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Opposite Regulation of IL-1β and Secreted IL-1 Receptor Antagonist Production by Phosphatidylinositide-3 Kinases in Human Monocytes Activated by Lipopolysaccharides or Contact with T Cells

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Opposite Regulation of IL-1β and Secreted IL-1 Receptor Antagonist Production by Phosphatidylinositide-3 Kinases in Human Monocytes Activated by Lipopolysaccharides or Contact with T Cells[1]

Nicolas Molnarfi, Lyssia Gruaz, Jean-Michel Dayer, and Danielle Burger[2]

The unbalanced production of IL-1β and its natural, specific inhibitor, the secreted IL-1R antagonist (sIL-1Ra), plays an important role in chronic/sterile inflammation. Relevant to this condition is direct cellular contact with stimulated T cells which is a potent inducer of cytokine production in human monocytes/macrophages. We previously demonstrated that activation of PI3Ks is a prerequisite of the transcription of the *sIL-1Ra* gene in human monocytes activated by IFN-β. In this study, we addressed the question of PI3K involvement in the production of IL-1β and sIL-1Ra in monocytes activated by cellular contact with stimulated T cells (mimicked by CHAPS-solubilized membranes of stimulated T cells (CE$_{sHUT}$)), and a crude preparation of LPS, to compare stimuli relevant to chronic/sterile and acute/infectious inflammation, respectively. In monocytes activated by either CE$_{sHUT}$ or LPS, the inhibition of PI3Ks abrogated sIL-1Ra transcript expression and sIL-1Ra production, demonstrating that PI3Ks control the induction of *sIL-1Ra* gene transcription. In contrast, PI3K inhibition increased the production of IL-1β protein in both CE$_{sHUT}$- and LPS-activated monocytes, the enhancement being drastically higher in the former. This was not due to changes in IL-1β mRNA steady-state levels or transcript stability, but to the involvement of PI3Ks in the repression of IL-1β secretion. The downstream PI3K effector, Akt, was implicated in this process. The present results demonstrate that PI3Ks are involved in the inhibition of IL-1β secretion and in the induction of sIL-1Ra production in human blood monocytes by controlling different mechanisms in conditions mimicking chronic/sterile (CE$_{sHUT}$) and acute/infectious (LPS) inflammation. *The Journal of Immunology,* 2007, 178: 446–454.

Interleukin-1β is a crucial mediator of the inflammatory response, playing an important part in the development of pathologic conditions that eventually lead to chronic inflammation. The production and effects of IL-1β are controlled at many levels, a critical one being the inhibition of its activities by the secreted form of the IL-1R antagonist (sIL-1Ra)[3] (1). Both IL-1β and sIL-1Ra belong to the *IL-1* gene family originating from the duplication of a common gene (2). IL-1β is synthesized as a precursor inactive protein that is devoid of a secretory signal sequence (3). Thus, IL-1β does not follow the classical endoplasmic reticulum-to-Golgi exocytic pathway (4) but it needs to be colocalized with and cleaved by caspase-1 into secretory lysosomes before being secreted to fulfill biological activity (5). The precursor of sIL-1Ra has a leader sequence requiring cleavage to be secreted as a mature protein (1). It is currently thought that an imbalance between IL-1β and sIL-1Ra production is conducive to the development of inflammatory diseases and resulting tissue damage. This has recently been highlighted by studies demonstrating the efficacy of sIL-1Ra administered to patients with systemic inflammatory diseases (6–8) or rheumatoid arthritis (RA) (9, 10). Upon inflammation, the main IL-1β-producing cells are the monocytes/macrophages that concomitantly produce sIL-1Ra (1, 11). In healthy individuals, the latter cells do not express IL-1β constitutively (12–14).

In acute inflammation, the expression of IL-1β by monocytes/macrophages is induced by microbial activators through binding to pattern recognition receptors of which members of the TLR family, as exemplified by LPS that bind to TLR4 (15). It was recently demonstrated that pure LPS induces the expression of the IL-1β transcript but not IL-1β processing and secretion (16). The latter processes require a second signaling by muramyl dipeptides, which are present in most commercial LPS preparations. Muramyl dipeptides induce IL-1β secretion via activation of the NALP3/cryopyrin inflammasome and subsequent cleavage and activation of procaspase-1 (16). Other components are involved in IL-1β secretion mechanisms including phospholipases C and A2, P2X$_7$R, and the ATP-binding cassette (ABC1) (5, 17–19). The activator(s) of IL-1β production in monocytes/macrophages upon chronic/sterile inflammation, i.e., in the absence of bacterial products, remain to be identified, but current evidence suggests that direct cellular contact with stimulated T cells might play a major part in this process, T cells and monocytes/macrophages being in close proximity at the inflammatory site (20). Indeed, contact-mediated activation of human monocytes/macrophages by stimulated T lymphocytes is a potent proinflammatory mechanism that triggers massive up-regulation of proinflammatory cytokine expression (21–23). T cell contact-mediated activation of monocytes/macrophages by

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stimulated T cells is comparable to LPS in inducing IL-1β and TNF production (24).

LPS and cellular contact with stimulated T cells induce the production of sIL-1Ra in monocytes/macrophages, at the same time as IL-1β (25). Considering that the production of IL-1β and sIL-1Ra is tightly controlled in time, the signaling pathways leading to their production have to be distinct while triggered by the same stimulus. We previously demonstrated that serine-threonine phosphatases play a part in the differential regulation of IL-1β and sIL-1Ra production by the monocytic cell line THP-1 when activated by contact with stimulated T cells (26). In contrast, IFN-β, which induces the production of sIL-1Ra without inducing that of IL-1β in isolated human blood monocytes (27), differentially modulates the production of cytokines induced by either cellular contact or LPS (25). Indeed, in LPS-activated monocytes, IFN-β enhances the production of the three cytokines IL-1β, TNF, and sIL-1Ra, whereas in T cell contact-activated monocytes, sIL-1Ra production was enhanced, that of TNF and IL-1β being inhibited. This implies that the signaling pathways leading to IL-1β and sIL-1Ra production may be different in monocytes activated by cellular contact and LPS. More recently, we demonstrated that the induction of sIL-1Ra production and gene transcription by IFN-β-activated human monocytes depends on the activation of PI3Ks (28). There are three classes of PI3Ks of which class I are the only enzymes capable of producing, in addition to phosphatidylinositol (3,4)bisphosphate (PtdIns(3,4)P$_2$), the crucial second messenger PtdIns(3,4,5)P$_3$ (29, 30). Located at the plasma membrane, these transiently synthesized lipidic products then provide docking sites for a subgroup of signaling proteins (31) that transmit downstream signaling events, the best-characterized protein kinase downstream class I PI3Ks being Akt.

