Research article

Cytochalasin B triggers a novel pertussis toxin sensitive pathway in TNF-alpha primed neutrophils

Johan Bylund[1,2], Sara Pellmé[1], Huamei Fu[1], Ulf-Henrik Mellqvist[3], Kristoffer Hellstrand[4], Anna Karlsson[1] and Claes Dahlgren*[1]

Address: [1]Department of Rheumatology and Inflammation Research, University of Göteborg, Göteborg, Sweden, [2]Department of Paediatrics, University of British Columbia, BC Research Institute for Children's and Woman's Health, Vancouver, British Columbia, Canada V5Z 4H4, [3]Department of Hematology, University of Göteborg, Göteborg, Sweden and [4]Department of Virology, University of Göteborg, Göteborg, Sweden

Email: Johan Bylund - jbylund@interchange.ubc.ca; Sara Pellmé - Sara.Pellme@microbio.gu.se; Huamei Fu - Huamei.Fu@microbio.gu.se; Ulf-Henrik Mellqvist - Ulf-Hendrik.Mellqvist@medic.gu.se; Kristoffer Hellstrand - Kristoffer.Hellstrand@microbio.gu.se; Anna Karlsson - Anna.Karlsson@microbio.gu.se; Claes Dahlgren* - Claes.Dahlgren@microbio.gu.se

* Corresponding author

Abstract

Background: Cytochalasin B does not directly activate the oxygen-radical-producing NADPH oxidase activity of neutrophils but transfers desensitized G-protein coupled receptors (GPCR) into an active signaling state by uncoupling GCPR from the cytoskeleton. The receptor uncoupling results in respiratory burst activity when signals generated by reactivated formyl peptide receptors trigger the NADPH-oxidase to produce superoxide anions.

Results: Tumor necrosis factor alpha (TNF-alpha) primes neutrophils for subsequent activation by cytochalasin B. Pretreatment with TNF-alpha induced mobilization of receptor-storing neutrophil organelles, suggesting that receptor up-regulation significantly contributes to the response, but the receptor mobilization was not sufficient for induction of the cytochalasin B sensitive state. The TNF-alpha primed state resembled that of the desensitized non-signaling state of agonist-occupied neutrophil formyl peptide receptors. The fact that the TNF-alpha primed, cytochalasin B-triggered activation process was pertussis toxin sensitive suggests that the activation process involves a GPCR. Based on desensitization experiments the unidentified receptor was found to be distinct from the C5a receptor as well as the formyl peptide receptor family members FPR and FPRL1. Based on the fact the occupied and desensitized receptors for interleukin-8 and platelet activating factor could not be reactivated by cytochalasin B, also these could be excluded as receptor candidates involved in the TNF-alpha primed state.

Conclusions: The TNF-alpha-induced priming signals could possibly trigger a release of an endogenous GPCR-agonist, amplifying the response to the receptor-uncoupling effect of cytochalasin B. However, no such substance could be found, suggesting that TNF-alpha can transfer G-protein coupled receptors to a signaling state independently of agonist binding.

Background

Human neutrophil granulocytes constitute an important part of the innate immune defense against microbial infections, and the bactericidal activities performed by these cells rely on their interaction with chemoattractants, cytokines and other inflammatory mediators [1]. The chemoattractants, including C5a, platelet activating factor (PAF), interleukin-8 (IL8) and formylated peptides, bind to specific receptors [2,3], all of which belong to a family of transmembrane G-protein coupled receptors (GPCRs). Activation of these receptors leads to directed migration, granule mobilization and activation of the neutrophil NADPH-oxidase [2]. The reactive oxygen species generated by the oxidase are of importance for microbial killing and for cell-cell-signaling [4].

Tumor necrosis factor-alpha (TNF-α) is one of the earliest cytokines produced at inflammatory sites by activated monocytes and macrophages. This cytokine affects neutrophil function mainly through binding to type I TNF receptor (TNFR1) [5]. The TNFR1 is a single transmembrane glycoprotein with several intracellular motifs with known functional significance, but it is not linked to any signaling G-protein [5-7]. Phosphorylation of TNFR1 occurs at a consensus MAPK site on its cytoplasmic domain or through tyrosine phosphorylation [6,7], although it is not fully understood how this phosphorylation control receptor signaling or processing.

The biological effects of TNF-α on neutrophil functions *in vitro* vary, as illustrated by the ability or inability of TNF-α to affect the neutrophil oxygen radical producing NADPH-oxidase. In order for TNF-α to trigger neutrophil superoxide production, cells need to adhere to a solid surface, and the magnitude of the response is determined by which protein that is coated on the surface [8]. TNF-α only weakly triggers the oxidase when the neutrophils are in suspension [8]; however, after exposure to TNF-α, these cells are primed with respect to NADPH-oxidase activation in response to other stimuli [9]. Thus, while TNF-α *per se* does not activate the NADPH-oxidase to any significant extent in nonadherent neutrophils, it induces a state of hyper-responsiveness to other stimuli.

Several mechanisms have been proposed to account for neutrophil priming [10-14], including receptor mobilization from intracellular granule stores [15-17]. The aim of this study was to characterize the primed state induced in human neutrophils by TNF-α, using an earlier described receptor uncoupling system [18]. We found exposure of new receptors to be a part of the priming process, but more importantly we found that neutrophils interacting with TNF-α were transferred into a novel state, in which the cytoskeleton disrupting compound cytochalasin B triggered activation. The TNF-α primed state shows many

similarities with that of neutrophils that have their formyl peptide GPCRs desensitized by a specific receptor agonist [18]. Isomerization of GPCRs, from an inactive to an active state, occurs normally as a result of ligand binding but can also occur independently of agonist [19] and our findings are suggestive of a TNF-α induced novel activation mechanism that is receptor agonist-independent.

Results

TNF-α primes the neutrophil NADPH-oxidase response to a subsequent stimulation/triggering with cytochalasin B

Cytochalasin B, a cytoskeleton disrupting compound, does not induce a neutrophil response by itself [18] but is known to augment the neutrophil response to many stimuli. We investigated whether this was true also for the minimal neutrophil response induced by a direct stimulation with TNF-α. We found that cytochalasin B had no effect on the NADPH-oxidase response when added to neutrophils prior to TNF-α treatment (data not shown). However, when the cells were first treated with TNF-α and subsequently challenged with cytochalasin B, a pronounced respiratory burst activity was noted (Fig 1). The time course of the induced response was similar to that seen with chemoattractants such as the formylated peptide fMLF, an agonist that activates cells through the G-protein coupled formyl peptide receptor, FPR. The peak of activity was reached 1–2 minutes after the addition of cytochalasin B and the response then rapidly declined to reach a base-line level after 3–4 min that remained constant throughout the observation period.

