TLR Costimulation Causes Oxidative Stress with Unbalance of Proinflammatory and Anti-Inflammatory Cytokine Production

Rosa Lavieri, Patrizia Piccioli, Sonia Carta, Laura Delfino, Patrizia Castellani and Anna Rubartelli

*J Immunol* 2014; 192:5373-5381; Prepublished online 25 April 2014;
doi: 10.4049/jimmunol.1303480
http://www.jimmunol.org/content/192/11/5373

---

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2014/04/25/jimmunol.1303480.DCSupplemental

**References**

This article cites 43 articles, 18 of which you can access for free at:
http://www.jimmunol.org/content/192/11/5373.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
TLR Costimulation Causes Oxidative Stress with Unbalance of Proinflammatory and Anti-Inflammatory Cytokine Production

Rosa Lavieri,1 Patrizia Piccoli,1 Sonia Carta, Laura Delfino, Patrizia Castellani, and Anna Rubartelli

IL-1β acts in concert with anti-inflammatory cytokines, in particular, IL-1R antagonist (IL-1Ra), to ensure the correct development and outcome of the inflammation: imbalance in the IL-1β/IL-1Ra ratio is implicated in many human diseases and may lead to dramatic consequences. In this article, we show that single TLR engagement induces IL-1β and, with a little delay, IL-1Ra. Differently, costimulation of TLR2, TLR4, and TLR7/8 enhances IL-1β secretion but severely inhibits IL-1Ra production. The IL-1β/IL-1Ra unbalance after activation of multiple TLRs depends on the insurgence of oxidative stress, because of enhanced production of reactive oxygen species and failure of the antioxidant systems. Increased reactive oxygen species levels increase ATP externalization by monocytes, resulting in enhanced inflammasome activation and IL-1β secretion. Oxidative stress then induces cell responses to stress, including inhibition of protein synthesis, which, in turn, is responsible for the impaired production of IL-1Ra. IL-1Ra secretion is restored by exogenous antioxidants that oppose oxidative stress. Similar effects are evident also on other cytokines: TNF-α is induced, whereas IL-6 is inhibited by costimulation. Our findings provide a molecular basis to the imbalance between proinflammatory and regulatory cytokine circuits that occur in various pathologic conditions, and suggest new strategies for controlling inflammation. The Journal of Immunology, 2014, 192: 5373–5381.

Recognition of microbial pathogens is mediated by germline-encoded pattern recognition receptors (1). Among these, TLRs detect a wide range of molecular structures broadly shared by pathogens, known as pathogen-associated molecular patterns (PAMPs) (1). In humans, a number of TLRs are expressed on the cell surface (TLR1, TLR2, TLR4, and TLR6); others (TLR3, TLR7, and TLR8) are located in intracellular vacuoles (1). TLR engagement activates signaling pathways, leading to induction of inflammatory mediators (2).

Several TLRs are likely triggered during a single infection because different pathogens may be involved and each comprises multiple PAMPs. Damage-associated molecular pattern molecules released by injured tissues may contribute to amplify TLR activation (3). How multiple TLR stimulation impacts on the subsequent inflammatory response is unclear. Synergistic effects on cytokine production prevail in macrophages and dendritic cells (4–7), although in human dendritic cells concomitant TLR2 stimulation blocks the induction of a subset of Th1 cytokines specifically induced by TLR4 or TLR3 (8). Fewer and discrepant results are available in primary human monocytes: triggering of TLR8, TLR2, and TLR4 increases cytokine responses (9–11), whereas TLR8 and TLR4 costimulation inhibit the production of IL-10 (10) and of chemokines such as CCL1 and CCL2 (11).

IL-1β is a major proinflammatory cytokine early induced in monocytes by TLR activation (12). Unlike most cytokines, IL-1β lacks a secretory signal sequence and accumulates intracellularly as an inactive precursor (pro–IL-1β) (13). Then, activation of the inflammasome triggers IL-1β processing and secretion (14). In murine macrophages and in macrophage cell lines, inflammasome activation requires a second signal such as exogenous ATP (14). Differently, exogenous ATP is dispensable in primary human monocytes because TLR activation triggers secretion of ATP, which autocrinally stimulates P2X7 receptors (P2X7R), resulting in inflammasome activation and secretion of mature IL-1β (15).

