the action of cytokines (2, 38). Following initial contact, leukocytes roll over the endothelial surface as a result of the interactions between L-selectin, expressed on leukocytes, and E- and P-selectin, expressed on the endothelial cell. The selectins bind to the endothelial cell surface at inflamed sites, with their ligands, Sialyl Lewis-X-containing glycoproteins (36). Binding causes rolling followed by firm adhesion, resulting from strong interactions between integrins and cell adhesion molecules located on the surface of leukocytes and endothelial cells, respectively (2, 35). Adhesion of leukocytes leads to migration through the endothelium in a process called diapedesis. Leukocyte transmigration requires a transient increase of intracellular free Ca2+ in endothelium at the region at which leukocyte transmigration will occur (2, 38). All of these events occur on postcapillary venules, where the hemodynamic conditions of blood flow and shear stress are adequate (24).

It has been suggested that gap junctions (GJs) might be involved in diverse processes of the adaptive immune system response, for instance, antigen presentation and lymphocyte maturation and proliferation. Moreover, components of GJs have also been found on the surface of cells that participate in the innate immune response, such as endothelial and epithelial cells, as well as monocytes/macrophages and polymorphonuclear cells (44). In reference to white blood cells, it has been shown that freshly isolated human polymorphonuclear cells treated with endothelial cell-conditioned medium plus proinflammatory molecules form aggregates, express the GJ proteins connexin (Cx) 40 (Cx40) and Cx43, and show transfer of Lucifer yellow, a fluorescent dye that permeates through GJs (6). Intercellular transfer of Lucifer yellow has also been described to occur between activated monocytes/macrophages (13), which, in addition to Cx43, also express Cx37 (51). Moreover, subpopulations of human lymphocytes show a differential expression of Cxs: Cx43 mRNA is expressed by peripheral blood and tonsil lymphocytes, whereas Cx40 mRNA is expressed in tonsil-derived T and B lymphocytes (41). The presence of Cx40, Cx43, and Cx37 in endothelial cells of different origins has been well documented, both in vivo (21, 33, 34, 45, 52) and in vitro (42, 49). Using an in vitro migration assay and flow cytometry, Oviedo-Orta et al. (40) showed that calcein-loaded human umbilical vein endothelial cells (HUVECs) transfer the fluorescent dye to adherent lymphocytes and the dye transfer was blocked by GAP27, a Cx

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mimetic peptide. It is noteworthy that transmigration was unaffected by GJ blockade (40). Other studies showed that human monocytes treated with tumor necrosis factor-α (TNF-α) plus interferon-γ became dye-coupled, and this condition was reversibly blocked with α-glycyrrhetinic acid (AGA), a GJ blocker (13). These authors also showed that AGA treatment reduced monocyte/macrophage transmigration in an in vitro model of blood-brain barrier (13). Moreover, transmigration of lymphoma cells across an endothelial cell barrier is partially attenuated by AGA (18). In contrast, Zahler et al. (53) showed that neutrophil transmigration through an HUVEC layer was increased after blocking GJ communication with specific Cx mimetic peptides, and this maneuver also enhanced endothelial monolayer permeability. In vivo, after ischemia-reperfusion, or topical platelet-activating factor application, leukocytes adhered to venules express Cx43 and form GJ-like structures at endothelial cell contacts (5, 23). Recent studies showed that the progression of atherosclerosis is reduced by 50%, and atherosclerotic lesions present less inflammatory cells in low-density lipoprotein receptor-deficient mice that are also heterozygous for Cx43, suggesting the participation of Cx43 in the infiltration process (30). In contrast, the lack of Cx37 hemichannels (one-half of GJ channels) enhances macrophages recruitment to atherosclerotic lesions by reducing the hemichannel-mediated ATP release, which normally acts as a negative modulator of adhesion (51).

Thus there is support for a role of GJs and hemichannels in the processes of leukocyte recruitment and activation during the inflammatory response, but their precise contribution is not well understood. To our knowledge, while the available evidence in vitro is controversial, there are no reports of in vivo models assessing the functional role of GJs on leukocyte adhesion and/or transmigration during the acute inflammatory phase. Therefore, the aim of this work was to study the effect of different GJ blockers and the specific deletion of Cx43 in endothelial cells on leukocyte adhesion and transmigration elicited by an acute inflammatory stimulus in vivo. We utilized the hamster cheek pouch and the mice cremaster muscle microcirculation as experimental models, and TNF-α as the inflammatory stimulus. The TNF-α-induced leukocyte adhesion and transmigration were greatly reduced by GJ blockade in the hamster cheek pouch and were also reduced in endothelial Cx43-deficient mice, pointing out a critical role of functional GJs in these two events of the inflammatory response.

MATERIALS AND METHODS

Animal and Reagent Sources

Male golden Syrian hamsters (Mesocricetus auratus) were obtained from the Research Animal Facilities of the Pontificia Universidad Católica de Chile and maintained with food and water ad libitum. Wild-type (WT) male mice C57 BL/6 from Hilltop Laboratory Animals, (Scottsdale, PA), and endothelial-specific Cx43 deficient mice (Cx43e−/−), developed in the laboratory of B. R. Duling using the Cre/loxP recombination system (31), were bred and maintained in the Research Animal Facilities of the University of Virginia. Experiments were conducted according to The Guiding Principles in the Care and Use of Laboratory Animals endorsed by the American Physiological Society. All studies were approved by the Institutional Bioethics Committee at the Pontificia Universidad Católica de Chile and the University of Virginia.