In agreement with the results reported by others [8,9] we found that TNF-α alone only poorly activated the neutrophil NADPH-oxidase, determined as the release of superoxide anions ($3.2 \pm 0.3 \times 10^6$ cpm; mean peak value $\pm$ SD, n = 3; corresponding to a maximum value that was less than 5% of that induced by fMLF). The oxidase activity induced by cytochalasin B in TNF-α-treated cells was of the same magnitude as that triggered by fMLF (Figs 1 and 2). In agreement with previous reports [20-22], TNF-α was found to prime neutrophils to a subsequent stimulation with fMLF (Fig 2).

The level of superoxide production induced by cytochalasin B was dependent on the concentration of TNF-α. (Fig 1) as well as on the duration of TNF-preactivation. When the time between the addition of TNF-α and cytochalasin B was less than 5 minutes, no respiratory burst activity was observed upon cytochalasin B challenge. The response to cytochalasin B gradually increased with the time allowed for TNF-α to interact with the neutrophils, reaching a plateau after approximately 20 min (Fig 3). The protein synthesis inhibitor cycloheximide did not inhibit the cytochalasin B-induced NADPH-oxidase activity in TNF-α primed cells (data not shown), suggesting that *de novo*
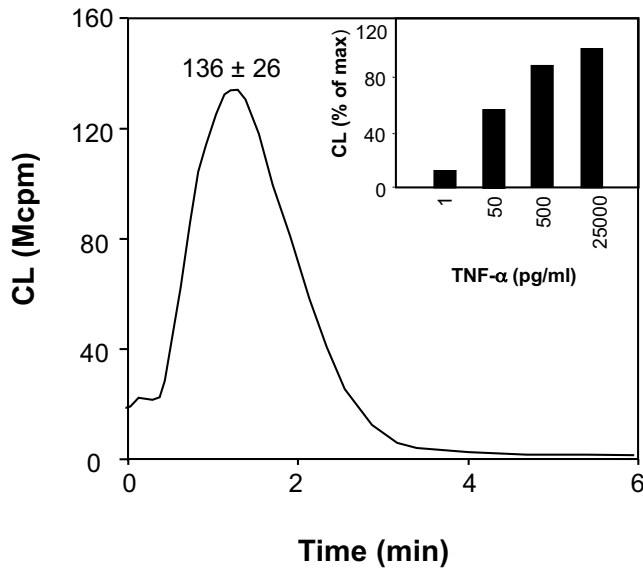
Figure 1
Cytochalasin B stimulation of TNF-α primed neutrophils. Neutrophils were pre-incubated with TNF-α (25 ng/ml, 20 min, 37°C) after which they were stimulated with cytochalasin B The extracellular release of superoxide anions was measured by isoluminol-amplifled chemiluminescence (CL) given as Mcpm (10^6 counts per minutes). The figure shows the kinetics of a representative experiment. The indicated value shows the mean peak value ± SD, n = 3. The ability of cytochalasin B to induce NADPH-oxidase activity was dependent on the priming concentration of TNF-α (inset; comparing the peak values of the responses at varying concentrations to the value obtained with 25 ng/ml of TNF-α).
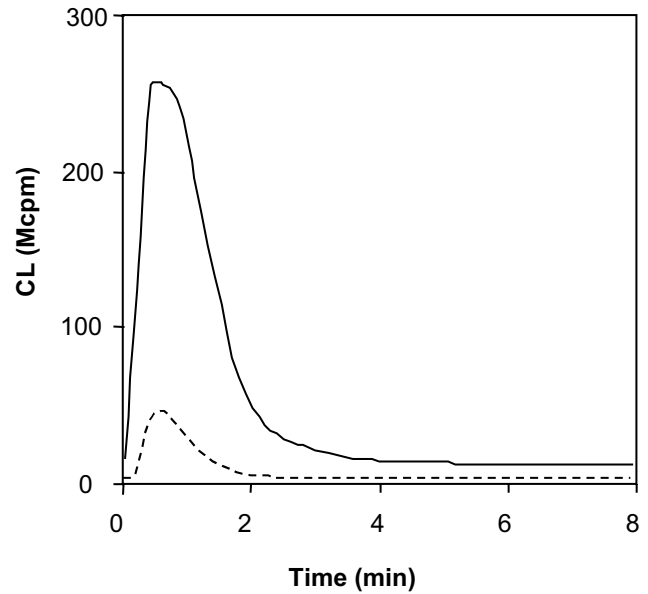

Figure 2
fMLF stimulation of TNF-α primed neutrophils. Neutrophils were pre-incubated in the absence (dashed line) or presence (solid line) of TNF-α (25 ng/ml) for 20 min at 37°C after which they were stimulated with fMLF. The extracellular production of superoxide anion after addition of the peptide (10^{-7}M) was measured by isoluminol-amplified CL. Responses of CL are given as Mcpm (10^6 counts per minutes). The figure shows the kinetics of representative experiments.

protein synthesis was not required for the observed neutrophil activation.

Reactivation of deactivated GPCR has implications for TNF-α priming
All neutrophil chemoattractant receptors including C5aR (for the complement component C5aR), PAFR (for platelet activating factor), CXCR1 and 2 (for IL-8), FPR (for fMLF) and FPRL1 (for WKYMVM) belong to the GPCR family [2]. Interaction between these receptors and their respective ligands results in activation of the neutrophil NADPH-oxidase (Table 1). Neutrophils that were allowed to interact with either of these agonists at 15°C and then transferred to 37°C became deactivated, i.e., there was no burst in oxidase activity, and the cells did not respond to further stimulation with the same chemoattractant. Furthermore, cytochalasin B induced a robust burst of oxidase activity in C5a- as well as in fMLF- and WKYMVM-deactivated cells i.e., these cells (or rather the FPR, FPRL1

and C5aR) were reactivated. In contrast, no such reactivation was induced by cytochalasin B in IL-8 or in PAF deactivated cells (Table 1).

The cytochalasin B induced superoxide production in TNF-α-primed neutrophils is similar to that induced by uncoupling and reactivation of occupied and deactivated FPR, FPRL1 or C5aR from the cytoskeleton, induced by the same drug. The most direct interpretation of these results is therefore that the TNF-α primed cells express a GPCR, of hitherto unknown identity, which gradually becomes occupied and then deactivated. When uncoupled from the cytoskeleton by cytochalasin B, the receptor is reactivated and the signals generated induce an activation of the oxidase. To test this hypothesis, we pretreated the cells with pertussis toxin, which inactivates the heterotrimeric G-proteins coupled to the GPCRs. Since no NADPH-oxidase activity could be induced by cytochalasin B in TNF-α primed cells that were first treated with pertussis toxin (Fig 4), our hypothesis is valid, i.e., a GPCR is involved in the TNF-α-primed cytochalasin B response. It should be noted that pertussis toxin had no effect on the
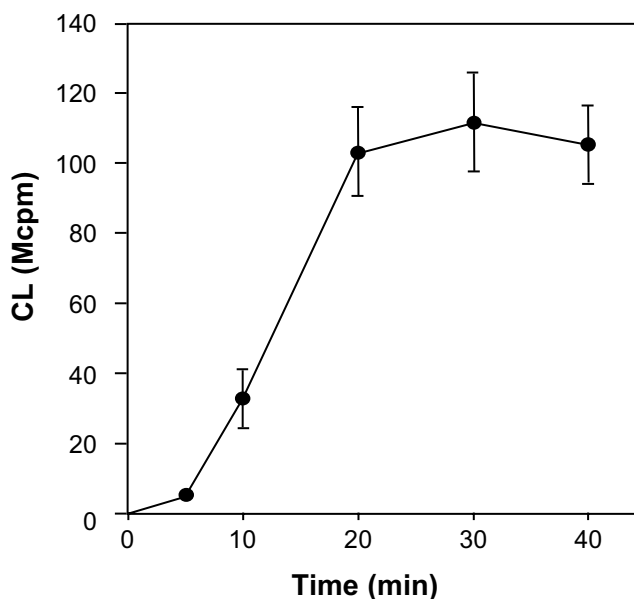
Figure 3
Time dependency of the TNF-α effect Responses to cytochalasin B after incubation with TNF-α for different periods of time. Neutrophils were pre-incubated with TNF-α (25 ng/ml, 37°C) under various time-periods (5–40 min), before they were stimulated with cytochalasin B. The production of superoxide anions was measured by isoluminol-amplified CL as described above.