Production of reactive oxygen species (ROS) and the consequent redox signaling are required for IL-1β induction (16, 17) and inflammasome-mediated IL-1β secretion (18). How redox signaling activates the inflammasome is, however, debated (13). We have shown that TLR agonist-induced IL-1β processing and secretion in human monocytes is regulated by a biphasic redox response consisting of a prompt burst of ROS and a delayed antioxidant response (19). The latter involves activation of the xc−antioxidant system (20), composed by xCT, the ROS-inducible transporter of oxidized cystine, and by intracellular oxidoreductases such as thioredoxin. The latter convert the internalized cystine to free cysteine that is then released and quantifiable extracellularly as a marker of the cell antioxidant capacity (13).
The involvement of both arms of the redox system (pro-oxidant and antioxidant) in IL-1β secretion and, in general, in inflammation has been confirmed by in vitro and in vivo studies (21, 22). IL-1β induces itself and several cytokines involved in the inflammatory cascade (12, 23, 24). Among these, IL-1R antagonist (IL-1Ra) is a natural inhibitor of IL-1 that smothers inflammation by antagonizing IL-1 binding to the IL-1R (25). Unlike IL-1β, the major form of IL-1Ra is a secretory protein (25) that upon transcription is synthesized into the endoplasmic reticulum lumen and secreted by default through the classical exocytotic pathway.

In this study, we investigated the effects of the simultaneous stimulation of different TLRs on the production of IL-1β and IL-1Ra by primary human monocytes. Our data show that costimulation induces IL-1β but inhibits IL-1Ra. Similarly, the early proinflammatory cytokine TNF-α (26) is enhanced by the costimulation, whereas the regulatory cytokine IL-6 (24), induced by both IL-1β and TNF-α, is inhibited. This perturbation of the cytokine network is due to an altered redox signaling in costimulated cells and has strong implications for the outcome of the inflammatory responses.

Materials and Methods

**Chemicals and PAMPs**

5,5′-Dithiobis-(2-nitrobenzoic acid), DTT, N-acetylcysteine (NAC), cysteine, brefeldin A, diphenylephrine iodonium (DPI), LPS, zymosan, oxidized ATP (oATP), and 6-N,N-diethyl-d-β-γ-dibromomethylene adenosine triphosphate (ARL 67156) were from Sigma-Aldrich; R848 was from Enzo life; apyrase was from BioLabs; Pam3CSK was from Invivogen; 2′,7′-dichlorofluorescein diacetate (H2DCF-DA) was from Invitrogen; and ac-YYAD-CMK was from Bachem.

**Cell cultures**

Human monocytes from healthy donors were isolated and cultured in RPMI 1640 medium (Sigma) supplemented with 5% FCS (Euroclone) at 37°C in 5% CO2, as described previously (15, 19), in the absence or presence of LPS, R848 and zymosan alone or in combination, at the indicated concentrations. The concentrations of the various TLR agonists able to provide a suboptimal to optimal IL-1β response, determined in earlier experiments (15, 19), were the following: LPS: 100 ng/ml; R848: 5 μg/ml; zymosan: 20 μg/ml; Pam3CSK: 2 μg/ml (referred to as standard dose in this article). When indicated, the following substances were added to cultures: ac-YYAD-CMK at 200 μM, ARL 67156 at 200 μM, DPI at 20 μM, oATP at 300 μM, apyrase at 2.5 μM, DTT at 0.25 mM, and NAC at 0.5 or 1 mM. For each substance a dose response was performed (data not shown) (15, 19, 27). The doses used correspond to the concentrations displaying the highest efficacy in the absence of cell toxicity evaluated by measuring cell death by trypan blue exclusion or lactate dehydrogenase (LDH) release in the supernatant (data not shown). At the end of the culture periods, death by trypan blue exclusion or lactate dehydrogenase (LDH) release was determined in 24-well plates in medium containing 5% FCS were reacted with 10 mM 5,5′-dithiobis-(2-nitrobenzoic acid), and the absorption measured at 412 nm (19, 27–29). Cysteine (Sigma-Aldrich) was used as external standards.