Numerous studies support the premise that PI3Ks play a pivotal role in regulating the production of pro- and anti-inflammatory mediators by monocytes/macrophages (32–37). However, results are controversial regarding the role of this pathway in regulating cytokine production, attributing pro- or anti-inflammatory functions to PI3Ks (for review, see Ref. 38). The present study was undertaken to determine the role of PI3Ks in the production by human monocytes of IL-1β and its natural inhibitor, sIL-1Ra, in conditions related to acute/infectious inflammation (crude preparation of LPS) and chronic/sterile inflammation (T cell contact). Because transfection techniques that had previously been used (28) resulted in the loss of monocyte ability to produce IL-1β and because monocytic cell lines do not display similar responses to freshly isolated human monocytes, the technical approach involves the use of pharmacological kinase inhibitors. The results show that PI3Ks dampen IL-1β secretion but induce the transcription of sIL-1Ra in human monocytes.

Materials and Methods

Materials

FCS, streptomycin, penicillin, L-glutamine, RPMI 1640, PBS free of Ca^{2+} and Mg^{2+} (Invitrogen Life Technologies); purified PHA (EY Laboratories); Ficoll-Paque (Pharmacia Biotech); LPS (crude preparation) from *Escherichia coli* 055:B5 (Difco Laboratories); Tri reagent, PMA, PMSF, endotoxin-free DMSO, pepstatin A, leupeptin, iodoacetamide, neuraminidase, BSA, actinomycin D (ActD), and polymyxin B sulfate (Sigma-Aldrich); and the kinase inhibitors SB203580, UO126, Ly294002, SH-5, i.e., Akt inhibitor II, and rapamycin (Calbiochem-Novabiochem) were purchased from the designated suppliers. All other reagents were of analytical grade or better.

Monocytes

Monocytes were isolated from buffy coats of blood from healthy donors provided by the Geneva Hospital Blood Transfusion Center as previously described (39). To avoid activation by endotoxin, polymyxin B sulfate was

added to all solutions during the isolation procedure (2 μg/ml) and in experiments (5 μg/ml), where monocytes were activated by stimuli other than LPS. Monocyte purity routinely consisted of >90% CD14$^+$ cells, <1% CD3$^+$ cells, and <1% CD19$^+$ cells as assessed by flow cytometry.

T cells and preparation of T cell plasma membranes

HUT-78, a human T cell line (40), was obtained from the American Type Culture Collection. Cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 50 μg/ml streptomycin, 50 IU/ml penicillin, and 2 mM -glutamine (complete RPMI medium) in a 5% CO$_2$-air humidified atmosphere at 37°C. HUT-78 cells (2 × 10^6 cells/ml) were stimulated for 6 h by PHA (1 μg/ml) and PMA (5 ng/ml). Plasma membranes of stimulated HUT-78 cells and their soluble CHAPS extract (CE$_{sHUT}$) were prepared as previously described (41). CE$_{sHUT}$ was equivalent to living HUT-78 cells (i.e., in cocultures) or fixed HUT-78 cells to activate monocytes (27, 39, 41). Alternatively, T lymphocytes from human peripheral blood were prepared and stimulated by PHA/PMA, and their membranes isolated and solubilized (CE$_{sT}$) as described previously (41). Proteins were determined by the method of Bradford (42) and cytokines as described below. IL-1β and IL-1Ra were not detected in CE$_{sT}$ and CE$_{sHUT}$ preparations.

Cytokine production

Monocytes (50 × 10^3 cells/well/200 μl) were preincubated for 60 min at the indicated concentration of kinase inhibitor and then activated for 24 h after the addition of CE$_{sHUT}$ (6 μg/ml proteins), CE$_{sT}$ (11 μg/ml proteins), or LPS (100 ng/ml) in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 50 μg/ml streptomycin, 50 U/ml penicillin, 2 mM glutamine (medium). When monocytes were activated by either CE$_{sHUT}$ or CE$_{sT}$, 5 μg/ml polymyxin B sulfate was added in cell culture. All conditions were conducted in triplicate. In indicated experiments, after removal of supernatants, cells were lysed in 200 μl/well PBS containing 1% Nonidet P-40. Culture supernatants or cell lysates were tested for the production of cytokines by commercially available enzyme immunoassay: IL-1β (Immunotech), sIL-1Ra and TNF (Quantikine; R&D Systems).

Caspase-1 secretion

Monocytes were activated as described above and culture supernatants were tested for caspase-1 content (Caspase-1 ELISA kit; Alexis).

Cytotoxicity assay

Monocytes (50 × 10^3 cells/well/200 μl) were preincubated for 60 min with 10 μM Ly294002 or SH-5 and then activated by CE$_{sHUT}$ (6 μg/ml proteins) or LPS (100 ng/ml). After 24 h of activation, 20 μl/well EZ4U dye solution (Biomedica) was added and cells were cultured for an additional 3 h. The cytotoxicity of kinase inhibitors was measured by absorbance at 450 nm.

mRNA

Monocytes (3 × 10^6 cells/well/3 ml) were cultured in 6-well plates for 60 min with 10 μM Ly294002 and then activated by CE$_{sHUT}$ (6 μg/ml proteins) or LPS (100 ng/ml) for the indicated time. When monocytes were activated by CE$_{sHUT}$, 5 μg/ml polymyxin B sulfate was added in cell culture. Alternatively, in transcriptional arrest experiments, 10 μg/ml ActD was added 3 h after monocyte activation. Total mRNA was prepared by Tri reagent and subjected to DNase digestion. Quantitative real-time duplex PCR analysis (TaqMan quantitative ABI PRISM 7900 Detection System; Applied Biosystems) was conducted after reverse transcription by the RQ1 kit (Promega). The levels of mRNA expression were normalized with the expression of a housekeeping gene (*18S*) analyzed simultaneously. IL-1β, sIL-1Ra, and 18S probes were obtained from Applied Biosystems. All measurements were conducted in triplicate.