Figure 4
Pertussis toxin (PtX) sensitivity of cytochalasin B induced superoxide anion production and CR3 upregulation in TNF-α primed neutrophils. Neutrophils were preincubated with TNF-α (25 ng/ml, 20 min, 37°C) before they were incubated with PtX (500 ng/ml) for various time-periods (20–140 min). The cells were either paraformaldehyde-fixed and analyzed by flow cytometry for CR3 upregulation (closed squares), or stimulated with cytochalasin B, and the superoxide anion production were measured by CL (open squares). All analyzed populations of PtX incubated cells were compared to TNF-α primed cells not exposed to PtX.

ability of TNF-α to mobilize CR3 to the neutrophil surface (Fig 4). On the basis of these results, we hypothesized that a preformed endogenous agonist may be released from neutrophils during interaction with TNF-α, and we therefore attempted to verify the existence of such an agonist.

Cytochalasin B induced reactivation of FPR is inhibited by the receptor antagonist cyclosporine H

To test the hypothesis of an endogenous GPCR-agonist, we first determined the basic reactivation characteristics of occupied and desensitized receptors, using a model system in which desensitized neutrophils were pre-incubated with fMLF at 15°C. When pre-warmed neutrophils (37°C for 20 min) were added to a measuring system containing desensitized cells, the oxidase of the non-desensitized cells was activated, suggesting that free ligands are present in the measuring system (data not shown). In order to determine if these free ligands are of importance for the FPR reactivation induced by cytochalasin B, we added the receptor specific antagonist cyclosporine H to fMLF-desensitized cells a few seconds before cytochalasin B. In agreement with the specificity of the antagonist for FPR, a reduced NADPH-oxidase activity was obtained. No
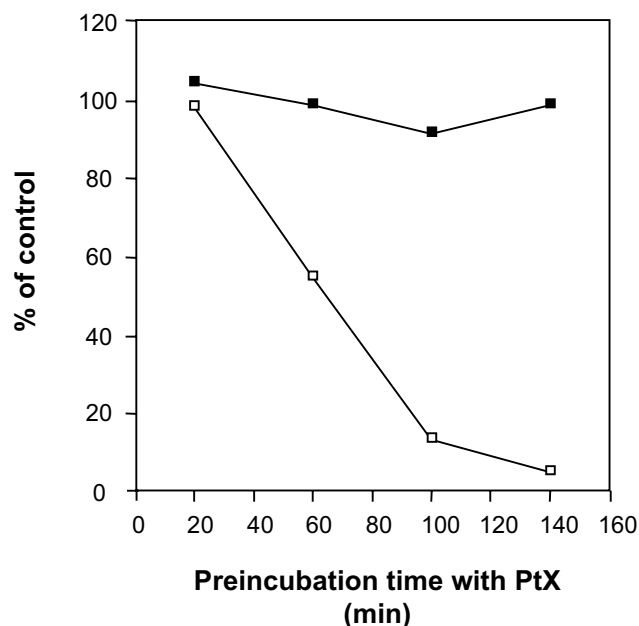
such inhibition by cyclosporine H was seen upon reactivation, when using cells that were incubated with the FPRL1 specific agonist WKYMVM or TNF-α (Fig 5).

Moreover, when the fMLF specific desensitization procedure was performed in a dense cell population (10^7 cells/ml desensitized with 10^{-7}M fMLF) and the cells were diluted to 10^5 cells/ml in pre-warmed measuring vials containing either a high concentration of peptide (final concentration of fMLF being 10^{-7}M) or no peptide (final concentration of fMLF being 10^{-9}M), the addition of cytochalasin B induced a burst in NADPH-oxidase activity only in samples with a high concentration of fMLF (Fig 6). Taken together these results suggest that in order for cytochalasin B to function as an inducer of respiratory burst, the fMLF desensitized neutrophils have to be continuously exposed to a high concentration of free ligand.

Table 1: Deactivation/reactivation properties of classical chemoattractants and their receptors.

		Effects on the neutrophil NADPH-oxidase*		
Agonist (conc.)	Receptor(s)	Activation	Deactivation	Reactivation
fMLF (10-7M)	FPR	+	+	+
WKYMVM (10-7M)	FPRL1	+	+	+
C5a (100 ng/ml)	C5aR	+	+	+
PAF (10-7M)	PAFR	+	+	-
IL8 (100 ng/ml)	CXCR 1/2	+	+	-

*Indicated chemoattractants were used to stimulate human neutrophils directly (activation), or added to the cells at 15 (according to the protocol described in Methods) after which these cells were transferred to 37 and stimulated with the same agonist (deactivation) or reactivated with cytochalasin B (reactivation). The superoxide anion production was measured by isolummol-enhanced CL. The + and - indicate whether or not the process occurred. The experiments were repeated between 2–10 times.

No endogenous neutrophil activator can be identified following incubation with TNF-α

Neutrophils contain several preformed agonists/chemoattractants [23,24] that could participate in an autocrine amplification loop. As described above, binding of neutrophil chemoattractants to their respective neutrophil receptors induces a rapid desensitization of the receptor, leading to an inability of the cells to respond to a second challenge by the same agonist. Accordingly, if TNF-α would induce secretion of an autocrine activator, this should be expected to induce a desensitized state of the specific receptor population involved. The finding that TNF-α triggered cells were primed rather than desensitized to fMLF, WKYMVM/m and C5a, suggests that the potential endogenous utilizes neither FPR, FPRL1 nor C5aR.