All biochemical reagents and mouse recombinant TNF-α were purchased from Sigma Chemical (St. Louis, MO), human recombinant TNF-α was obtained from Calbiochem (La Jolla, CA), and chemicals of analytic grade were obtained from Merck (Darmstadt, Germany).

Intravital Microscopy

Hamster cheek pouch. Hamsters (110–130 g) were anesthetized with pentobarbital sodium (65 mg/kg ip). The trachea and left jugular vein were cannulated, and the cheek pouch was prepared for intravital microscopy, as described (4). The right cheek pouch was immobilized with a Lucite plate introduced through the mouth, the tissue was exposed through a skin incision, and the nonvascular layer of connective tissue was cleared. A lucite observation chamber (1.7-cm internal diameter) was placed on the external surface of the pouch, and the tissue was transilluminated with a fiber-optic bundle. The hamster was placed on the stage of a Nikon Optiphot microscope connected to a television camera and a video display and recording system. The exposed cheek pouch area within the chamber was superfused at 1 ml/min by a peristaltic pump with a bicarbonate buffer. HEPES (152 mM NaCl, 4.7 mM KCl, 1.17 mM MgSO4, 2 mM CaCl2, 20 mM NaHCO3), equilibrated with 95% N2-5% CO2, pH 7.4, and kept at 37°C. The observation chamber was isolated from room air by a glass coverslip. After the surgical procedures were completed, each cheek pouch was equilibrated for 45 min under superfusion with bicarbonate buffer. Supplementary doses of anesthetic were given through the jugular vein (usually 10–15 mg/kg iv, every 25–30 min). At the end of the experiment (3.5 h of observation), the animal was killed by an anesthetic overdose (~150 mg/kg iv).

Mouse cremaster muscle. Mice (22–30 g) were anesthetized with pentobarbital sodium (80 mg/kg ip, diluted in isotonic saline to 10 mg/ml), and the cremaster muscle was prepared as described previously (15). The cremaster muscle was spread and pinned on a Sylgard pedestal. The mouse was placed on the stage of an Olympus microscope (BX 50 WI, Gibraltar Platform), and the cremaster muscle was continuously superfused at 3 ml/min with a bicarbonate-buffered saline solution (131.9 mM NaCl, 4.7 mM KCl, 2.0 mM CaCl2, 1.2 mM MgSO4, 20.0 mM NaHCO3) kept at 35°C. The buffer solution was equilibrated with 95% N2-5% CO2 to yield a pH 7.35–7.45. Each preparation was allowed to stabilize for 30 min before the observation period was started. Throughout the experiment, supplemental doses of anesthetic in isotonic saline (20 mg/kg ip) were administrated as appropriate. At the end of each experiment, the animal was killed by an anesthetic overdose (~120 mg/kg ip).

Leukocyte dynamics. Leukocyte rolling (LR), adhesion, and transmigration were studied in venules of 25–50 μm in diameter. Before starting the observation period, a few suitable vessels in this size range (1–3 venules in each hamster cheek pouch and 2–5 venules in each mouse cremaster muscle) were chosen and analyzed throughout the experiment. Thus each vessel served as its own control. Rolling flux was determined by counting the number of leukocytes that crossed an imaginary line during 1-min interval and were moving next to the vessel wall at a significantly slower pace than the mainstream flux of red blood cells (denoted by LR, cell/min) (19). Leukocyte adhesion was evaluated by counting the number of leukocytes adhered to a venular segment of ~100-μm length (LAV, cells/100 μm). Only leukocytes that remained stationary for >30 s were considered adherent, as described elsewhere (10, 19). To assess leukocyte transmigration, we counted white cells present in the 50-μm-wide strips adjacent to each side of the observed venular segment (total area of 100 × 100 μm = 10,000 μm²). The number of leukocytes located at this perivenular region was denoted by LPV (cells/10,000 μm²).

We performed measurements every 30 min during the experimental period. Vessels diameter and length were measured using an electronic caliper (Texas A&M).

Hemodynamic parameters. In some experiments, microvascular blood flow in the hamster cheek pouch was determined by the
clearance of a highly diffusible tracer, as previously reported (4, 14). Briefly, after the equilibration period, an intravenous injection of sodium-22 radioisotope ($^{22}$Na, $2 \times 10^{-6}$ counts/min) was given. After 20 min for tracer equilibration, the cheek pouch superfusate started to be collected at 2.5-min intervals. The carotid artery was cannulated to record arterial pressure on a Grass polygraph, and to withdraw blood samples (in 20-$\mu$L microhematocrit tubes) at 0, 90, and 180 min. Radioactivity of superfusate and plasma samples was determined in a gamma counter (Wallac-Turku), and clearance of $^{22}$Na was calculated for each sampling interval. Venular and arteriolar diameters were measured in selected fields where leukocyte adhesion was determined.

**Choice of Test Drugs**

**Inflammatory stimulus.** We used topical TNF-$\alpha$ as the primary stimulus, because this 17-kDa polypeptide is considered a major mediator of acute inflammation. This cytokine produces diverse biological effects, including expression of cell adhesion molecules in endothelial cells, neutrophil activation, cellular differentiation, and induction of other cytokines (1). In vivo, treatment with TNF-$\alpha$ induces a dramatic increase in leukocyte adhesion and subsequent transmigration in the microcirculation (19, 35). Homologous TNF-$\alpha$ was used in the mouse experiments, and the human recombinant form was used in the hamster cheek pouch.