**Western blot analysis**

Triton X-100 cell lysates were resolved on 12% SDS-PAGE and electrotransferred (15, 19, 27–29). Filters were probed with the following Ab: 3ZD anti–IL-1β mAb (IgG1; obtained from the National Cancer Institute Biological Resources Branch); anti-human thioredoxin mAb (clone 2B1; gift from Prof. F. Clarke, Griffith University, South Brisbane, QLD, Australia); anti-human phospho-εIF2α mAb from Cell Signaling; and anti-human GAPDH mAb (Novus Biologicals) followed by the relevant secondary Ab (Dako) and developed with ECL-plus (GE Healthcare). Densitometric analyses of the Western blots were performed using the Quantity One 1-D Analysis Software (Bio-Rad).

**ELISA**

IL-1β, TNF-α, IL-6, and IL-1Ra in supernatants and pro–IL-1β in cell lysates (27) were determined by ELISA (R&D Systems). In some experiments, secretion was expressed as percent of control to account for interpatient variability.

**Real-time PCR**

Total mRNA was isolated from cells using TriPure Isolation Reagent (Roche Applied Science) and reverse-transcribed with the QuantiTect Reverse Transcription Kit (Qiagen). Real-time PCR was performed using Platinum SYBR green qPCR SuperMix-UDG (Invitrogen). The specific primers for IL-1β, cytokine transporter (xCT), and GAPDH have been described elsewhere (27, 29). The specific primers for TNF-α, IL-1Ra, and IL-6 were designed by PRIMER 3 (v.0.4.0; sequences available on request). Target gene levels were normalized to that of GAPDH mRNA, and relative expression was determined using the 2-ΔΔ-Ct method as described previously (27, 29).

**Biosynthetic labeling**

Monocytes were endogenously labeled with 0.5 Ci/ml [35S]methionine/cysteine (Perkin Elmer, Monza, Italy) for 45 min at various times from exposure to TLR agonists (28). Cells were then lysed and aliquots of cell lysates precipitated in 25% cold trichloroacetic acid. Insoluble radioactivity was measured in beta counter (28).

**Statistical analysis**

Data were analyzed by using the one-way ANOVA test, followed by Bonferroni posttest, using GraphPad software as previously described (27–29). Significance is expressed as *p < 0.05, **p < 0.01, ***p < 0.001.

**Results**

**TLR costimulation enhances IL-1β secretion by primary human monocytes**

The production of IL-1β was studied in monocytes from 15 donors after stimulation of TLR4, TLR2, or TLR7/8 with the proper ligands LPS (100 ng/ml), zymosan (20 μg/ml), or R848 (5 μg/ml). The concentrations used (standard doses) were determined as those providing a suboptimal to optimal IL-1β response in the different subjects (data not shown) (15, 19). IL-1β secretion was induced by all PAMPs in variable amounts in the various subjects and was strongly increased by costimulation with two and, to a greater extent, with three TLR agonists (Fig. 1A and 1B). The increased secretion was not a simple dose-dependent effect as single stimulation of each TLR with a triple dose of its agonist induced less IL-1β than