To identify the hypothetical endogenous agonist, we prepared cell free supernatants of TNF-α treated neutrophils and added these to new populations of primed or unprimed cells. No oxidase activation could be induced by compounds secreted from the TNF-α primed cells (data not shown). As stated above, neutrophils added to a population of fMLF desensitized cells were rapidly activated to generate superoxide by the fMLF present in the medium. When repeating this experiment with TNF-primed instead of fMLF desensitized cells, no such activity was seen (Fig 7). Furthermore, the newly added cells (non TNF-α primed) were not triggered by the addition of cytochalasin B (Fig 7), as illustrated by the finding that the NADPH-oxidase activity in mixed population of cells (50% primed and 50% non-primed) was only half of that of a cell population (with the same number of cells) in which all the cells were primed. Taken together these data suggest that no free agonist is present in the TNF-α primed cell suspension that could be responsible for transfer of the cells into a cytochalasin B-sensitive state.

TNF-α induces mobilization of receptor-storing granules

Previous studies have shown that different receptor structures as well as potential activating agonists are stored in secretory granules of peripheral blood neutrophils [17,25,26], suggesting that mobilization of these organelles will prime neutrophils to certain stimuli. We monitored the amount of the complement receptor 3 (CR3) and found that TNF-α-primed cells exposed an increased number of CR3 on their surface (Fig 8). In addition, TNF-α priming was accompanied by an increased specific binding of radiolabeled fMLF (10.2 ± 3.3 moles/10^6cells bound to TNF-α treated cells compared to 4.0 ± 1.6 fmoles/10^6 cells for corresponding control cells; mean ± SEM, n = 6), reflecting an increased amount of formyl peptide receptors (FPR) on the neutrophil surface. Hence, FPR and integrin-storing organelles were significantly mobilized upon treatment of neutrophils with TNF-α. We also found it of interest to determine whether induction of the cytochalasin B sensitive state is unique for TNF-α, or if it occurs also with other secretagogues. To investigate the precise role of granule mobilization in induction of the cytochalasin B sensitive state, we monitored the amount of the complement receptor 3 (CR3) on the surface of neutrophils. The finding that neutrophil receptors for IL-8 were not reactivated by cytochalasin B (Table 1) suggested that IL-8 is a suitable control priming agent in these experiments. No respiratory burst was obtained in response to cytochalasin B when TNF-α was replaced by IL-8 (Table 1), despite an induction of storage organelle mobilization also by IL-8 (Fig 9). It should be pointed out that the activation potency in relation to cytochalasin B was retained also when the concentration of TNF-α was reduced to concentrations that gave a level of CR3 mobilization similar to that induced by IL-8.
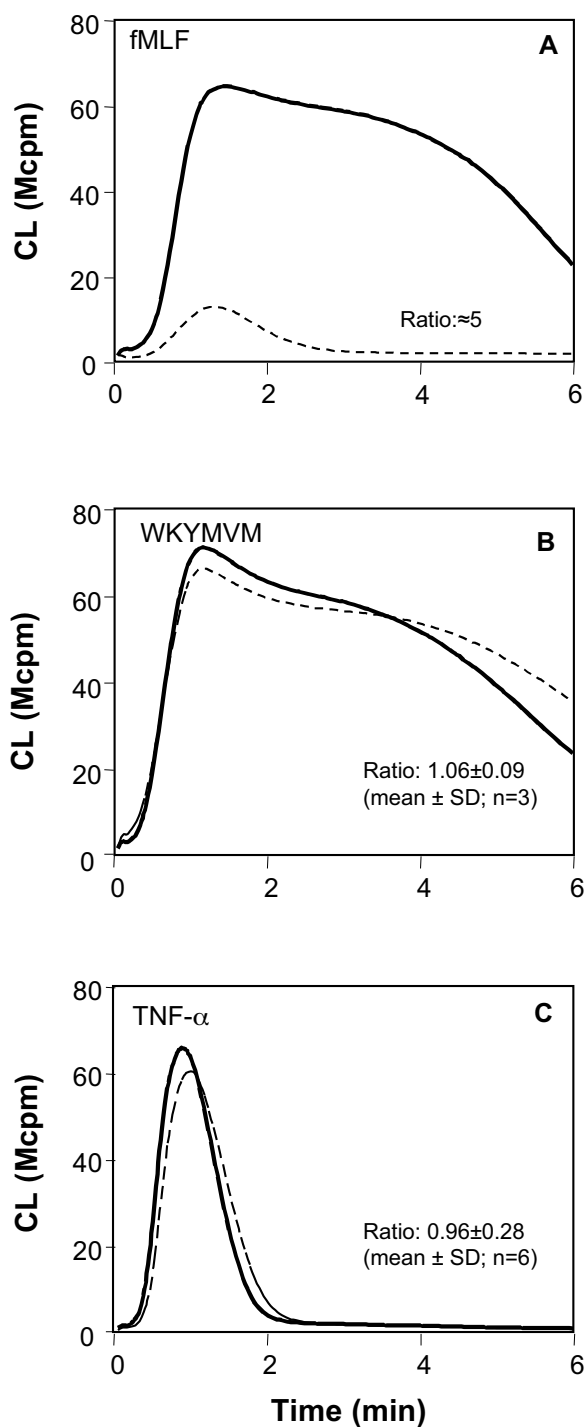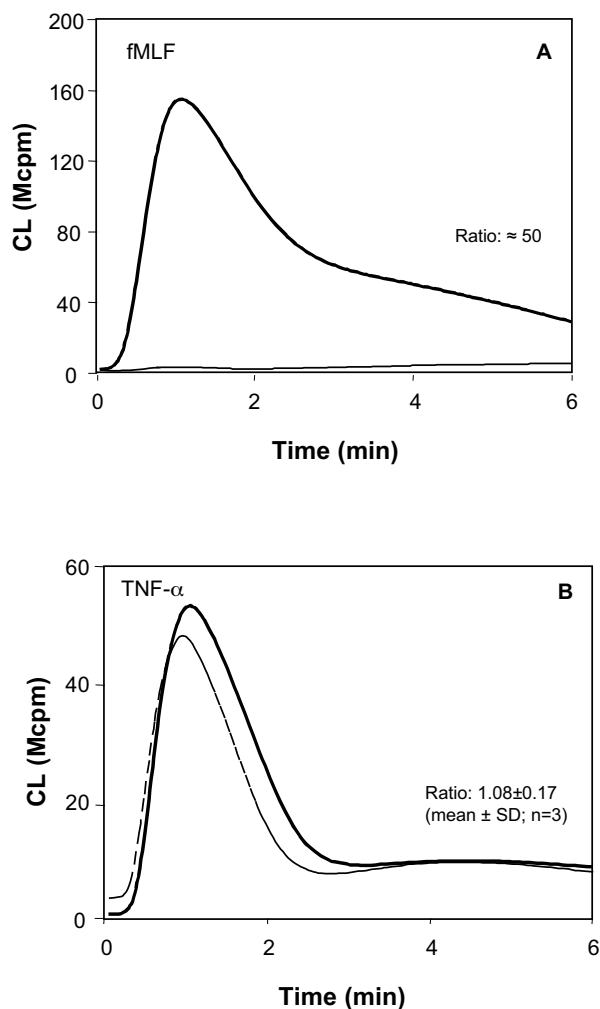
Figure 5
Cyclosporin H sensitivity of cytochalasin B induced superoxide anion production in fMLF desensitized, WKYMVM desensitized or TNF-α primed neutrophils. Neutrophils were preincubated with fMLF (10^{-7}M; 10 min A), WKYMVM (10^{-7}M; 10 min B) or TNF-α (25 ng/ml; 20 min C). The cells were challenged with cytochalasin B in samples without (solid lines) and with cyclosporine H (final concentration in A, 10^{-7}M and in B and C, 10^{-6}M; dashed lines) that was added 1 min before cytochalasin B. The production of superoxide anions was measured by isoluminol-amplified CL as described above. The figure shows the kinetics of representative experiments and the ratios between the peak response in the absence and presence of cyclosporine H are given.