**GJ blockade.** To evaluate a possible function of GJs during the inflammatory response, we used primarily 75 $\mu$M 18-AGA. Many studies have shown that this agent reversibly blocks intercellular communication between leukocytes and endothelial cells in vitro at that concentration (6, 13, 53). We also conducted a concentration-response curve on the effects of AGA in the 7.5–75 $\mu$M range. In separate experiments, we assessed the effect of other structurally related GJ blockers, 50 $\mu$M 18-$\beta$-glycyrrhetinic acid (BGA) and 75 $\mu$M carbenoxolone (Cbxn).

AGA, BGA, and Cbxn are synthetic structural analogs of glycyrrhizic acid, a natural substance also known as glycyr rhizin (Gly). All of these compounds are carbohydrate analogs to the tetrasaccharide sialyl Lewis X, which is the most characterized ligand for selectins (25). Thus the possibility exists that the used blockers may interfere with LR, the initial interaction between white blood cells and endothelium (28). Previous studies had shown that, in fact, some of these compounds decrease neutrophil adhesion to HUVEC monolayers and are also effective in reducing myocardial injury after ischemia-reperfusion (27). Therefore, we used Gly (75 $\mu$M), which is devoid of GJ blocking properties (46), as a further control to assess for possible effects of glycyrrhetic acid derivative unrelated to GJ inhibition. Moreover, we used 100 $\mu$M oleamide (Oleam), a GJ blocker with chemical structure unrelated to Gly derivatives (3), as an additional control for the specificity of GJ blockade on leukocyte dynamics during the inflammatory response.

**Experimental Protocols**

**Studies in hamsters.** After the 45-min equilibration period, the experimental observation was started with a 15-min baseline determination of rolling flux, LAV, and LPV. Next, TNF-$\alpha$ (human recombinant, 150 ng/ml) was applied topically during 15 min. The superfusate flow was stopped, and the chamber content was replaced by bicarbonate buffer containing TNF-$\alpha$, or just buffer in time control experiments. Thereafter, superfusion was resumed for an additional 165 min, to complete a 195-min observation period. In all protocols, the beginning of stimulation with TNF-$\alpha$ or its vehicle was considered as time zero. Leukocyte adhesion was determined at $-15$, 0, 15, 30, 60, 90, 120, 150, and 180 min. All GJ blockers and Gly were first dissolved in DMSO and then diluted in the superfusion buffer to the reported effective concentration and applied starting on the first baseline observation, i.e., minute $-15$, and maintained until the end of the experiment, except when specifically stated otherwise. In all cases, the final concentration of the vehicle was 78 mM (1:1,000 vol/vol). Control responses were evaluated in the presence of DMSO alone.

In an additional protocol, we tested the effect of initiating GJ blockade after the leukocyte adhesion response was well established. In this case, 90 min after TNF-$\alpha$ application, the superfusion solution was changed to one containing AGA, or, in some cases, Gly was used as control. We also tested the effect of removing the GJ blocker 90 min after TNF-$\alpha$ stimulation.

**Studies in mice.** LR, adhesion, and transmigration in response to cytokine stimulation were observed in the cremaster muscle of endothelial Cx43e$^{-/-}$ and WT mice, following reported methods (10). Briefly, after induction of anesthesia, TNF-$\alpha$ (mouse form, 0.5 $\mu$g) or just its vehicle (0.3 ml isotonie saline) was injected intrascrotal. A 30-min period was allowed before beginning to exteriorize the cremaster muscle, and another 50–60 min were used to complete the surgical procedures. Thereafter, each tissue was equilibrated for 30 min under constant superfusion, before starting the experimental observation period, which lasted 3.5 h. By convention, the moment of intrascrotal injection of TNF-$\alpha$ or its vehicle was considered as time zero; thus, leukocyte adhesion and transmigration was started 2 h after the beginning of stimulation.

**Data analysis.** Results are presented as means ± SE. Comparisons within and between groups were made using two-way ANOVA, followed by Bonferroni posttest or Newman-Keuls posttest for multiple comparisons (Graph Pad Prism, version 4.0). The level of statistical significance was set at $P < 0.05$.

**RESULTS**

**TNF-$\alpha$ Stimulates Leukocyte Adhesion and Transmigration in the Hamster Cheek Pouch in Vivo**

Combining data from all series of experiments, after the surgical procedures and the equilibration period, the basal rolling flux ranged between 25 and 75 cells/min, LAV was 2.5 ± 0.2 cells/100 $\mu$m of venule length ($n$ = 82 vessels from 69 animals), and basal LPV was 2.6 ± 0.2 leukocytes/10,000 $\mu$m$^2$ ($n$ = 58 vessels from 50 animals). In nonstimulated tissues, in the ensuing 180 min, while rolling flux was maintained relatively constant (Fig. 1A), LAV rose slightly to reach 3.6 ± 0.8 cells/100 $\mu$m (Fig. 1B). Likewise, LPV almost doubled at the end of the 180-min observation period (to 5.6 ± 0.6 leukocytes/10,000 $\mu$m$^2$; Fig. 1C). These findings reflect a basal rate of adhesion and transmigration in the exposed and superfused cheek pouch.

Topical stimulation with 150 ng/ml TNF-$\alpha$ for 15 min caused a steady increase in LAV, which reached 29.4 ± 3.6 cells/100 $\mu$m after 180 min, which is ∼12 times above its basal level and 8 times above the corresponding time controls (Fig. 1B). Concomitantly, stimulation with TNF-$\alpha$ significantly increased the rate of leukocyte transmigration. At 180 min posttreatment, LPV was ∼3.5 times above its basal value (13.4 ± 1.6 vs. 2.9 ± 0.5 leukocytes/10,000 $\mu$m$^2$) and 2.5 times above that of time controls (Fig. 1C). The changes in adhesion and transmigration were independent of changes in rolling flux, because LR showed only small fluctuations with a tendency to increase toward the end of the experiment (Fig. 1A). These results confirm that, in the hamster cheek pouch microcirculation, TNF-$\alpha$ produces a major effect on leukocyte activation, as described in previous studies (10, 19).