Western blot analysis

Human monocytes were starved for 16 h at 37°C in RPMI 1640 medium supplemented with 1% heat-inactivated FCS in nonadherent conditions, i.e., in polypropylene tubes (Falcon). Cells were harvested, resuspended at 8 × 10^6 cells/ml in medium supplemented with 1% heat-inactivated FCS, and 500 μl was placed in 2-ml polypropylene tubes (Eppendorf) at 37°C. After 1 h, inhibitors were added for 45 min and then cells were activated by CE$_{sHUT}$ (6 μg/ml proteins) or LPS (100 ng/ml). At the indicated time, the reaction was stopped by the addition of 800 μl of ice-cold PBS and centrifugation. Total cell lysate was prepared and subjected to Western blot analysis as described previously (28). The blots were probed with anti-p44/42 MAPK, anti-Akt/protein kinase B, anti-phospho-Akt/protein kinase

Table I. *Production of IL-1β and sIL-1Ra by isolated human monocytes*

Stimulus	Inhibitor	IL-1β (pg/ml)	IL-1Ra (pg/ml)
Medium		Not detected	823 ± 605
Medium + 5 μg/ml polymyxin		Not detected	825 ± 844
LPS (100 ng/ml)		2,931 ± 1,758	3,251 ± 2,976
LPS (100 ng/ml)	SB203580	673 ± 391	2,438 ± 656
LPS (100 ng/ml)	UO126	875 ± 486	1,879 ± 819
CE_{sHUT} (6 μg/ml)		541 ± 134	9,399 ± 1,434
CE_{sHUT} (6 μg/ml)	SB203580	253 ± 52	4,402 ± 620
CE_{sHUT} (6 μg/ml)	UO126	29 ± 46	4,144 ± 3,323
CE_{sT} (11 μg/ml)		503 ± 156	19,118 ± 9,259
CE_{sT} (11 μg/ml)	SB203580	138 ± 195	8,663 ± 4,172
CE_{sT} (11 μg/ml)	UO126	22 ± 23	4,302 ± 3,098

B (pS^{473}; Cell Signaling Technology), and anti-β-tubulin (Sigma-Aldrich). Secondary HRP-conjugated goat anti-rabbit or goat anti-mouse Abs were from DakoCytomation. Ab-bound proteins were detected by the Uptilight Hrp Blot Chemiluminescent substrate (Uptima; Interchim).

Statistics

When required, significance of differences between groups was evaluated using the Student paired *t* test.

Results

PI3K inhibition enhances IL-1β but decreases sIL-1Ra production in monocytes activated by LPS and CE_sHUT

Before assessing the role of PI3Ks in the induction of IL-1β and sIL-1Ra, the activity of the different stimuli used in this study was ascertained. The cytokine production varied in the different preparations of monocytes, depending on individual donors (Table I). In the absence of stimulus, IL-1β was not detectable in any of the cell supernatants, whereas sIL-1Ra production was similar in the presence or absence of the endotoxin inhibitor, polymyxin B sulfate (Table I). Because high doses of CE_{sT} and CE_{sHUT} displayed cytotoxicity (25), suboptimal doses of CE_{sT} (11 μg/ml proteins) and CE_{sHUT} (6 μg/ml proteins), but an optimal dose of LPS (100 ng/ml) were used throughout this study. In these conditions, LPS was more efficient in increasing IL-1β production than CE_{sT} and CE_{sHUT} (Table I). In contrast, CE_{sT} and CE_{sHUT} were more potent inducers of sIL-1Ra production than LPS. These results confirm that CE_{sT} and CE_{sHUT} (i.e., cell-cell contact with stimulated T cells) are potent means of activating the secretion of large amounts of IL-1β and sIL-1Ra. Because the levels of cytokine production varied considerably as a function of monocyte preparation (Table I), the results below are presented as percentages of cytokine production in the absence of inhibitor.

To assess the role of PI3Ks in the production of IL-1β and sIL-1Ra by human monocytes, freshly isolated cells were stimulated by CE_{sT}, CE_{sHUT}, or LPS in the presence or absence of an optimal dose of Ly294002 (data not shown). Ly294002 inhibited the induced production of sIL-1Ra, while enhancing that of IL-1β regardless of the monocyte stimulus (Fig. 1). In the presence of Ly294002, the production of sIL-1Ra induced by CE_{sT} and CE_{sHUT} was reduced by 81 ± 1% and 77 ± 13%, respectively, while it was reverted to basal level upon LPS stimulation (Fig. 1). The inhibition of PI3Ks increased the production of IL-1β when monocytes were activated by either CE_{sT}, CE_{sHUT}, or LPS, although this enhancement was higher when monocytes were activated with CE_{sT} (7-fold) or CE_{sHUT} (14-fold) than with LPS (1.5-fold). The enhancement of IL-1β in culture supernatant was not due to a putative cytotoxic effect of Ly294002, as determined by the tetrazolium reduction assay EZ4U (data not shown). This dem-
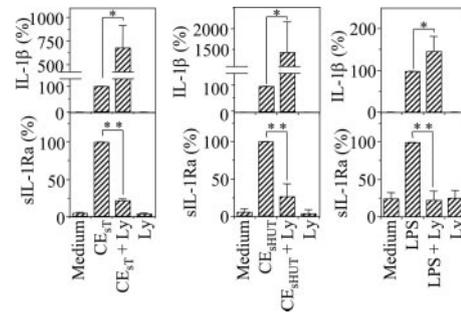

FIGURE 1. PI3K inhibition induces differential production of sIL-1Ra and IL-1β in human monocytes activated by CE_{sHUT} or LPS. Isolated monocytes (5 × 10⁴ cells/200 μl) were preincubated for 60 min in the presence or absence of 10 μM Ly294002 (Ly) and then stimulated or not with either CE_{sHUT} (6 μg/ml proteins) or LPS (100 ng/ml) for 24 h in 96-well plates. Experiments were performed in the presence polymyxin B sulfate (5 μg/ml) when monocytes were activated by CE_{sT} or CE_{sHUT}. sIL-1Ra and IL-1β production was assessed in supernatants of triplicate cell cultures as described in *Materials and Methods*. Results obtained with monocytes from three different donors are presented as mean ± SD of percentage of sIL-1Ra and IL-1β production induced by CE_{sHUT} or LPS in the absence of inhibitor; ∗, *p* < 0.05, and ∗∗, *p* < 0.01, as determined by Student's *t* test.

onstrates that IL-1β and its specific inhibitor sIL-1Ra were inversely regulated by PI3Ks upon activation by LPS or contact with stimulated T cells as mimicked by CE_{sT} or CE_{sHUT}. In the absence of stimulus, Ly294002 did not affect cytokine production by monocytes (Fig. 1). Similar results were obtained when 1 μg/ml rIL-1Ra (Amgen) was added to culture medium (data not shown), suggesting that the enhancement of IL-1β production in the presence of Ly294002 was not consecutive to a lack of feedback repression by sIL-1Ra. Consequently, PI3K signaling prompted the differential modulation of the production of sIL-1Ra and IL-1β being required for the induction of the former and the repression of the latter. This opposite effect was restricted to PI3Ks, because similar results were obtained with another PI3K inhibitor, wortmannin (data not shown), and because inhibitors of MAPK-signaling pathways such as SB203580 (p38 MAPK) and UO126 (MEK1/2) concomitantly diminished the production of both IL-1β and sIL-1Ra (Table I). Furthermore, the premise that comparable results were obtained with CE_{sT} and CE_{sHUT} demonstrates that freshly isolated T lymphocytes and the lymphocytic cell line HUT-78 cells displayed similar activity. For this reason, CE_{sHUT} were used in the experiments described below.