Figure 6
Dilution effects on cytochalasin B induced activation Neutrophils were incubated in the presence of fMLF (10^{-7}M; A) or TNF-α (25 ng/ml; B) for 20 min at 37°C and then diluted in measuring vials containing the same concentration (high; solid lines) or no agonist (low; dashed lines), respectively. The cells were then immediately challenged with cytochalasin B. The production of superoxide anion was measured by isoluminol-amplified CL as described above. The figure shows the kinetics of representative experiments and the ratios, determined from the peak response in the presence of high and low concentrations of agonist.

Effects of the MAPK inhibitor SB 203580
There is a well described link between TNF-α-induced activation of the p38 mitogen-activated protein kinase (MAPK) and superoxide anion formation in adherent neutrophils (reviewed by Berton [27]). We investigated the effect of the specific p38 MAPK inhibitor SB203580 in our model system. As shown in Fig 8, SB203580 significantly attenuated the TNF-α priming, whereas no reduction was observed when neutrophils were first incubated with SB203580 and then stimulated with PMA, a phorbol ester known to activate the NADPH oxidase directly

through protein kinase C (Fig 10A). Furthermore, the p38 MAPK inhibitor attenuated the effect of TNF-α induced granule mobilization, as determined by a reduced exposure of CR3 on the surface of cells challenged with TNF-α in the presence of SB 203580 (Fig 10B). Hence, p38MAPK is involved not only in the TNF-α-induced NADPH-oxidase activation in adherent cells, but also in the priming effects (degranulation and cytochalasin B-sensitivity) induced by the cytokine.
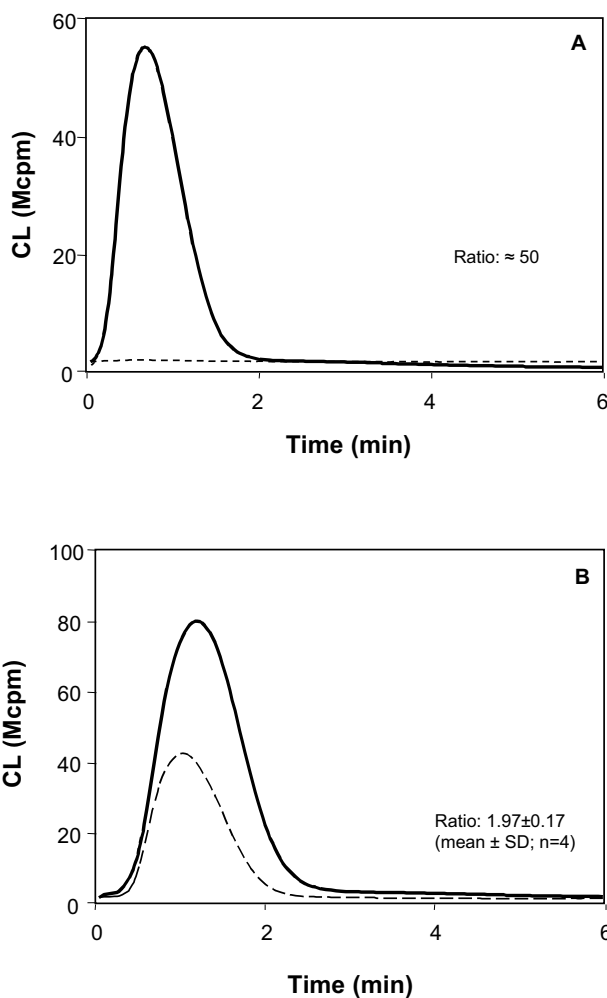
Figure 7
Addition of non-primed cells to TNF-α primed cells and activation by cytochalasin B. Neutrophils incubated for 20 min at 37°C for 20 min were added to a population of TNF-α primed cells. The amount of superoxide release by the newly added cells was determined (A; dashed line). For comparision the NADPH-oxidase activity induced by cytochalasin B when added to the TNF-primed cells (A; solid line) is also shown. The NADPH-oxidase activity induced by cytochalasin B in a cell population where all the cells were primed with TNF-α for 20 min (B; solid line) was compared to that of a cell population where 50% of the cells were primed with TNF-α for 20 min and the other 50% for 1 min (B; dashed line). The production of superoxide anions was measured by isoluminol-amplified CL as described above. The figure shows the kinetics of representative experiments and the ratios between the peak responses in the populations are also given.

Discussion

A prominent feature of TNF-α is its capacity to prime neutrophils to other stimuli. In this study, we show that TNF-α-treated neutrophils are primed for activation by the cytoskeleton-disrupting drug cytochalasin B. The molecular mechanisms underlying priming of the NADPH oxidase response have been extensively studied. In previous reports, we have forwarded the hypothesis that mobilization of receptors, stored within granules/ves-

icles, is a major mechanism involved in priming of the neutrophil response [15-17]. Other proposed mechanisms include alterations of intracellular signaling pathways (increased protein phosphorylation [28], phospholipase activity [29], intracellular Ca^{2+} changes [30]), cross-talk between Ca^{2+} increase and tyrosine phosphorylation [31], altered assembly of the oxidase [32], and proteolytic processing of cell surface proteins [33]. Although we can not exclude that multiple mechanisms
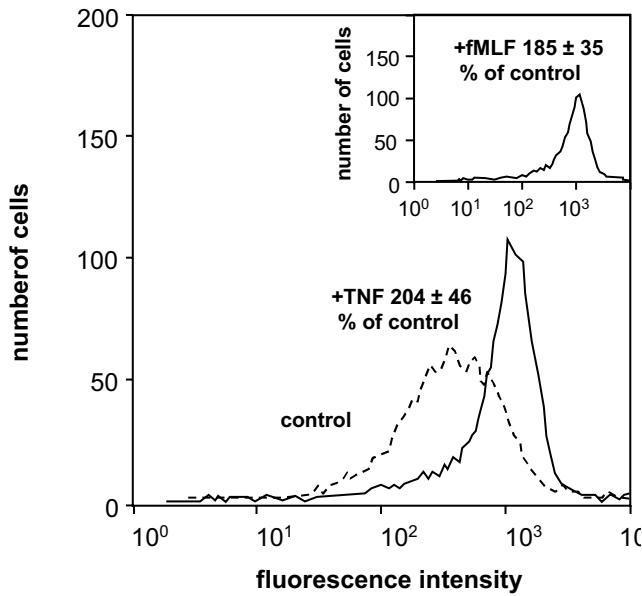
Figure 8
CR3-upregulation in TNF-α primed neutrophils. Neutrophils were incubated with TNF-α (25 ng/ml, 20 min, 37°C, solid line) and were paraformaldehyde-fixed, incubated with phycoerythrin-conjugated anti-CR3 antibodies (CD18/CD11b) and analysed by flow cytometry and compared with control cells (dashed line). Representative histograms of binding are shown, and the inset shows the exposure of CR3 on fMLF-primed neutrophils. The numbers indicate the mean fluorescence intensity as percent of control ± SD, n = 3.