*Science*
AGA, a GJ Blocker, Inhibits TNF-α-induced Increase in Leukocyte Adhesion and Transmigration in a Concentration-dependent and Flow-independent Manner

In tissues superfused with 75 μM AGA, stimulation with TNF-α failed to increase LAV and LPV at all (Fig. 1, B and C). Indeed, both variables were kept equal to that of nonstimulated tissues: at 180-min post-TNF-α, LAV was 5.9 ± 0.8 cells/100 μm, and LPV was 5.0 ± 1.0 leukocytes/10,000 μm². The striking differences in LAV and LPV cannot be ascribed to changes in rolling flux, since this variable was similar in AGA-treated and control tissues (Fig. 1A). Application of AGA alone did not modify any of these indexes for leukocyte dynamics, which followed the same pattern as in nonstimulated tissues (data not shown).

In marked contrast to the findings with AGA, the leukocyte response to TNF-α was practically unaffected when this cytokine was applied in the presence of 75 μM Gly (Fig. 1, B and C). At 180 min poststimulation, LAV and LPV were equal in tissues treated with Gly or vehicle (DMSO). As in the previous cases, LR did not change during the observation period (Fig. 1A).

Besides the relatively constant LR flux, no noticeable changes in venular diameter or venular blood flow were evidenced by video microscopy in all of the experiments summarized in Fig. 1. Nevertheless, because leukocyte dynamics are dependent on venular blood flow, we performed an additional experimental series to determine microvascular flow and microvessel diameters before and after stimulation with TNF-α in vehicle and AGA-treated tissues.

Plasma exchangeable flow in the perfused cheek pouch area, as assessed by clearance of 22Na, was similar in tissues treated with DMSO or AGA throughout the experiment (Fig. 2A). Baseline flow was 4.00 ± 0.94 and 3.82 ± 0.73 μl/min in control and AGA-treated animals, respectively. Because superfusate flow was stopped during TNF-α application, it was not possible to measure microvascular blood flow at the precise moment of cytokine treatment. Nevertheless, the net 22Na clearance determined by averaging the 15-min stop-flow period plus the 15-min period immediately after resuming superfusion was 2.71 ± 0.75 μl/min in DMSO and 2.78 ± 0.53 μl/min in AGA. Thus TNF-α caused a moderate, transient reduction in exchangeable plasma flow of similar magnitude in both groups (accounting for a 25 ± 8% decrease relative to basal value, P < 0.01: TNF/DMSO vs. nonstimulated. ††† P < 0.001: TNF/Gly vs. nonstimulated (two-way ANOVA, Bonferroni posttest). This transient reduction in flow was due to TNF-α application, because, in control experiments, the average 22Na clearance was kept identical before, during, and after performing a 15-min stop-flow without changing the superfusion buffer (4.04 ± 0.44, 3.82 ± 0.65, and 4.29 ± 0.71 μl/min; n = 6). Most importantly, no significant differences in microvascular flow were detected between the groups treated with AGA and vehicle in the period following TNF-α stimulation (60–180 min) when the most striking differences in leukocyte adhesion and transmigration were found.

Fig. 1. α-Glycyrrhetinic acid (AGA), a gap junction (GJ) blocker, inhibits leukocyte adhesion and transmigration induced by tumor necrosis factor (TNF)-α in the hamster cheek pouch microcirculation. A: number of leukocytes observed rolling (LR) on the wall of 25- to 40-μm diameter venules is shown as a function of time. At time zero, a 15-min stimulus with TNF-α (150 ng/ml, horizontal bar) was applied to tissues superfused with vehicle (TNF/DMSO; ●), 75 μM AGA (TNF/AGA; ▲), or 75 μM glycyrrhizin (Gly) (TNF/Gly; ○). Time control tissues did not receive TNF-α (nonstimulated; ○). Microvascular flow was determined by averaging the 15-min stop-flow period plus the 15-min period immediately after resuming superfusion (4.04 ± 0.44, 3.82 ± 0.65, and 4.29 ± 0.71 μl/min; n = 6). Most importantly, no significant differences in microvascular flow were detected between the groups treated with AGA and vehicle in the period following TNF-α stimulation (60–180 min) when the most striking differences in leukocyte adhesion and transmigration were found.

AJP-Heart Circ Physiol • VOL 295 • SEPTEMBER 2008 • www.ajpheart.org
Throughout the experiment, arteriolar and venular diameters were kept similar between groups and relatively constant over time (Fig. 2, B and C). With the sampling rate used, it was not possible to detect a probable transient arteriolar constriction that may have explained the observed reduction in $^{22}$Na clearance during TNF-$\alpha$ application. In both groups, TNF-$\alpha$ application caused a moderate but significant increase in venular diameter that was sustained until the end of the observation period (amounting to a 13.8 ± 5.2 and 11.9 ± 3.9% increase in control and AGA groups, respectively; $P < 0.05$ vs. baseline, paired $t$-test, Fig. 2C). Altogether, the results of $^{22}$Na clearance and microvessel diameter indicate that the major differences observed in LAV and LPV with and without the GJ blocker (Fig. 1) were independent of hemodynamic changes involving venular size, venular blood flow, or venular rolling flux.