**FIGURE 1.** Costimulation of TLR2, TLR4, and TLR7/8 increases IL-1β secretion. Monocytes from 15 healthy donors were cultured untreated (UNT) or with LPS, R848, Zymosan (Zym) at the standard doses, alone or in double (LPS+R848 [LR]; LPS+Zym [LZ]; R848+Zym [RZ]; n = 6) or triple (LPS+R848+Zym [LRZ]; n = 15) combination. Intracellular pro–IL-1β and secreted IL-1β were quantified by ELISA. (A) IL-1β in 18-h supernatants. (B) Kinetics of IL-1β secretion (one representative experiment, n = 8). (C) IL-1β in 18-h supernatants of monocytes, single-stimulated with standard (1×) or triple dose (3×) of each TLR agonist or costimulated with the standard dose of the three agonists (LRZ). Data are expressed as percent of the IL-1β secretion induced by each agonist, at standard (1×) or triple (3×) dose, with respect to IL-1β secretion induced by LRZ (mean ± SEM, n = 5). (D) Monocytes were stimulated as indicated in the presence or absence of ac-YVAD-CMK. The percent of inhibition of IL-1β secretion by ac-YVAD-CMK is shown (mean ± SEM, n = 3). (E) RT-PCR of IL-1β mRNA at various times from the exposure to TLR agonists. Data are expressed as fold changes versus untreated monocytes at time 0. One representative experiment (same donor as in (B), n = 4). Despite the high variability among donors, the kinetics were consistently very similar in all individuals tested. (F) Intracellular pro–IL-1β at 18 h from exposure to the stimuli. Mean ± SEM (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001.

The costimulation with the standard dose of the three TLR agonists (Fig. 1C). IL-1β secretion induced by single or combined stimulation was prevented by the caspase-1 inhibitor ac-YVAD-CMK (Fig. 1D) or by downmodulating NLRP3 with siRNA (data not shown), confirming (27) its caspase-1 and NLRP3 inflammasome dependency. Despite the strong enhancement of secreted IL-1β, IL-1β mRNA expression was only slightly higher in monocytes triggered by the three TLR agonists (Fig. 1E). Moreover, at the end of the stimulation time (18 h), costimulated monocytes showed less intracellular pro–IL-1β than single-stimulated cells (Fig. 1F).

Because zymosan triggers the phagocytic receptor Dectin-1 in addition to TLR2 (1), we tested also the synthetic TLR2-specific ligand Pam3CSK. The results (Supplemental Fig. 1A) indicate that when used in single stimulation, Pam3CSK induced less IL-1β than zymosan, LPS, or R848. However, in association with LPS and R848, it displayed similar synergistic effects as zymosan.

**Release of endogenous ATP is enhanced by the increased ROS levels in costimulated monocytes and mediates IL-1β secretion**

IL-1β secretion by human monocytes is mediated by endogenous ATP, released in response to TLR triggering, which engages the P2X7R on monocyte surface resulting in autocrine NLRP3 inflammasome activation (15). As shown in Fig. 2A, the amount of released ATP by costimulated cells was dramatically higher than that induced by single stimuli, suggesting a mechanistic link among increased ATP release, P2X7R activation, and increased IL-1β secretion in costimulated cells. This link was, in fact, demonstrated by the following observations: 1) the increase of the extracellular ATP levels obtained by treating monocyte cultures with the ectonucleotidase inhibitors ARL 67156 that prevent ATP hydrolysis (15) (Fig. 2A) was paralleled by increased secretion of IL-1β (Fig. 2B); and 2) in turn, the block of the P2X7R by αATP (15) or the hydrolysis of the released ATP by apyrase (15) strongly inhibited IL-1β secretion (Fig. 2B).

To understand the mechanism connecting TLR costimulation and increased release of endogenous ATP, we investigated the role of TLR agonist-induced ROS. TLR-induced ROS production (19) was significantly increased by costimulation (Fig. 2C and Supplemental Fig. 1B). We then stimulated monocytes in the presence of DPI, a compound that blocks ROS production (19, 27). The results show that DPI decreased ROS production (Fig. 2D), ATP externalization (Fig. 2E), and IL-1β secretion (Fig. 2F) by either single-stimulated or costimulated cells with percent of inhibition consistently >60%. In contrast, the inhibitory effect on pro–IL-1β biosynthesis induced by single or associated TLR agonists was modest (from 0 to 30%, data not shown; Fig. 2G). Together, these data indicate that the higher ROS levels in costimulated cells account for the higher secretion of ATP and IL-1β.