Akt is involved in the differential production of sIL-1Ra and IL-1β

To ascertain that the activity of PI3Ks was inhibited by Ly294002, the phosphorylation of Akt on Ser^{473} was determined. As shown in Fig. 2, both CE_{sHUT} and LPS induced the phosphorylation of Akt. When monocytes were activated by CE_{sHUT}, the phosphorylation of Akt was observed after 5 min of activation and reached a maximum at 60 min before tapering off (Fig. 2A). Upon LPS activation, Akt phosphorylation also peaked at 60 min, but was observed after 30 min of activation. Thus, both stimuli triggered the activation of the PI3K/Akt-signaling pathway, the latter being activated early and persistently by CE_{sHUT}, whereas 30-min activation by LPS was required to induce the transient phosphorylation of Akt. In the presence of Ly294002, Akt phosphorylation was abolished in both CE_{sHUT}- and LPS-activated monocytes (Fig. 2B), demonstrating that activation of the PI3K/Akt pathway was suppressed.
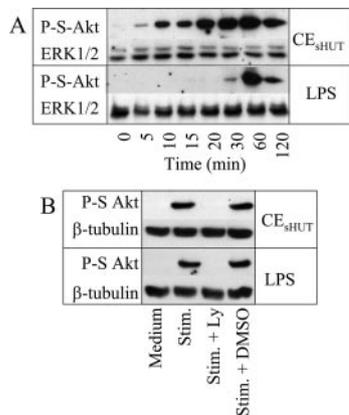
FIGURE 2. CE$_{sHUT}$ and LPS induce Akt phosphorylation via PI3K activation. *A*, Isolated monocytes (4×10^6 cells/500 μl) were incubated or not with either CE$_{sHUT}$ (6 μg/ml proteins) or LPS (100 ng/ml) in 2-ml tubes for the indicated time. Experiments were performed in the presence polymyxin B sulfate (5 μg/ml) when monocytes were activated by CE$_{sHUT}$. Cell lysates were analyzed by Western blot as described in *Materials and Methods* with Abs to phosphorylated Ser^{473}-Akt (P-S-Akt) and Abs to ERK1/2 as a loading control. *B*, Isolated monocytes (4×10^6 cells/500 μl) were treated or not with 10 μM Ly294002 (Ly) and 1/1000 dilution of DMSO for 45 min and then stimulated with either CE$_{sHUT}$ or LPS as described above in 2-ml tubes for 60 min. Experiments were performed in the presence (CE$_{sHUT}$) or absence (LPS) of polymyxin B (5 μg/ml). Cell lysates were analyzed by Western blot as described using Abs to phosphorylated Ser^{473}-Akt and mAbs to β-tubulin as a loading control. The presented autoradiographs are typical of three different experiments.

To assess the involvement of Akt in the control of IL-1β and sIL-1Ra production downstream of PI3Ks, monocytes were preincubated with an optimal dose of the cell-permeable Akt inhibitor SH-5 that prevents PtsIns(3,4,5)P$_3$ formation and binding to Akt, and then stimulated with either CE$_{sHUT}$ or LPS. SH-5 inhibited sIL-1Ra production by $54 \pm 25\%$ and $32 \pm 16\%$ in monocytes activated by CE$_{sHUT}$ and LPS, respectively (Fig. 3). The production of IL-1β by monocytes activated with LPS and CE$_{sHUT}$ was enhanced 1.5- and 2.3-fold, respectively, in the presence of SH-5 (Fig. 3). The enhancement of IL-1β in culture supernatant was not due to any cytotoxic effect of SH-5 as determined by using the tetrazolium reduction assay EZ4U (data not shown). Contrary to LPS-activated monocytes, in which the production of IL-1β was enhanced to similar extents in the presence of Ly294002 or SH-5, in CE$_{sHUT}$-activated monocytes, the inhibition of Akt was less efficient than that of PI3Ks in enhancing the production of IL-1β (Figs. 2 and 3). The inference from these results is that Akt plays a part downstream of PI3Ks in regulating both sIL-1Ra and IL-1β production upon the activation of monocytes by either LPS or CE$_{sHUT}$. To assess the involvement of p70 S6-kinase (p70S6K) another PI3K downstream element, monocytes were treated with 1 nM rapamycin before activation. The production of sIL-1Ra was not affected by the p70S6K inhibitor, whereas IL-1β was inhibited by 30 and 60% when monocytes were activated by LPS and CE$_{sHUT}$, respectively (data not shown). This demonstrates that the opposite regulation exerted by PI3Ks on the production of IL-1β and sIL-1Ra did not occur through p70S6K activation.

PI3Ks control sIL-1Ra *gene transcription*

To investigate the molecular mechanisms underlying the control of sIL-1Ra production, we ascertained whether the decreased sIL-1Ra production in Ly294002-treated monocytes would be accompanied by changes in sIL-1Ra mRNA steady-state levels. No sIL-1Ra mRNA was detectable in the absence of stimulus (Fig. 4*A*).
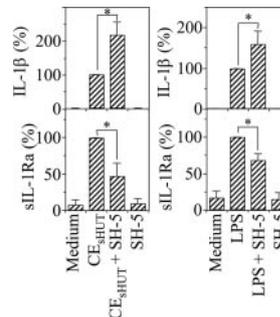

FIGURE 3. Akt inhibition differentially regulates production of sIL-1Ra and IL-1β in human monocytes activated by CE$_{sHUT}$ or LPS. Isolated monocytes were cultured as described in Fig. 1 using 10 μM Akt inhibitor II (SH-5) instead of Ly294002 as indicated. sIL-1Ra and IL-1β production was assessed in supernatants of triplicate cell cultures. Results obtained with monocytes from three different donors are presented as mean $\pm$ SD of percentage of sIL-1Ra and IL-1β production induced by CE$_{sHUT}$ or LPS in the absence of inhibitor; *, $p < 0.05$ as determined by Student's *t* test.