Inhibition of leukocyte adhesion and transmigration was directly related to the concentration of AGA utilized. Superfusion with 25 $\mu$M AGA was equally efficient as 75 $\mu$M AGA to block the increase in LAV and LPV induced by TNF-$\alpha$. However, when applied at the concentration of 7.5 $\mu$M, AGA did not reduce significantly LAV and LPV relative to control (Fig. 3). In no case were changes in venular diameters detected (data not shown). These results support a specific blockade of AGA, and thus, the concentration of 75 $\mu$M was used in all experiments involving this drug or Gly, unless specifically stated differently.

**Different GJ Blockers Decrease the TNF-$\alpha$-induced Leukocyte Adhesion and Transmigration**

To further confirm that the inhibitory effect of AGA on leukocyte adhesion and transmigration was due to GJ blockade, we tested three additional blockers: BGA (50 $\mu$M) and Cbnx (75 $\mu$M), two known Gly derivates, and Oleam (100 $\mu$M)....
and 75%, respectively (at 180 min, LAV was 13.2/11011 m2, values similar to those of nonstimulated tissues. Cbnx and Oleam reduced LAV by 50 and 75%, respectively (at 180 min, LAV was 13.2 ± 1.1 and 8.7 ± 2.6 cells/100 μm2, respectively, Fig, 4A). The effect of these two blockers on transmigrated leukocytes was less pronounced. At 180 min, only Cbnx-treated tissues presented a reduced number of LPV compared with control. Nevertheless, both blockers reduced the net accumulation in LPV above their respective baseline (Fig. 4B).

The Inhibitory Effects of GJ Blockers on Leukocyte Adhesion Are Reversible and Independent of the Moment of Application

Since in the previous protocols GJ blockers were applied continuously throughout the experiment, it was not feasible to know whether GJ blockade at the moment of stimulation was effective or sufficient to prevent the TNF-α-induced leukocyte responses. Therefore, we assessed the effects of removing or applying AGA at 90 min poststimulation with TNF-α. As a control, we applied Gly to equivalent preparations at 90 min poststimulation with TNF-α.

LAV did not increase when TNF-α was applied in the presence of AGA. However, immediately after removal of the GJ blocker, leukocytes began to adhere to the venular endothelium at a similar rate to that observed after TNF-α application in the absence of the blocker, even though stimulation had occurred 90 min before (Fig. 5B). Removal of AGA at 90 min caused a modest increase in LPV (Fig. 5C), suggesting that perhaps adhesion and transmigration might have different time windows poststimulus.

In tissues superfused with vehicle, TNF-α stimulation caused an increase in LAV, as previously seen. However, LAV decreased dramatically immediately after AGA was applied at 90 min (Fig. 5B). In contrast, addition of Gly at minute 90 did not affect the slope of LAV vs. time curve, confirming that this non-GJ blocking analog does not interfere with leukocyte adhesion (Fig. 5B). Application of AGA at 90 min also blocked the increase in LPV, whereas Gly had no effect (Fig. 5C).

The major and opposite changes on leukocyte adhesion elicited by adding or removing AGA 90 min postcytokine stimulation were not accompanied by any significant modification in LR (Fig. 5A). Furthermore, as in the previous series, the changes in adhesion were observed in the absence of variations in venular diameter. These results suggest that GJs are key to sustain leukocyte adhesion, independent of the time elapsed after triggering the inflammatory response.

Leukocyte Adhesion and Transmigration Does Not Increase After TNF-α Stimulation in the Microcirculation of Cx43e−/− Mice

Throughout the observation period, LR was kept constant and at a similar level in control and TNF-stimulated WT mice (see Fig. 7A). Intrascrotal injection of TNF-α induced a significant increase on leukocyte adhesion to venular endothelium of WT mice cremaster muscle in absence of changes in LR (Figs 6A and 7, A and B). At the start of the observation period, LAV was threefold higher in TNF-α-treated animals compared with controls injected only with saline (16.8 ± 2.5 vs. 4.5 ± 0.6 cells/100 μm2 venule). The maximal effect (30.7 ± 3.9 cells/100 μm2) was observed ~4 h after TNF-α application, i.e., 2 h after the observation was initiated (Fig. 7B). Leukocyte transmigration was also increased considerably by the cytokine stimulus (Fig. 7C). At the beginning of observation, LPV was almost three times higher in TNF-α-treated mice compared with control animals (14.5 ± 2.4 and 5.1 ± 0.8 cells/10,000 μm2, respectively). The peak LPV was observed 4 h after stimulation, reaching 29.7 ± 3.6 cells/10,000 μm2.
Contrary to the observation in WT mice, intrascrotal injection of TNF-α failed to increase leukocyte adhesion and transmigration in Cx43e−/−; this happened even though LR flux was higher in Cx43e−/− mice than in WT (Figs. 6B and 7).

At the beginning of observation, the flux of rolling leukocytes was higher in both groups of Cx43e−/− compared with their WT counterparts, regardless of whether they were treated with saline or TNF-α (Fig. 7A). During the observation period, while LR was maintained elevated in saline-treated Cx43e−/− mice, in cytokine-treated animals this variable suffered a significant reduction, reaching values below that of WT mice toward the end of the experiment (Fig. 7A).

At the start of the observation period, LAV tended to be higher in saline-treated Cx43e−/− mice compared with WT controls, although this was not statistically significant at any time point when comparing all four groups by ANOVA (Fig. 7A). During the whole observation period, LAV was similar in Cx43e−/− mice treated with TNF-α compared with Cx43e−/− injected only with saline. Interestingly, at the peak response, LAV was significantly lower in Cx43e−/− animals treated with TNF-α than in WT mice similarly stimulated with TNF-α.