**TLR costimulation induces oxidative stress and cell responses to stress**

Cells producing high levels of ROS upregulate antioxidant systems to prevent the oxidative stress (30). In keeping with previous results (19), the xCT antioxidant system was upregulated by single TLR agonists as indicated by the increase of its hallmarks xCT (Fig. 3A), thioredoxin (Fig. 3B), and extracellular free cysteine release (Fig. 3C). In contrast, xCT and thioredoxin were markedly less induced (Fig. 3A and 3B), and cysteine release was strongly inhibited (Fig. 3C and Supplemental Fig. 1C) by TLR costimulation, indicating the insurgence of oxidative stress. Costimulation of the three TLRs, but not high doses of single agonists, induced the oxidative stress, as demonstrated by the maintenance of the extracellular cysteine levels in monocytes single stimulated with triple dose of each PAMP (Fig. 3C).

An important cell response to stress aimed at attenuating new protein synthesis is the inactivation of the translation initiation factor eIF2α through phosphorylation (31, 32). As shown in Fig. 3D and 3E, phospho-eIF2α was induced in costimulated cells earlier, at 30 min from stimulation, and at higher levels than in single-stimulated cells. Accordingly, protein synthesis was slowed down by costimulation (Fig. 3F). In particular, the peak of [35S] amino acid incorporation induced at 3 h from stimulation with either of the three TLR agonists was absent in costimulated monocytes.

The Journal of Immunology 5375

Downloaded from http://www.jimmunol.org/ by guest on June 13, 2017

---

The Journal of Immunology 5375

Downloaded from http://www.jimmunol.org/ by guest on June 13, 2017
costimulation was required for the inhibitory effect. Despite the lower secretion of the cytokine, in costimulated cells, IL-1Ra mRNA was comparable or even higher than in cells triggered by single agonists (Fig. 4D). IL-1Ra expression peaks at 6–9 h (Fig. 4D), when protein translation is downmodulated in costimulated monocytes (Fig. 3F), suggesting that the decreased IL-1Ra secretion in the face of unaffected gene transcription is due to a block in the biosynthesis of the cytokine.

Oxidative stress and breakdown of IL-1Ra production after TLR costimulation are prevented by low doses of TLR agonists or by antioxidants

Lower doses of each TLR agonist (1:100 or 1:200 of the standard doses) in single administration were highly inefficient in inducing
IL-1β (Fig. 5A and 5B) and IL-1Ra secretion (Fig. 5C and 5D). In contrast, costimulation with the lower doses of the three agonists together induced less IL-1β than costimulation with the standard doses (Fig. 5A and 5B) but rescued the defect in IL-1Ra secretion (Fig. 5C and 5D). Also, the antioxidant response was restored in monocytes costimulated with the lower amounts of TLR agonists, as indicated by the dose-dependent recovery of cysteine release (Fig. 5E).

The effects of the exogenous antioxidants DTT and NAC on cytokine secretion were then investigated. In keeping with our previous observations (27), antioxidants reduced the levels of TLR agonist-induced ROS (Fig. 6A) but did not significantly affect the kinetics were consistently very similar in all individuals tested. *p < 0.05, **p < 0.01, ***p < 0.001.

TLR costimulation also modulates TNF-α and IL-6 secretion in a stress-dependent way

The effects of single and multiple stimulation of TLRs were then investigated on the early induced proinflammatory factor TNF-α (26) and on IL-6, a regulatory cytokine that like IL-1Ra is induced by TLR triggering with a delay with respect to TNF-α and IL-1β (24).