may be involved in TNF-α-induced priming, it is important to point out that a prerequisite for priming through the mechanisms described above is that a second agonist is required, in addition to the priming agent, in order to disclose the increased potential for neutrophils to respond.

The cytoskeleton disrupting molecule cytochalasin B has generally been regarded as a substance which lacks the ability to activate neutrophils by itself, and the results described here could possibly be explained by an ability of cytochalasin B to induce a state of receptor reactivation [18]. This is defined as the transfer of a deactivated/desensitized receptor into an actively signaling state, achieved by uncoupling of the receptor from the cytoskeleton. Using the ligand-receptor pair fMLF-FPR as a model, it has been shown that the processing of neutrophil chemoattractant receptors includes highly regulated events that occur in a given chronological order. The binding of the agonist to its inactive cell surface receptor (FPR) generates an actively signaling receptor-ligand complex (FPR*). Shortly after binding of the chemoattractant to its

receptor, the receptor-ligand complex associates with actin or other cytoskeletal proteins [34-36], inducing a physical segregation of the signaling G-protein and the receptor into different plasma membrane domains [37]. This leads to termination of the response by a direct cessation of the transmembrane signals, and the receptor-ligand complex is thus deactivated/desensitized (FPR^*_{des}). The addition of cytochalasin B to cells with such deactivated/desensitized receptors leads to uncoupling of the receptor-ligand complex from the cytoskeleton, resulting in regained signaling capacity of the receptor (FPR^{re*}), and the signals generated activate the oxidase [18].

The cytochalasin B induced activation of the NADPH-oxidase in TNF-α primed neutrophils is very similar to that achieved by an uncoupling of FPR^*_{des} from the cytoskeleton and a transfer of the receptor into an FPR_{re}^* state. The assumption that GPCRs are involved also in TNF-α/cytochalasin B induced oxidase activity was validated by experiments using pertussis toxin, a specific inhibitor of the heterotrimeric G-proteins linked to all the neutrophil chemoattractant receptors yet characterized [2]. The cytochalasin B-induced oxidase activity in TNF-α primed cells was clearly pertussis toxin sensitive; in contrast, the toxin did not affect TNF-α induced mobilization of CR3 to the cell surface, which is in agreement with the fact that the TNF-α receptor itself is not a member of the GPCR family [38]. It is interesting to note that cytoskeleton-dependent receptor reactivation is not achieved with all neutrophil GPCRs. FPR^*_{des}, $FPRLl^*_{des}$ and $C5aR^*_{des}$ were all reactivatable by cytochalasin B. No such reactivation was however obtained with the occupied receptors for IL-8 and PAF. Neither the $IL\text{-}8\text{-}R^*_{des}$ nor the $PAF\text{-}R^*_{des}$ could thus be reactivated with cytochalasin B (Table 1). These data suggest that these two receptor-ligand pairs should be excluded from being responsible for the TNF-α induced change in sensitivity for cytochalasin B, but more importantly the data also suggest that there are fundamental differences with respect to signaling between different groups of chemoattractant receptors. This is in agreement with recently presented data describing distinct signaling pathways for GPCRs that mediate directional migration by chemoattractants, guiding the neutrophils out of the vasculature (i.e., interleukins and lipid mediators) and those that guide the cells through the interstitium to a site of infection (i.e. bacterial chemoattractants and activated complement factors) [39]. Our results suggest that TNF-α induces a neutrophil state that shares basic the signaling properties with the FPR^*_{des}, $FPRLl^*_{des}$ and $C5aR^*_{des}$, i.e., a state that is reactivated when the cytoskeleton is disrupted by cytochalasin B.

Neutrophils contain known, and probably also unknown, secretable chemoattractants [24,40], as well as known and possibly not yet identified receptors for such agonists (i.e.,
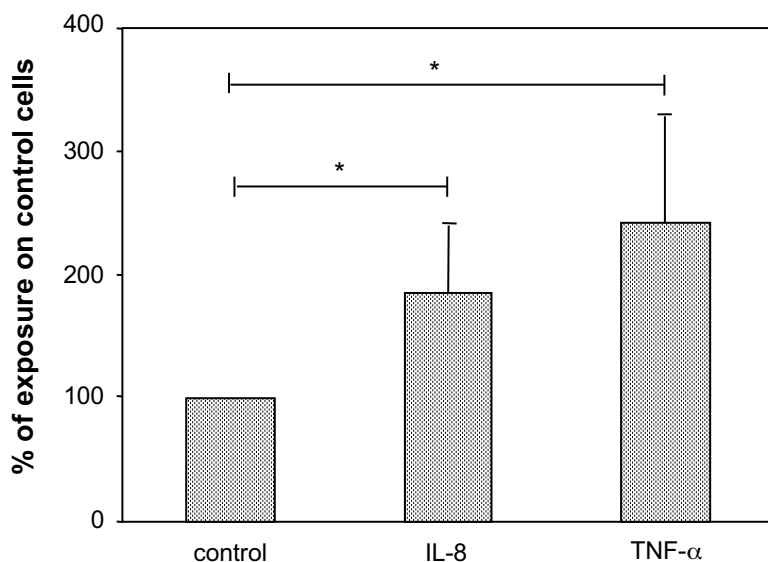
Figure 9
CR3-upregulation in IL-8 and TNF-α primed neutrophils. Neutrophils were incubated with or without IL-8 (100 ng/ml) or TNF-α (25 ng/ml) for 20 min at 37°C. The cells were then paraformaldehyde-fixed, incubated with phycoeryterin-conjugated anti-CR3 antibodies (CD18/CD11b), analysed by flow cytometry and compared with control cells. The data is calculated from the mean fluorescence intensity of each cell population and expressed as percentage of the value obtained in control cells, n = 6.