LPV also tended to be higher in Cx43e−/− animals than in saline-injected WT mice (Fig. 7C); however, similar to adhesion, LPV remained invariant throughout the observation period, regardless of whether the animals were injected with TNF-α or saline (Fig. 7C). Again, for the most part of the experiment, LPV was significantly lower in Cx43e−/− animals treated with TNF-α, compared with WT mice submitted to similar stimulus.

These results constitute the first in vivo observations of leukocyte dynamics during acute inflammation in endothelial Cxs-deficient mice, and they are in agreement with a role of GJs in early events of the inflammatory response.

DISCUSSION

The major finding of this work is that leukocyte adhesion and extravasation induced by TNF-α in the in vivo microcirculation are greatly reduced or prevented in normal animals treated with GJ blockers and are not elicited in animals lacking Cx43 expression in the endothelium. These results support the notion that Cx43 is critical for the establishment of GJ communication between activated leukocytes and endothelial cells, and that this heterocellular communication favors the adhesion and extravasation of leukocytes.

Our results are consistent with other studies showing that TNF-α induces leukocyte recruitment in mammalian postcapillary venules (10, 19, 47). In the presence of AGA, a GJ blocker, leukocyte adhesion induced by TNF-α was inhibited in proportion to the concentration of the blocker (Figs. 1–3), and similar results were obtained using two other structural

![Fig. 5. Inhibitory effect of AGA on adhesion was completely reversed after removal of the blocker. A and B: number of LR and LAV are shown as a function of time. Details are as in Fig. 1. At time zero, a TNF-α stimulus (horizontal bar) was applied to tissues superfused with AGA (●) or vehicle (DMSO 1:1,000, other two curves). At minute 90 (arrow), the superfusate solution was replaced by one containing vehicle (▲), AGA (♦), or Gly (○). n = x/y, number of analyzed vessels/animals. No significant differences were detected in LR at any time point. In the control, vehicle-treated group, the increase in LAV in response to TNF-α was not modified by addition of Gly at 90 min (Control/Gly curve). Conversely, when AGA was added to control tissues, LAV decreased dramatically (Control/AGA curve). In tissues treated with AGA, at the moment of stimulation, LAV did not increase until the GJ blocker was removed at minute 90; from this point on, LAV started to increase at a similar rate as in nonblocked tissues (AGA/Control curve). Statistical analysis of the LAV vs. time curves in the 90- to 180-min period indicates that Control/Gly and AGA/Control curves have the same slope, whereas Control/AGA was different from the other two curves. *P < 0.001 with respect to Control/Gly and AGA/Control (one-way ANOVA Newman-Keuls posttest). C: number of LPV for the same vessels studied in A and B. In control tissues (open bars), addition of Gly at 90 min did not modify the increase in LPV in response to TNF-α. In contrast, addition of AGA at 90 min stopped the normal progression of LPV, which remained constant until 180 min (shaded bars). In tissues treated with AGA at the moment of stimulation, LPV showed just a modest increase after washing the GJ blocker (solid bars). The bars marked as "Net incr. (180–90)" represent the change in LPV determined in each venule between 90 and 180 min. n = x/y, number of analyzed vessels/tissues. *P < 0.05, †P < 0.01, ‡P < 0.001 with respect to Control/Gly (two-way ANOVA, Bonferroni posttest).]
analogs, BGA and Chnx, at similar concentrations as AGA (Fig. 4). An equimolar concentration of Gly, a structural analog to AGA, BGA, and Chnx without GJ blocker action, did not alter the leukocyte response to TNF-α, making it unlikely that the observed reduction in leukocyte adhesion observed with AGA, BGA, and Chnx was the result of an effect unrelated to their GJ blocking action. Direct observation of LR indicates that this parameter was similar in tissues treated with AGA or Gly, compared with control (Figs. 1 and 5). This issue is relevant, because glycyrrhetinic acid analogs may interfere with LR due to their resemblance with Sialyl Lewis-X, one of the primary ligands for P-selectin (25). In addition, LAV was also decreased by Oleam, a GJ blocker that is not structurally related to Gly. Therefore, we interpret these results as evidence that interference with selectin ligand did not play a major role in the inhibition of leukocyte adhesion in the model of acute inflam-

**Fig. 6.** TNF-α increases leukocyte adhesion and transmigration in cremaster muscle venules of wild-type (WT) mice but not in connexin (Cx) 43e−/− mice. Representative fields are shown of the cremaster muscle microcirculation of WT mice (C57 bl/6) or endothelium Cx43-deficient mice (Cx43e−/−) photographed at the beginning and toward the end of the experimental period. Mice were injected intrascrotally with isotonic saline solution or with TNF-α (0.5 μg in 0.3 ml isotonic saline) at time zero, and intravital microscopic observation started 2 h later. A: a clear increase in venular leukocyte adhesion (arrows) and transmigration (arrow heads) was found in TNF-α-treated WT animals, whereas, in saline-treated controls, adhesion and transmigration increased weakly over time. B: at difference with the observations in WT animals, leukocyte adhesion (arrows) and transmigration (arrow heads) were similar in Cx43e−/− mice treated with TNF-α or with saline. In addition, these variables were kept relatively constant over time.