Expression (Fig. 7A) and secretion (Fig. 7B and 7C) of TNF-α was induced early after single TLR agonist at variable extent in the different donors. TNF-α secretion, like IL-1β, was synergically induced by TLR costimulation (Fig. 7E, 7C, and Supplemental Fig. 1E). However, the mechanism of costimulation-mediated synergy was different. In the case of IL-1β, mRNA expression was only slightly increased by TLR costimulation (Fig. 1E), and the enhancement of secretion was mediated by a posttranslational mechanism (Fig. 2). Differently, TLR costimulation induced
TNF-α at the transcriptional level, as indicated by the strong increase of mRNA paralleled by increased secretion of the cytokine (Fig. 7A–C). In addition, both TNF-α expression (Fig. 7D) and secretion (Fig. 7E) induced by single and triple TLR agonists were strongly (≥80%) downmodulated by DPI. Under the same conditions, IL-1β secretion was also highly inhibited (Fig. 7F), whereas IL-6 mRNA expression was only slightly decreased (Fig. 7F).

Expression (Fig. 8A) and secretion (Fig. 8B and 8C) of IL-6 started later but lasted longer than that of TNF-α after single TLR triggering. Despite higher induction of mRNA (Fig. 8A), IL-6 secretion, like IL-1Ra, was inhibited by costimulation (Fig. 8B and 8C). Monocytes were then single-stimulated or costimulated in the presence of brefeldin A, a drug that inhibits secretion of classical secretory proteins, and intracellular accumulation of IL-6 was analyzed by FACS. As shown in Fig. 8D, the intracellular IL-6 was effectively less abundant in costimulated monocytes (Fig. 8D), indicating that inhibition of IL-6 secretion is due to the translation halt consequent to the TLR costimulation-induced oxidative stress.

Lower doses of agonists or exogenous antioxidants rescue IL-6 secretion in costimulated monocytes

Lower doses of TLR agonists (1:100 and 1:200 of the standard doses), alone or in combination, were dramatically less efficient than the standard doses in the induction of TNF-α (Fig. 9A and 9B). However, like for IL-1Ra, the inhibition of IL-6 secretion induced by the standard doses of the agonists together was rescued by costimulation with lower doses (Fig. 9C and 9D).

The addition of the antioxidants DTT (Fig. 9E and 9G) or NAC (Fig. 9F and 9H) did not affect or rather decreased the secretion of both cytokines after single TLR stimulation. By contrast, in costimulated monocytes, antioxidants increased at a little extent TNF-α secretion (Fig. 9E and 9F) and restored the secretion of IL-6 (Fig. 9G and 9H).

Discussion

Human monocytes represent a major source of cytokines that modulate acute and chronic inflammation (10, 33, 34). However, the balance between proinflammatory and anti-inflammatory cytokines is lost in various inflammatory conditions is largely unclear. In this article, we show that the TLR agonists LPS, zymosan, and R848 administrated one by one trigger the secretion of IL-1β and IL-1Ra. When provided simultaneously, however, they strongly increase the secretion of IL-1β but antagonize that of IL-1Ra. Costimulation-induced synergy and antagonism are not restricted to the IL-1β/IL-1Ra couple, but affect the production of other mediators of inflammation, such as TNF-α, which is enhanced, and IL-6 that is inhibited. All these effects are mediated by redox responses, although with different mechanisms. Despite the high interdonor variability in the amount of the induced cytokines, likely related to polymorphisms in TLR or inflamm-
some genes (35, 36), the observed results were consistent in all subjects studied.

We have previously shown that IL-1β secretion induced by single PAMPs is mediated by autocrine stimulation of P2X7R by released ATP (15). In this study, we demonstrate that ATP release is induced by ROS, as it is inhibited by the ROS inhibitor DPI. Because TLR costimulation triggers a stronger production of ROS, higher amounts of endogenous ATP are externalized, leading to increased secretion of IL-1β. The involvement of ATP-mediated P2X7R activation in IL-1β secretion was confirmed by the strong inhibitory effect of the P2X7R blocker αATP. Thus, the enhancing effects on IL-1β by costimulation occurs at the post-translational level, through ATP-mediated increase of IL-1β processing and secretion. This justifies the strong enhancement in IL-1β secretion by costimulated cells in the face of a little increase in IL-1β mRNA, and explains the depletion of intracellular pro-IL-1β, secondary to the increased secretion.