CXCR1 and 2 for IL-8 and FPRL1 for LL37). The combined TNF-α/cytochalasin B-dependent activation process was pertussis toxin sensitive suggesting that the activation process involves a heterotrimeric G-protein, possibly (but not known for certain) linked to a receptor. An attractive hypothesis that directly explains our results would be that TNF-α induces secretion of an endogenous agonist which binds back to a neutrophil receptor sharing its basic signaling properties with the cytoskeleton-regulated group of GPCRs. This endogenous agonist would occupy its surface receptors and being a ligand-receptor pair of the cytoskeleton-binding type, the receptors would then become desensitized upon agonist binding and the cells would be transferred into a cytochalasin B-activated state. Although we can not exclude this possibility, the experimental evidence presented is inconsistent with this scenario. On the one hand pretreatment with secretagogues induces mobilization of neutrophil storage organelles, but on the other hand, such a mobilization is insufficient for induction of the cytochalasin B sensitive state as illustrated by the finding that IL8, a potent secretagogue (Fig 9), failed to prime neutrophils for subsequent activation by cytochalasin B (Table 1). In addition, we assumed that provided that a hypothetical agonist secreted in response to TNF-α has a binding affinity for its receptor, that is of the same magnitude as the hitherto identified/characterized neu-

trophil activators, it should be present in the extracellular environment, but despite the use of several experimental approaches we were unable to find any evidence for the existence of such an agonist.

Over the past few years, studies on the biology of GPCR have been focused on the activation achieved through binding of a specific agonist to its receptor. It has, however, become increasingly clear that GPCRs can be transferred from a non-signaling R state to an actively signaling R* state also in the absence of any bound ligand [19,41]. It is obvious that the R* state can not be constitutively active, and irrespectively of whether the receptor reaches the R* state following agonist binding or if this state is reached independently of any activating agonist, the deactivation mechanisms appears to be put in action. The physical separation of the R* from the G-protein occurring through a linkage of the receptor to the cytoskeleton, constitute an important termination mechanism, and R^*_{des} state can be reversed through an uncoupling from the cytoskeleton [18]. The fact that we were unable to find any secreted components that could fulfill the role of a receptor agonist in the TNF-α/cytochalasin B dependent activation system, suggests that TNF-α may transfer neutrophil GPCR's to a cytoskeleton associated R^*_{des} state independently of agonist binding.
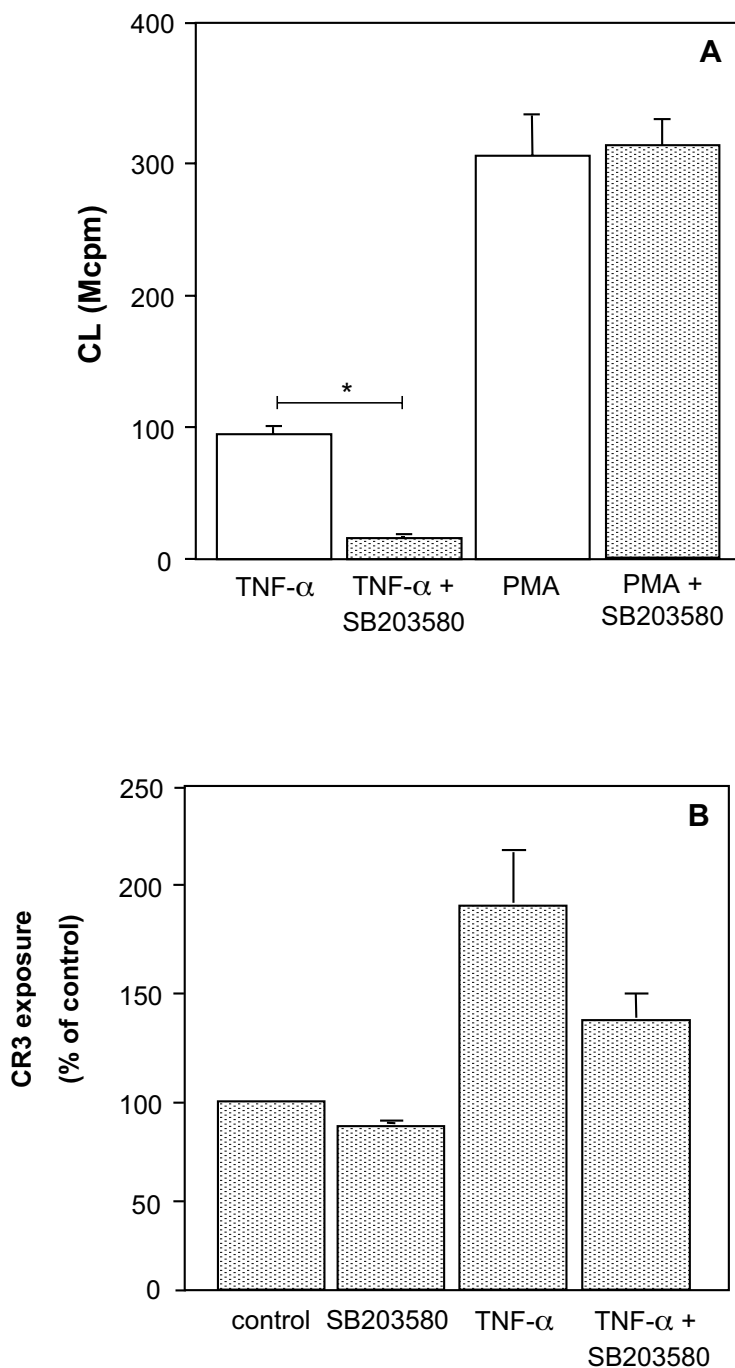
Figure 10
Effects of SB203580 on TNF-α priming. Neutrophils were primed by TNF-α (25 ng/ml, 20 min, 37°C) in the absence or presence of the p38 MAPK inhibitor SB203580 (1 μM). (A) The cells were stimulated with cytochalasin B (5 μg/ml) and the extracellular production of superoxide anion was measured by CL. The effect of SB203580 on PMA-induced superoxide anion production was used as control. (B) Neutrophils were incubated with TNF-α (25 ng/ml) in the absence or presence of SB203580 (1 μM)), or with SB203580 alone (20 min, 37°C) and were paraformaldehyde-fixed, incubated with phycoerythrin-conjugated anti-CR3 antibodies (CD18/CD11b), analysed by flow cytometry and compared with control cells. Calculation were made from the mean fluorescence intensity of each cell population and expressed as percentage of the value obtained in control cells. The results are given as mean ± SE of three independent experiments.

A transformation from R to R* can, thus, occur in the absence of a specific ligand, but very little is known about the regulatory mechanisms that determines the rate of transfer or the levels of the two forms at equilibrium. The R/R* equilibrium differs for individual wild-type isoforms of a given receptor, and the ratio between the two forms can also be changed both by defined point mutations localized to several different intracellular receptor domains, as well as by the degree of glycosylation of the receptor [19]. It seems reasonable to hypothesize that the R/R* transformation rate and/or ratio at equilibrium can be changed not only through direct structural changes in the receptor protein, but also indirectly through signals generated by a second receptor such as the one for the TNF-α (receptor communication), or through a direct receptor-receptor interaction in the membrane.