**Fig. 7.** TNF-α does not increase leukocyte adhesion and transmigration in Cx43e−/− mice. A: number of LR on the wall of venules (25–40 μm in diameter), in 4 groups of mice: WT injected with saline (○), or with TNF-α (●), and Cx43e−/− mice injected with saline (●), or with TNF-α (▲). Two-way ANOVA, Bonferroni posttest indicates significant differences (P < 0.05) as follows: *Cx43e−/−-Saline vs. Cx43e−/−-TNF; †Cx43e−/−-Saline vs. Cx43e−/−-TNF and Cx43e−/−-Saline vs. WT-Saline and WT-TNF; §Cx43e−/−-Saline vs. all other groups; #WT-Saline vs. Cx43e−/−-TNF. No significant differences were detected between WT-Saline and WT-TNF at any time point, nor between WT-TNF and Cx43e−/−-TNF. B: number of LAV registered in 100-μm-long segments of the same venules analyzed in A. Two-way ANOVA, Bonferroni posttest indicates significant differences (P < 0.05) as follows: *WT-TNF vs. WT-Saline; †WT-TNF vs. WT-Saline and WT-TNF; §WT-TNF vs. all other groups; ‡Cx43e−/−-Saline vs. WT-TNF; ††Cx43e−/−-Saline vs. WT-Saline and WT-TNF; †‡Cx43e−/−-Saline vs. all other groups; †WT-Saline vs. Cx43e−/−-TNF. No significant differences were observed between WT-Saline and WT-TNF at any time point. C: number of LPV outside the same venular segments analyzed in A and B, determined at different time points for WT injected with saline (white bars), WT injected with TNF-α (dark gray bars), Cx43e−/− injected with saline (light gray bars), and Cx43e−/− injected with TNF-α (black bars). †P < 0.05 and ††P < 0.01, WT-TNF vs. WT-Saline. §P < 0.05, WT-TNF vs. all other groups. ‡P < 0.05, WT-Saline vs. Cx43e−/−-TNF. No significant differences were found between Cx43e−/− treated with saline and TNF. n = x/y, number of venules/animals analyzed.
mation used. The substantial differences in leukocyte adhesion brought out by GJ blockers do not appear to be due to variations in microvascular hemodynamics, as treatment with AGA did not affect arteriolar and venular diameters, nor blood flow (Fig. 2). A role for the participation of GJ in leukocyte adhesion and transmigration in inflammation is strengthened by the observation that TNF-α was devoid of effect in the cremaster muscle microcirculation of mice that do not express endothelial Cx43 but are otherwise normal and did not receive any pharmacological treatment.

It is important to note that, in the present study, the extent of cell-cell communication was not directly assessed. However, it is reasonably to think that, at the concentrations of blockers used in the cheek pouch (25–75 μm), GJ blockade was effective, because at similar concentrations AGA and the other blockers are very effective in preventing dye coupling in vitro (6, 13, 53). In addition, effective pharmacological action of these GJ blockers at the concentrations used in the present study have been established in various in vivo experimental models (8, 9, 39, 48). Thus it is reasonable to assume that, in our study, the different compounds caused total or partial inhibition of GJ communication in the hamster cheek pouch. Since, except for AGA, the other blockers were used at a single concentration, we cannot calculate the strength of inhibition, but, among the structurally related compounds, Cbnx was the less effective blocker of leukocyte adhesion.

Cx43 and Cx37 are present in both polymorphonuclear cells and monocyte/macrophages (6, 12, 51), and in these cells Cx hemichannels mediate the release of ATP, which is believed to reduce the adhesive and inflammatory properties of leukocytes (12, 51). Thus the inhibition of Cx hemichannels is expected to increase leukocyte adhesion and extravasation, as observed in atherosclerotic mice that are also Cx37 deficient (51). Therefore, the reduction in LAV induced by all of the blockers used in the present study is likely to reflect predominance of GJ rather than hemichannel inhibition.

Firm adhesion of activated leukocytes in response to inflammatory cytokines occurs subsequent to rolling. Binding to activated endothelium is mediated primarily by two integrins: Mac-1 (CD11b/CD18) and leukocyte function-associated antigen-1 (CD11a/CD18) and their ligands ICAM-1 (CD54) and VCAM-1. TNF-α activates translocation of integrins to the neutrophil surface and upregulates ICAM-1 on the endothelium surface (50). We suggest two possible ways by which GJ blockers impede the firm adhesion of leukocytes. First, uncoupling of endothelial cells may diminish the expression or exposure of adhesion molecules on the venular endothelial surface. Such a mechanism would imply that the venular response is less robust when endothelial cells are not communicating to each other, despite the fact that the inflammatory stimulus was applied to the whole tissue. Alternatively, junctional uncoupling might operate by impeding the formation of GJs between leukocytes and endothelium. Perhaps the extra-cellular loops of connexons act as additional adhesion molecules between both cells types, contributing to anchoring leukocytes to endothelial cells in the face of hydrodynamic forces, as recently demonstrated for radial migration in the brain cortex (11). The presence of Cx hemichannels at the endothelial cell surface facing the lumen, in addition to the accepted location at cell-cell interfaces, is supported by a report documenting that functional hemichannels on the surface of bovine corneal endothelial cells partake in the propagation of Ca^{2+} waves, contributing to ATP release in response to mechanical stimulation (17).

It should be noted that the two preceding hypotheses are not mutually exclusive, but the reversibility of the effect of GJ blockers on leukocyte adhesion (Fig. 5) perhaps supports the second hypothesis. On the one hand, the finding that adding the GJ blocker once the adhesion response had been fully established causes a rapid detachment of leukocytes adhered to the venular walls suggest a mechanical role of GJs by providing a direct leukocyte-endothelium interaction, since it is unlikely that application of GJ blockers would cause such a rapid (5–10 min) reduction in the exposure of endothelial adhesion molecules. On the other hand, and by the same reasoning, it is unlikely that restoring interendothelial GJ coupling would cause a rapid increment in the exposure of adhesion molecules at the endothelial surface, despite the fact that TNF-α had been applied 90 min earlier. Glycyrhetinic acid does alter connexon particle packing in GJs plaques (16), which might reduce the adhesion between connexons. However, once formed, GJ plaques are difficult to dissociate, and it has been reported that the uncoupling agent heptanol does not change the fate of intercellular GJ plaques in cell cultures (7). Therefore, some functional aspect of GJ cell-cell communication may be involved beyond a mere contribution to cellular adhesion. Additional studies are required to distinguish between the various possibilities, and it will be particularly important to determine the effect of GJ blockers on the abundance of cell adhesion molecules exposed at the endothelial surface.