Although several mechanisms mediating ATP release have been proposed in different cell types (37), how ATP is externalized by monocytes is undefined. The lack of correlation with cell death, however, indicates that ATP release by costimulated monocytes is an active process.

Differently from IL-1β, the increased secretion of TNF-α by costimulated monocytes is regulated at the transcriptional level and is mediated by ROS, as indicated by the dramatic inhibition of TNF-α mRNA expression in cells exposed to DPI. Interestingly, IL-1β transcription was only marginally inhibited by DPI, indicating that TNF-α gene expression is more sensitive to ROS levels than IL-1β.

The impaired IL-1Ra and IL-6 production depends on the oxidative stress caused by TLR costimulation. Whereas the ROS increase induced by LPS, zymosan, or R848 alone is followed by upregulation of the xcc− antioxidant system (19), TLR costimulation induces a stronger ROS production followed by a collapse of the xcc− system resulting in oxidative stress. In addition, costimulated monocytes display cell responses to stress such as phosphorylation of eIF-1α and downmodulation of protein synthesis (31, 32), which occur earlier than in single-stimulated monocytes. The halt in protein synthesis is likely responsible for the impaired secretion of IL-1Ra and IL-6 despite the increased mRNA expression, as production of the two cytokines reaches significant levels after 3 h from TLR cotriggering, when stressed monocytes are already under translational arrest. In agreement,
inhibitors (45) may be successful both in acute inflammatory disease. A combination of redox modulators and TLR (44) or P2X7R (46) restores the production of IL-1Ra and IL-6. However, reducing agents increase also IL-1β. Furthermore, in healthy monocytes that display a balanced redox state at baseline (27–29), costimulation of multiple TLRs is needed to derange the cytokine network leading to uncontrolled inflammation. Also, the dose of the single agonists is relevant. Unlike sustained doses of TLR agonists, low doses, unable to activate monocytes when provided one by one, do not induce oxidative stress when provided together, but rather lower the threshold of monocyte activation allowing a correct inflammatory response.

The mechanisms described in this article, leading to perturbation in the inflammatory cytokine network, may play a pathogenic role in chronic syndromes that exhibit inflammation and redox alterations. As an example, oxidative stress, cell responses to stress, and inflammation feature type 1 and 2 diabetes (38, 39): the presence of stressed monocytes could explain the increased IL-1β and decreased IL-6 production upon LPS stimulation reported in subjects at risk for diabetes development (40).

Our results may provide a new clue to understand the pathogenesis of sepsis, which is due to persistent inflammation coupled to immunosuppression (33, 41). Generation of oxidative stress by massive stimulation of different TLRs in sepsis would result in the oxidative stress inhibiting the late production also of these cytokines. The increase of major proinflammatory cytokines by antioxidants explains the uncertain therapeutic utility of NAC to control inflammation (43). Rather, novel therapeutic approaches based on a combination of redox modulators and TLR (44) or P2X7R inhibitors (45) may be successful both in acute inflammatory diseases such as sepsis and in chronic pathologic conditions including autoimmune diseases, diabetes, chronic heart failure, and cancer.

Acknowledgments

We thank the National Cancer Institute (Biological Resources Branch) for the anti–IL-1β 3D2 mAb, Prof. F. Clarke for the anti-antithioredoxin mAb, Caterina Pellecchia for technical assistance, and the Blood Center of IRCCS Azienda Ospedaliera Universitaria San Martino-IST, Istituto Nazionale per la Ricerca sul Cancro for buffalo costs.

Disclosures

The authors have no financial conflicts of interest.

References


5. Re, F. and J. L. Strominger. 2014. IL-10 released by concomitant TLR2 stimulation blocks the induction of a subset of Th1 cytokines that are specifically induced by TLR4 or TLR3 in human dendritic cells. J. Immunol. 173: 7548–7555.