Activation of monocytes by both LPS and CE$_{sHUT}$ increased sIL-1Ra mRNA levels as a function of incubation time, the steady state being not reached after 9 h of activation. In contrast, in Ly294002-treated monocytes, sIL-1Ra mRNA remained undetectable after 7 h of activation by either stimulus (Fig. 4*A*). Interestingly, in the presence of Ly294002, sIL-1Ra mRNA levels in monocytes activated for 9 h with CE$_{sHUT}$ were detectable although low. This is consistent with the results of Fig. 1 in that the production of sIL-1Ra in monocytes activated by CE$_{sHUT}$ for 24 h did not revert to basal level, contrary to LPS-activated monocytes. The latter result could also be due to a differential stability of sIL-1Ra mRNA upon monocyte activation by LPS or CE$_{sHUT}$. This hypothesis was tested by blocking transcription by ActD after 3 h of activation. As
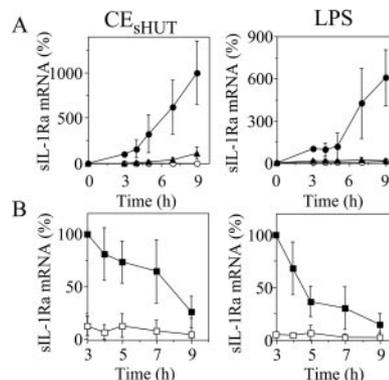

FIGURE 4. Time course of sIL-1Ra mRNA induction and stability in monocytes activated by CE$_{sHUT}$ or LPS. *A*, Isolated monocytes (3×10^6 cells/3 ml) were preincubated for 60 min in the presence (▲) or absence (●) of 10 μM Ly294002 (Ly) and then stimulated or not (○) with either CE$_{sHUT}$ (6 μg/ml proteins) or LPS (100 ng/ml) in 6-well plates, as described in *Materials and Methods*. *B*, Cells were stimulated for 3 h before the addition of 10 μg/ml ActD in conditions containing (□) or not (■) 10 μM Ly294002. Cell culture was stopped at the indicated time and sIL-1Ra mRNA levels were determined by duplex quantitative real-time PCR analysis of triplicates normalized to the levels of the 18S mRNA as described in *Materials and Methods*. Experiments were performed in the presence of polymyxin B sulfate (5 μg/ml) when monocytes were activated by CE$_{sHUT}$. The relative expression levels of sIL-1Ra mRNA are presented as mean $\pm$ SD of percentage of relative sIL-1Ra mRNA expression induced by CE$_{sHUT}$ or LPS. The value of mRNA levels in 3 h-stimulated monocytes being arbitrarily considered as 100%. Results are presented as mean $\pm$ SD of three distinct experiments.
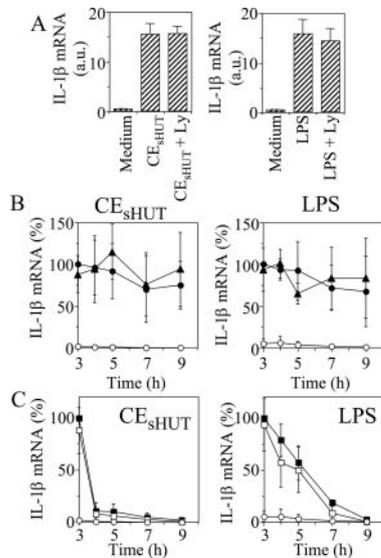
FIGURE 5. PI3K inhibition does not affect IL-1β mRNA steady-state levels or stability in monocytes activated by CE$_{sHUT}$ or LPS. *A*, Isolated monocytes (3×10^6 cells/3 ml) were preincubated for 60 min in the presence or absence of 10 μM Ly294002 (Ly) and then stimulated or not with either CE$_{sHUT}$ (6 μg/ml) or LPS (100 ng/ml) for 3 h as indicated. *B*, Isolated monocytes (3×10^6 cells/3 ml) were preincubated for 60 min in the presence (▲) or absence (●) of 10 μM Ly294002 (Ly) and then stimulated or not (○) with either CE$_{sHUT}$ (6 μg/ml proteins) or LPS (100 ng/ml) in 6-well plates, as described in *Materials and Methods*. *C*, Cells were stimulated for 3 h before the addition of 10 μg/ml ActD in conditions containing (□) or not (■) 10 μM Ly294002. Cell culture was stopped at the indicated time, and IL-1β mRNA levels were determined by duplex quantitative real-time PCR analysis of triplicates normalized to the levels of the 18S mRNA as described in *Materials and Methods*. *A*, Results are presented as mean ± SD of relative IL-1β expression (arbitrary units (a.u.) induced by CE$_{sHUT}$ or LPS from triplicate cultures. *B* and *C*, Results are presented as mean ± SD of percentage of relative IL-1β transcript expression induced by CE$_{sHUT}$ or LPS. The value of mRNA expression in 3-h-stimulated monocytes being arbitrarily considered as 100%. Results are presented as mean ± SD of three distinct experiments.

shown in Fig. 4*B*, sIL-1Ra transcript was more stable when induced by CE$_{sHUT}$ (half-life = 4–6 h) than by LPS (half life = 1–2 h). This observation also supports the data of Table I showing that cellular contact induced higher sIL-1Ra production than did LPS. According to these data, PI3Ks regulate the induction of sIL-1Ra production in activated monocytes at the level of gene transcription.

PI3Ks do not control steady-state levels or stability of IL-1β mRNA

To determine the mechanism(s) by which PI3K inhibition enhanced the production of IL-1β, the effect of Ly294002 on IL-1β mRNA levels was assessed. IL-1β mRNA reached steady-state levels after 3 h of stimulation by either CE$_{sHUT}$ or LPS (data not shown), both stimuli inducing similar levels of IL-1β transcript (Fig. 5*A*). At this time point, the levels of IL-1β mRNA induced by either LPS or CE$_{sHUT}$ were not affected by the presence of the PI3K inhibitor (Fig. 5*A*), suggesting that PI3Ks were not involved in the control of IL-1β mRNA steady-state levels. This was confirmed in time-course experiments because IL-1β transcript levels remained unchanged in the presence or absence of Ly294002 for at least 9 h after the addition of either LPS or CE$_{sHUT}$ (Fig. 5*B*). Independent of the stimulus and the presence of Ly294002, the steady-state levels of the IL-1β transcript remained stable up to

9–12 h before slowly declining (data not shown), suggesting that the enhancement of IL-1β production upon PI3K blockade was not due to new, late transcription induction or mRNA stabilization. To gain insight into the mechanisms by which PI3K inhibition increased the production of IL-1β in monocytes, the effect of Ly294002 on IL-1β mRNA stability was assessed in time-course experiments in the presence or absence of the transcription inhibitor ActD. As shown in Fig. 5*C*, the presence of Ly294002 did not affect the rate of IL-1β mRNA decay in the presence of ActD, regardless of whether monocytes were activated by CE$_{sHUT}$ or LPS. Thus, the increase in IL-1β production observed upon PI3K inhibition could not be attributed to either an enhancement of mRNA steady-state levels or mRNA stabilization. The results of Fig. 5*C* also indicate that IL-1β transcripts induced by LPS were more stable (half-life = 3–4 h) than those induced by CE$_{sHUT}$ (half-life <1 h). This supports the data of Table I showing that cellular contact induced lower IL-1β production than did LPS.