The finding that specific deletion of endothelial Cx43 prevents the response to TNF-α is additional evidence supporting the second hypothesis that the leukocyte and endothelium form heterocellular GJs. The expression of endothelial adhesion molecules has not been characterized in the Cx43−/− model, but some differences may exist because rolling flux was higher in nonstimulated Cx43−/− than in WT mice (Fig. 7). Nonetheless, the lack of response to cytokine stimulation in Cx43−/− does not seem to be due to a reduced leukocyte delivery, because in these animals rolling flux was similar or even higher than that of WT mice during most of the observation period. Our results demonstrate that endothelial Cx43 is required for normal adhesion in response to cytokine stimulation. However, because Cx43−/− mice express other Cxs, their endothelial cells may still be coupled to each other. Based on previous reports, we can expect that these knockout animals present Cx43 and Cx40 on the leukocyte surface after TNF-α stimulation (6); thus, conservatively thinking, one can presume that only homotypic Cx43 GJ communication between endothelial cells and leukocytes was prevented in these mice. Nevertheless, recent evidence indicates a strong interrelation between vascular endothelial Cx43 and Cx40 (21). In endothelial cells of mice devoid of Cx40 (Cx40 null), Cx43 is redistributed from the plasma membrane to the cytoplasm and perinuclear space and may, therefore, be unable to form GJ. It is possible that deletion of endothelial Cx43 affects the cellular distribution of Cx40, thereby impeding any possible GJ between endothelial cells and leukocytes, which express only these two types of Cxs.

GJs possess the capacity to transfer signals from one type of cells to the other, perhaps allowing for complementary cytoskeleton changes during extravasation. In vitro studies dem-
onstrate that neutrophils and monocytes stimulated by cytokines respond with a rise in endothelial intracellular Ca\(^{2+}\), and this increase is required for leukocyte migration (20, 26). A GJ-mediated coordination of Ca\(^{2+}\) signals between endothelium and attached white cells is an interesting possibility, particularly related to the forthcoming transmigration process, which requires prior firm adhesion. Several authors had shown dye transfer via GJs between lymphocytes or neutrophils and endothelial cells in the process of transmigration through endothelial (HUVeC) monolayers in vitro (13, 40, 53). However, it is not clear from these studies whether intercellular communication mediated by GJ channels played a role in diapedesis, because GJ blockade did not affect lymphocyte transmigration (40), increased neutrophil transmigration (53), or reduced monocyte migration through a model of brain-blood barrier of HUVeC cocultured with astrocytes (13).

In general, our results show a very good agreement between the effect of different treatments or conditions on leukocyte adhesion and transmigration. The number of leukocytes found in the outside vicinity of venules was reduced in the presence of GJ blockers roughly in the same proportion as adhesion was diminished (Figs. 1, 3, and 4). The same relationship was detected when comparing Cx43e and WT mice treated with TNF-\(\alpha\) (Fig. 7). The correlation was not as clear in the reversion experiments. As mentioned, adding AGA after 90 min rapidly reduced the number of sticky cells induced by TNF-\(\alpha\), but LPV remained constant. This discrepancy is probably due to the fact that LPV represents an accumulative process, and the clearance of extravasated leukocytes occurs rather slowly compared with the rate at which the white cells might detach from the endothelium and return to the circulation. Conversely, the rapid increase in adherent leukocytes observed after removing AGA at minute 90 was not associated with an obvious increment in LPV, suggesting that there is a time lag between adhesion and transmigration. Since a reduced transmigration may be a direct consequence of the reduced adhesion, our results provide indirect evidence for a role of leukocyte-endothelial cell GJ communication on diapedesis, as it was demonstrated in breast tumor cells in culture (43).

Based on our results, we hypothesize that GJ communication might modulate the firm adhesion between leukocytes and endothelial cells, perhaps defining whether leukocytes are kept stationary at the damaged site, allowing them to migrate, or are released back to the blood flow. Our findings in acute inflammatory events agree with previous reports showing that GJs play a role in the long-term development of atherosclerosis and leukocyte infiltration. Expression of vascular Cx37, Cx40, and Cx43 is altered during experimental induction of atherosclerosis (29). Furthermore, mice with reduced levels of Cx43 (Cx43\(^{-/-}\)) presented a lesser progress of atherosclerotic lesion (29). In addition, downregulation of Cx43 at wound sites in mice reduced the number of neutrophil and macrophage infiltration, limiting the extent of the inflammatory response (37), further supporting a critical role of this Cx in inflammation. These mechanisms might be common to other migratory cells, including metastatic tumor cells. Accordingly, melanoma cells expressing Cx26 form functional GJs with endothelial cells and are more metastatic than melanoma cells deficient in Cx expression (22).

In summary, by combining pharmacological and genetic techniques, the present work demonstrates that GJ communication, at least based on endothelial Cx43, plays a role in the development of leukocyte adhesion and transmigration in acute inflammation in vivo. The results support the hypothesis that communication through GJ channels between leukocytes and endothelial cells during the adhesion event helps to stabilize the cellular attachment and favors the subsequent migration into the damaged tissue.

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