Conclusions

Neutrophils triggered with TNF-α mobilize receptor storing organelles and concomitantly but independently, the cells are primed to respond to the microfilament disrupting toxin cytochalasin B. These findings are suggestive of two mechanisms involved in neutrophil priming by TNF-α. While the enhanced response to fMLF is explained by recruitment of new formyl peptide receptors to the cell surface, the cytochalasin B-sensitive state is achieved through a novel mechanism that transfers G-protein coupled receptors to a primed state which is fully activated when cytochalasin B uncouples the receptors from the cytoskeleton. Such a reaction could possibly be induced by an endogenous secreted receptor agonist. We were, however, unable to find any components that could fulfill the role of an agonist in the TNF-α/cytochalasin B dependent activation system. The presented data support the recently introduced concept that receptor activation can occur independently of a specific receptor ligand [19]. Accordingly, we suggest that TNF-α transfers neutrophil receptors to a desensitized and cytoskeleton associated state independently of agonist binding.

Methods
Isolation of neutrophils
Leukocytes were isolated from freshly prepared leukopacks (The Blood Center, Sahlgrenska University Hospital, Göteborg) obtained from healthy blood donors. After removal of erythrocytes through dextran sedimentation, the leukocyte-rich supernatant was carefully layered onto Ficoll-Paque (Lymphoprep, Nyegaard, Norway). After centrifugation at 380 × g for 30 minutes, the pellet was resuspended in Krebs-Ringer phosphate buffer (KRG, pH 7.3; 120 mM NaCl, 5 mM KC1, 1.7 mM KH_2PO_4, 8.3 mM $NaHPO_4$ and 10 mM glucose) supplemented with Ca^{2+} (1 mM) and Mg^{2+} (1.5 mM), and kept on melting ice until used in experiments [42].

Neutrophil activators
The hexapeptide Trp-Lys-Tyr-Met-Val-Met-NH_2 (WKYMVM) was synthesized and HPLC-purified by Alta Bioscience (University of Birmingham, Birmingham, United Kingdom). The formylated peptide N-formylmethionyl-leucyl-phenylalanine (fMLF), the chemotactic fragment of complement factor 5 (C5a), platelet activating factor (PAF), and TNF-α were from Sigma Chemical Co., St. Louis. Human recombinant IL-8 was provided by R&D Systems, Oxon, UK. The peptide agonists were dissolved in dimethyl sulfoxide to 10^{-2}M and stored at -70°C until use. Further dilutions were made in KRG.

Neutrophil priming and NADPH-oxidase activity
Neutrophils (1–2 × 10^6cells/ml) were incubated at 37°C for 5–40 minutes in the presence or absence (control) of a priming agent. The NADPH-oxidase activity of these cells was then recorded using luminol/isoluminol-enhanced chemiluminescence (CL) systems [43]. The CL activity was measured in a six-channel Biolumat LB 9505 (Berthold Co. Wildbad, Germany), using disposable 4-ml polypropylene tubes with a 0.90-ml reaction mixture containing 1–2 × 10^6 neutrophils. In order to differentiate between intracellularly and extracellularly generated reactive oxygen species, two different reaction mixtures were used. Tubes used for the measurement of extracellular release of superoxide anion contained neutrophils, horseradish peroxidase (HRP; a cell impermeable peroxidase; 4 U) and isoluminol (a cell impermeable CL substrate; 2 × 10^{-5}M). Tubes used for measurement of intracellular generation of reactive oxygen species contained neutrophils, SOD (a cell impermeable scavenger for O_2^-; 50 U), catalase (a cell impermeable scavenger for H_2O_2; 2000 U), and luminol (a cell permeable CL substrate; 2 × 10^{-5}M). The tubes were equilibrated at 37°C, after which the stimulus (0.1 ml) was added. The light emission was recorded continuously.

Determination of receptor exposure
The amount of fMLF-receptors expressed on the cell surface in the different neutrophil populations was determined by incubating the cells with radiolabeled fMLF in the presence or absence of unlabeled fMLF. Unbound peptide was removed by centrifugation of the cells through an oil layer. The oil layer which was composed of a mixture of dibutylphtalate and dinonylphtalate (10:3, v/v; 100 μl) was layered on top of 10 μl of urea (6 M) in Eppendorf tubes. The radiolabeled peptide (50 μl [^{3}H]fMLF; 8 × 10^{-8}M) was then layered on top of the oil followed by 50 μl of unlabeled fMLF (4 × 10^{-5}M) in KRG or vehicle alone. Neutrophils (2 × 10^6, 100 μl) were added to the fMLF-solution and the tubes were incubated on melting ice for 1 h. After centrifugation at 9000 × g for 15 seconds in a Beckman microfuge (Beckman Instruments, Fullerton, CA), the bottom of the centrifuge tubes (con-

taining the pelleted cells) was excised and collected for determination of radioactivity.

To measure surface exposure of CR3, the cells were labeled with phycoerythrin-conjugated monoclonal antibodies specific for CD11b (DAKO M741; 10 μl to a cell pellet of 10^6). The cells were examined by use of flow cytometry (FACScan; Becton Dickinson, Mountain View, CA).

Deactivation and reactivation

In order to deactivate neutrophils to GPCR agonists, the cells were first equilibrated for 10 min at 15°C. The agonist, e.g., the chemoattractant fMLF (final concentration 10^{-7}M) was added and the incubation was continued at 15°C for an additional 5 min [44]. The cells were then transferred to 37°C for 5 minutes, and reactivation was subsequently achieved by adding cytochalasin B (5 μg/ml).

Reagents

Human recombinant TNF-α, fMLF, luminol, isoluminol, cycloheximide, Pertussis toxin, SB 203580 and cytochalasin B were obtained from Sigma Chemical Co., St. Louis. Superoxide dismutase (SOD), catalase and horse radish peroxidase (HRP) were from Boehringer-Mannheim, Germany. Radiolabeled fMLF was from Du Pont NEN (Boston, Mass).

Statistical analysis

The Student's *t*-test (two-tailed) was performed to determine statistical significance.

List of abbreviations

CD11b, cluster of differentiation number 11b; CL, chemiluminescence, GPCR, G-protein coupled receptor, CR3, complement receptor 3, CXCR, the IL 8 (CXC cytokine) receptor, C5a, the chemotactic split product from complement factor 5; C5aR, the C5a receptor, fMLF, formylmetleu-phe; FPR, the formyl peptide receptor; FPRL1, the formyle peptide like receptor 1; IL-8, interleukin 8; SOD, superoxide dismutase; TNF, tumor necrosis factor, TNFR, the TNF-recepor; WKYMVM, Trp-Lys-Tyr-Met-Val-Met.

Authors contributions

The scientific question raised in the paper was formulated during discussions between all the authors, about the mechanisms behind the priming phenomenon in relation to receptor resensitization (see [18]). JB, SP, HF and UMH performed all the experiments using techniques developed by CD, JB and AK. All authors participated in the planning of the work and in analyzing the results. UMH and CD wrote the first version of the paper, but contributions from all authors were important for the final outcome of the paper.

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