PI3K/Akt pathway dampens IL-1β secretion

Considering that PI3Ks did not control IL-1β production–because they failed to affect its mRNA steady-state level or stability–they might affect its secretory processes. To assess this hypothesis, monocytes were activated by LPS and CE$_{sHUT}$ in the presence or absence of Ly294002 and tested for the production of both secreted and intracellular IL-1β. As illustrated in Fig. 6*A*, in both CE$_{sHUT}$- and LPS-activated monocytes, the intracellular IL-1β content was diminished by ∼50% in the presence of Ly294002, whereas the extracellular IL-1β production was enhanced in a similar way as in Fig. 1. Furthermore, the total IL-1β production (i.e., the addition of intra- and extracellular IL-1β) induced by CE$_{sHUT}$ was not significantly affected in the presence of PI3K inhibitor reaching 13.0 ± 7.2 and 12.2 ± 3.9 ng/ml in the presence and absence of Ly294002, respectively. Similarly, when monocytes were activated by LPS, the total production of IL-1β remained unchanged reaching 9.5 ± 6.2 and 9.4 ± 5.9 ng/ml in the presence and absence Ly294002, respectively. It follows that the secretion of IL-1β was boosted upon PI3K blockade, implying that PI3Ks dampened the secretion of IL-1β by monocytes activated by LPS and CE$_{sHUT}$.

To investigate whether the regulation of IL-1β secretion by PI3Ks depended on the activity of Akt, monocytes were activated by LPS and CE$_{sHUT}$ in the presence or absence of SH-5 and tested for the production of secreted and intracellular IL-1β. As shown in Fig. 6*B*, in both CE$_{sHUT}$- and LPS-activated monocytes, the extracellular concentration of IL-1β was enhanced in the presence of SH-5 as described in Fig. 3. The total IL-1β production (i.e., the addition of intra- and extracellular IL-1β) induced by CE$_{sHUT}$ was not significantly affected in the presence of the Akt inhibitor reaching 11.7 ± 3.3 and 11.8 ± 4.5 ng/ml in the presence and absence of SH-5, respectively. Similarly, when monocytes were activated by LPS, the total production of IL-1β remained unchanged reaching 8.2 ± 5.0 and 9.4 ± 5.9 ng/ml in the presence and absence SH-5, respectively. As shown in Fig. 6*B*, the decrease in the intracellular level of IL-1β in LPS-activated monocytes matched that observed with Ly294002 (Fig. 6*A*). This did not apply to CE$_{sHUT}$-activated monocytes in which IL-1β intracellular pools were diminished by 12 ± 2% in the presence of SH-5 (Fig. 6*B*), whereas Ly294002 triggered 51 ± 10% inhibition (Fig. 6*B*). This corroborates the differences observed in the extracellular production of IL-1β in the presence of Ly294002 and SH-5, the enhancement being higher with the PI3K inhibitor than with the Akt inhibitor
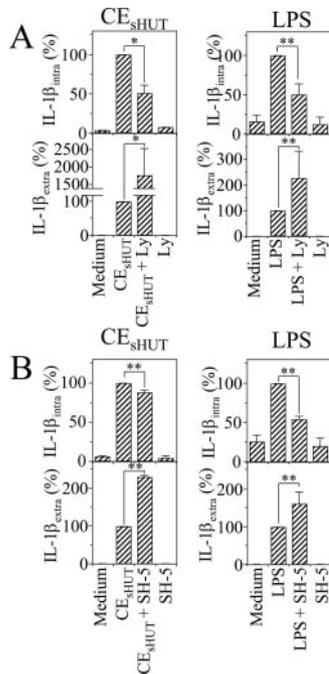
FIGURE 6. The PI3K/Akt pathway regulates IL-1β secretion in human monocytes activated by CE$_{sHUT}$ or LPS. Isolated monocytes (5×10^4 cells/200 μl) were preincubated for 60 min in the presence or absence of (*A*) 10 μM Ly294002 (Ly) or (*B*) 10 μM SH-5 and then stimulated or not with either CE$_{sHUT}$ (6 μg/ml proteins) or LPS (100 ng/ml) in 96-well plates. Experiments were performed in the presence of polymyxin B sulfate (5 μg/ml) when monocytes were activated by CE$_{sHUT}$. After 24 h, the supernatant was harvested and tested for IL-1β production as in Fig. 1. Cells were lysed in PBS containing 1% Nonidet P-40 and tested for the production of intracellular (IL-1β$_{intra}$) in triplicate cell cultures as described in *Materials and Methods*. Results obtained with monocytes from three different donors are presented as mean ± SD of percentage of IL-1β production induced by CE$_{sHUT}$ or LPS in the absence of inhibitor; *, $p < 0.05$, and **, $p < 0.01$, as determined by Student's *t* test.

FIGURE 7. The PI3K/Akt pathway regulates caspase-1 secretion in human monocytes activated by CE$_{sHUT}$ or LPS. Culture supernatants of monocytes activated as described in Fig. 6 were analyzed for their content in caspase-1. Results obtained with monocytes from three different donors are presented as mean ± SD of percentage of caspase-1 production induced by CE$_{sHUT}$ or LPS in the absence of inhibitor; *, $p < 0.05$, as determined by Student's *t* test.

(Figs. 1 and 3). These results suggest that in LPS-activated monocytes, the secretion of IL-1β is regulated by PI3Ks via Akt activation, whereas in CE$_{sHUT}$-activated monocytes only part of the PI3K IL-1β-regulatory signal is transmitted by Akt.

Because the secretion of IL-1β is accompanied by that of its converting enzyme, caspase-1 (43), the enhancement of IL-1β production upon PI3K and Akt blockade should be reflected by differences in caspase-1 secretion. Therefore, to confirm that the PI3K/Akt pathway was involved in the control of IL-1β secretion, the release of caspase-1 was assessed in monocyte culture supernatants. Caspase-1 secretion was higher in LPS- than in CE$_{sHUT}$-activated monocytes reaching 33 ± 13 and 26 ± 4 pg/ml (mean ± SD, $n = 5$), respectively. This correlated with differences in IL-1β production (Table I). As shown in Fig. 7, the release of caspase-1 was enhanced in the presence of Ly294002 in both CE$_{sHUT}$- and LPS-activated monocytes reaching 201 ± 67 and 79 ± 4 pg/ml, respectively. Upon addition of SH-5, caspase-1 secretion was enhanced to 51 ± 19 pg/ml when monocytes were activated by CE$_{sHUT}$. Although the enhancement of caspase-1 induced by LPS in the presence of SH-5 did not reach statistical significance ($p = 0.07$), caspase-1 was reproducibly enhanced in all experiments, reaching 40 ± 7 pg/ml. Similar to the effect of Ly294002 on IL-1β, the effects of Ly294002 on caspase-1 secretion were stronger than those of Akt, corroborating results of Fig. 6. Furthermore, although proportionally dissimilar, the enhancement of caspase-1 in the presence of kinase inhibitors was more important when
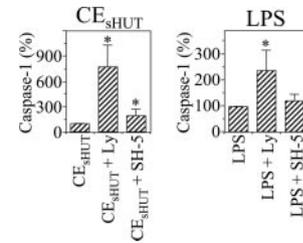
monocytes were activated by CE$_{sHUT}$ as compared with LPS. This further demonstrates that PI3Ks and Akt regulate IL-1β secretory mechanisms.

Discussion

This study reveals that PI3Ks represent key signaling effectors controlling the differential production of IL-1β and sIL-1Ra in human peripheral blood monocytes in conditions related to chronic/sterile and acute/infectious inflammation. Indeed, PI3Ks control different mechanisms leading to the induction of sIL-1Ra expression and the repression of IL-1β secretion. PI3K activation proved mandatory to the induction of *sIL-1Ra* gene transcription when monocytes were activated by either LPS or cellular contact with stimulated T cells (CE$_{sHUT}$). In contrast, PI3Ks did not affect IL-1β mRNA expression levels or stability but displayed dampening effects on IL-1β secretion which correlated to caspase-1 release. These opposite effects on the production of the proinflammatory cytokine and its specific, natural inhibitor are unique to PI3Ks, because MAPKs such as p38 and MEK1/2 positively regulate IL-1β and sIL-1Ra production (Table I). This is consistent with reports advancing that MAPK activation is necessary for both *IL-1β* and *sIL-1Ra* gene transcription upon activation by various stimuli including LPS (44–46).

Both LPS and CE$_{sHUT}$ activate PI3Ks that are instrumental to *sIL-1Ra* gene transcription in human monocytes. This is reminiscent of a recent study of ours demonstrating that the induction of sIL-1Ra transcription in human monocytes activated by IFN-β is dependent on PI3K activation (28). Taken together, these results emphasize the importance of PI3K activity in the control of *sIL-1Ra* gene transcription in human monocytes upon various stimulatory conditions. The regulation of sIL-1Ra transcription by PI3Ks requires the downstream activation of Akt in both LPS- and CE$_{sHUT}$-activated monocytes. However, there was a difference in the extent of sIL-1Ra inhibition achieved by PI3K and Akt inhibitors. A possible reason could be that PI3K downstream effectors other than Akt and/or that class II or III PI3Ks which do not generate PtdIns(3,4,5)P$_3$ were responsible for sIL-1Ra triggering (47–49). However, inhibition of p70S6K did not affect the production of sIL-1Ra, suggesting that p70S6K was not involved downstream of PI3Ks.

In contrast with the data presented in this study, in septic/LPS-adapted monocytic THP-1 cells, LPS-induced PI3K activation contributed to elevate translation of sIL-1Ra, without effect at the transcriptional level (50). Hence, depending on the stage of maturation/differentiation of monocytes or monocytic cell line, the PI3K pathways control different mechanisms to ensure sIL-1Ra production.

PI3K inhibition increased IL-1β production in culture supernatants of monocytes activated by either CE$_{sHUT}$ or LPS due to its effect on posttranscriptional processes. This is supported by the premise that neither steady-state levels nor stability of IL-1β mRNA were affected by the inhibition of PI3Ks. Moreover, the increase in extracellular levels of IL-1β was accompanied by a decrease in intracellular IL-1β levels in monocytes whose PI3Ks were inhibited. These observations demonstrate that PI3Ks dampen the mechanism(s) involved in IL-1β secretion. In LPS-activated monocytes, most of the effects of PI3Ks are due to the downstream activation of Akt. In contrast, in CE$_{sHUT}$-activated monocytes the production of extra- and intracellular levels of IL-1β was less affected by SH-5 than by Ly294002, suggesting either the involvement of PI3K downstream effector(s) other than Akt or that of different classes of PI3Ks. Indeed, it was recently reported that class III PI3Ks, which do not generate PtdIns(3,4)P$_2$ and PtdIns(3,4,5)P$_3$, may regulate cytokine production in murine macrophages (49). However, inhibition of p70S6K diminished the production of IL-1β, demonstrating that p70S6K did not reflect the effect of PI3Ks.

The repression of IL-1β secretion by the PI3K/Akt pathway might be the result of interference in one of the multiple steps leading to IL-1β release (8), e.g., by impairing translocation of IL-1β into specialized secretory lysosomes (5) and/or activation of caspase-1 that is elicited through the inflammasome complex and required for the maturation of pro-IL-1β into IL-1β (43). Our results show that inhibition of PI3K and Akt enhanced the secretion of both caspase-1 and IL-1β, suggesting that the PI3K/Akt pathway reduces the secretion of both the cytokine and its activating enzyme. In accordance with this, a recent study demonstrated that a direct link may exist between PI3Ks and inflammasome activation, i.e., caspase-1 activation and in turn IL-1β maturation and secretion (51).

Contrary to the present data demonstrating that PI3Ks control posttranscriptional regulation of IL-1β, the inhibition of PI3Ks up-regulates IL-1β mRNA in THP-1 monocytic cells activated by LPS (52). Furthermore, PI3K-dependent pathways inhibit the activation of the transcription factors that regulate TNF and tissue factor gene expression in LPS-stimulated THP-1 cells (33). Thus, depending on the type of monocytic cell or readout products, PI3Ks might control different mechanisms limiting the production of proinflammatory factors.

The enhancement of IL-1β production upon PI3K inhibition is reminiscent of a previous study showing that, in vivo, the pharmacological inhibition of PI3Ks results in increased serum levels of cytokines like IL-1β in septic mice (53). The differential control of pro- and anti-inflammatory factors by PI3Ks was also described in vitro in monocytes activated by *Porphyromonas gingivalis* LPS, i.e., through TLR2. In that system, the inhibition of PI3Ks diminished the production of anti-inflammatory cytokine IL-10 and concomitantly enhanced that of IL-12, a proinflammatory cytokine (36). The repressing effects of PI3Ks on proinflammatory cytokine production have also been described in monocytes/macrophages activated by contact with stimulated T cells. Indeed, cytokine-activated T cells and synovial T cells isolated from RA patients induced TNF production in human monocytes/macrophages, which was enhanced upon PI3K inhibition (54). In contrast, IL-10 production induced by cytokine-activated or synovial T cells in monocytes/macrophages was suppressed upon inhibition of PI3Ks (55). In our system, the production of TNF was significantly diminished in the presence of Ly294002 in LPS-stimulated monocytes (data not shown). This contrasted with previous observations in THP-1 cells or PBMC (33), suggesting that this effect might depend on the type of monocytes or cell preparation (41). The

effect of PI3K blockade on TNF production by CE$_{sHUT}$-activated monocytes was less clear, the latter being either unchanged or slightly inhibited (10–20%). This suggests that, in terms of PI3K regulation of TNF production, CE$_{sHUT}$-activated monocytes display an intermediate behavior between monocytes activated by anti-CD3-stimulated and cytokine-stimulated T cells (54). Together with the above studies, our results clearly support anti-inflammatory functions of PI3Ks in human peripheral blood monocytes/macrophages in chronic inflammatory conditions.

In addition to shedding light on the role of PI3Ks in cytokine production by monocytes, the present results reveal that IL-1β transcripts have a shorter half-life in CE$_{sHUT}$- than in LPS-activated monocytes. The instability of IL-1β transcripts indicates that the CE$_{sHUT}$-induced transcription rate has to be higher to maintain steady-state levels comparable with those induced by LPS (see Fig. 5A). In agreement with this, β$_2$ integrins that might play a part in the production of IL-1β in monocytes activated by cell-cell contact with stimulated T cells also induced very unstable IL-1β transcripts in monocytes (45). The mechanisms underlying this phenomenon are unclear, but because AU-rich elements (ARE) are present on IL-1β transcript 3′ untranslated region, one could hypothesize that different ARE-binding proteins affecting mRNA stability are induced by either LPS or CE$_{sHUT}$. Contrasting with IL-1β mRNA, the half-life of sIL-1Ra mRNA was longer in CE$_{sHUT}$- than in LPS-activated cells (see Fig. 4B), consistent with the levels of sIL-1Ra mRNA which are higher in monocytes activated by CE$_{sHUT}$ than in LPS-activated cells after 9 h activation (see Fig. 4A). Consequently, a different posttranscriptional regulation of sIL-1Ra mRNA decay in monocytes activated by LPS or CE$_{sHUT}$ could account for the differences observed in the production of sIL-1Ra (Table I). Although differential sIL-1Ra transcript stability has been observed in THP-1 cells pretreated or not with LPS (56), the mechanisms underlying this stimulus-dependent mRNA stability remain elusive because sIL-1Ra mRNA does not contain ARE (57). The premise that contact-mediated activation of monocytes induced the expression of the stable sIL-1Ra transcript correlates with the tremendous amounts of sIL-1Ra which are often seen in the inflamed synovium of RA patients, i.e., a chronic inflammatory disease (58, 59). The high stability of IL-1β mRNA induced by LPS might favor physiological inflammatory responses to infectious agents; in contrast, the unbalanced overproduction of IL-1β participates in the development of septic shock and systemic inflammatory diseases (60). Because contact-mediated activation of monocytes by stimulated T cells is thought to be relevant of chronic inflammation, i.e., pathological mechanisms, the instability of the IL-1β transcript arising in the latter conditions might be part of a protective mechanism set in by the cells to counterbalance inappropriate inflammatory processes. Moreover, comparable levels of IL-1β mRNA expression, but dissimilar levels of IL-1β secretion, were observed upon the activation of monocytes by LPS and CE$_{sHUT}$ (Table I and Fig. 5A). This suggests that not only the stability of IL-1β transcripts but also the more tightly restrained secretory processes elicited by CE$_{sHUT}$ might be part of mechanisms aimed at tempering inflammatory responses related to chronic/sterile conditions.

As stated above (see Introduction), controversial results as to the pro- of anti-inflammatory functions of PI3Ks have been reported. Recently, attention has increasingly been paid to two PI3Ks of class I, i.e., PI3Kγ and PI3Kδ, that are mainly expressed in cells of the hemopoietic system. Thus, the blockade of the class IB PI3K isoform (PI3Kγ) in animal models of RA suppresses leukocyte recruitment and subsequent joint inflammation and damage (61). Furthermore, both PI3Kγ and PI3Kδ are involved in the induction

of respiratory burst in human neutrophils (62). This does not corroborate the present results and those of others showing that the activity of PI3Ks limits inflammatory responses (36, 54, 55). Accordingly, a PI3Kγ inhibitor (5-(2,2-Difluoro-benzo[1,3]dioxol-5-ylmethylene)-thiazolidine-2,4-dione; Calbiochem) did not affect IL-1β and sIL-1Ra production in monocytes activated by either LPS or CE$_{sHUT}$ (data not shown). It follows that it would be of biological and therapeutic interest to identify specific classes and isoforms of PI3Ks implicated in the regulation of cytokine production in human monocytes activated by stimulated T cells and/or LPS.

In conclusion, this study affords new insights into the molecular mechanisms controlling the production of IL-1β and its natural specific inhibitor sIL-1Ra in human monocytes activated in conditions related to acute/infectious and chronic/sterile inflammation. The activation of PI3Ks represses IL-1β production by controlling mechanisms leading to its secretion and induces *sIL-1Ra* gene transcription in monocytes activated by LPS and direct cellular contact with stimulated T cells. By reducing IL-1β and enhancing sIL-1Ra production in human monocytes, PI3K activity may lead to the resolution of the inflammatory responses.

Disclosures

The authors have no financial conflict of interest.

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