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Oxidation-dependent maturation and survival of explanted blood monocytes via Bcl-2 up-regulation

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ABSTRACT

Monocytes isolated and cultured according to standard procedures from the blood of 22 healthy donors display an activation process, monitored as adhesion and increased exposure of CD11. Starting from very early time points, monocytes undergo a deep redox modulation, i.e., they increase reactive oxygen species (ROS) formation and decrease glutathione content; at the same time, the anti-apoptotic protein Bcl-2 is substantially up-regulated. The cause-effect relationship between these parameters was investigated. On the one side, pharmacological glutathione depletion with BSO further increases ROS formation and Bcl-2 levels. On the other side, scavenging of ROS by Trolox prevents Bcl-2 up-regulation. Two lipoxygenase (LOX) inhibitors (CAPE or AA861) prevent ROS increase and, accordingly, also prevent Bcl-2 up-regulation. All this evidence supports the redox-sensitivity of Bcl-2 regulation. Trolox, CAPE and AA861, i.e., all treatments that abolish ROS increase and prevent Bcl-2 up-regulation, increase the rate of cell loss, whereas BSO, increasing Bcl-2, reduces cell loss and induces chemo-resistance. Thus, explanted healthy monocytes seem to undergo an oxidation-dependent maturation implying increased survival via Bcl-2 up-regulation, perhaps mimicking physiological activation.

1. Introduction

Monocytes are among the main effectors of innate immunity; they are solicited to the inflammatory site by cytokine released by tissue-resident dendritic cells, mast cells and/or neutrophils, and participate to the progression of the inflammatory response by undergoing a set of activation processes that enable them to phagocytosis, release of active oxygen species, and production of inflammatory cytokines with the aim of reinforcing/re-addressing and progress the response [1]. Maturation is accompanied by the expression of surface markers such as the integrin CD11 [2], adhesion molecules that promote attachment to endothelial cells thus allowing diapedesis and enabling escape from the blood flow. Another important marker of activation is the ability to respond to specific stimuli by the oxidative burst, where H_2O_2 and superoxide anion are produced by NAPDH-oxidase [3,4] with an anti-microbial aim. The oxidative status of monocytes/macrophages is a very intriguing issue. Pathological conditions such as HIV infection increase the basal level of reactive oxygen species (ROS) in circulating monocytes, probably due to a generalized/systemic inflammatory status [5]. Basal oxidative levels are influenced by the cytokine-mediated activation of other pro-oxidant enzymes such as lipoxygenases (LOX) and cycloxygenases (COX), which process the arachidonic acid liberated by phospholipase A2 (PLA_2), whose

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up-regulation plays a key role in the inflammatory process [6,7]. LOX and COX play a pivotal role in inflammation, due to the promotion of an oxidant status and mostly for the production of arachidonate-derived inflammatory mediators such as leukotrienes and prostaglandins [8]. Lipoxygenase has been shown to be involved in promoting adhesion of monocytes, especially to endothelial cells [9]; this is a physiological process that, if deregulated, may prime/facilitate the onset of atherosclerosis. On the other hand, exogenous H_2O_2 facilitate monocyte adhesion [4], and also promotes the activation of arachidonate/PLA_2-dependent enzymes [10]: this suggests that a cycle of oxidation/activation is generated to promote and reinforce the activation process, pointing to a key role of oxidations in monocyte activation.

A corollary of the inflammatory response is that the cell actors of the innate immunity must display increased survival thus allowing a longer anti-microbial action at the lesion site; this is a fascinating issue, since the environmental conditions are intrinsically harsh, mainly due to increased oxidation, and the activated cells in order to survive must build up resistance strategies to avoid/delay cell apoptosis. The life span of circulating neutrophils is very short, but is greatly increased upon activation in the inflammatory site due to down-regulation of the pro-apoptotic protein Bax [11]. Un-stimulated monocytes are also prone to apoptosis; upon activation, the Fas ligand/Fas receptor system is neutralized [12]; in addition, many different pro-inflammatory stimuli such as lipopolysaccharide, interleukin 1 and tumor necrosis factor-alpha affect monocyte proneness to apoptosis [13] and prolong survival.

Intracellular pathways built up to cope with oxidative stress are mostly aimed at scavenging the surplus of reactive oxygen species, dealing with the over-expression of antioxidant enzymes such as catalase, superoxide dismutase or glutathione (GSH) peroxidase [14] and the general potentiation of the glutathione system. Glutathione plays important roles in scavenging free radical, in maintaining correct redox equilibrium, and in cell detoxification. The antioxidant role is performed by scavenging the oxidized molecules via the redox cycling of reduced (GSH) and oxidized (GSSG) glutathione, achieved enzymatically by GSH peroxidase and GSSG reductase at the expense of the NADP+/NADPH redox couple. Maintenance of the redox asset is achieved by the ability of the GSH reactive cysteine to form or substitute disulfide bridges with protein cysteine residues with the aid of tutoring enzymes such as thioredoxin or disulfide isomerases [15,16]. Cell detoxification is achieved by GSH-S-transferase (GST), which covalently links nucleophilic molecules (usually xenobiotics) with GSH [17]; the complex is then released outside the cells via the multi drug resistance (MDR) complexes [17], thus freeing the cells from toxicants at the expense of GSH. Detoxification may be achieved also in the absence of GST; in this instance the toxicant is co-charged with GSH on the MDR without covalent link, and co-extruded as separate molecules [17]. This modality of extrusion allows re-use of the extruded GSH via the extracellular GSH metabolism; the ecto-enzyme gamma-glutamyl-transpeptidase (GGT) [18] cleaves the glutamyl residue of GSH, and the residual cysteinyl-glycine may be recovered and re-imported within the cell [18], thus providing building material for re-synthesis of GSH though the so-called salvage pathway, bypassing the bottleneck of the main GSH biosynthetic enzyme, gamma-glutamyl cysteine synthase GGCS). GGT cleaves also many GSH-S-conjugates, such as leukotriene C4 [19] and nitrosoglutathione (GSNO) [20], thus participating to the processing of important mediators of the inflammatory response derived by the metabolism of LOX and nitric oxide synthase (NOS). The efflux of GSH has also an extracellular role, providing body fluids of the required redox equilibrium; the main providers of extracellular GSH are hepatocytes, where GSH efflux occurs via canalicular or sinusoidal carriers that may be inhibited by monochlorobimanes and cystathionine or methionine, respectively [21].

A different/complementary strategy for surviving oxidative damage is the building up of survival pathways, aimed at antagonizing apoptosis. NF-kappaB, LOX, NOS, the MAP kinase network participate to eliciting pathways culminating with the up-regulation of anti-apoptotic protein. The over-expression of Bcl-2 is one of the most effective way of inhibiting damage-induced apoptosis, especially in cancer cells, even though in most instances Bcl-2 is over-expressed as a result of gene rearrangement [22]. However, several evidences show that Bcl-2 can be up-regulated as a NF-kappaB target [23–25]. We have demonstrated that slow GSH depletion by the GGCS inhibitor buthionine sulfoximine (BSO) up-regulates Bcl-2 in the monocytic cells U937 [26] by triggering a non-canonical NF-kappaB pathway (Cristofanon et al., submitted for publication); the increased Bcl-2 levels allow survival to oxidant conditions [26,27]. Interestingly, some GSH depleted cells, due to Bcl-2 over-expression, even develop chemo-resistance in spite of the GSH depletion [26].

In this study, we describe that survival and activation of human monocytes depend on the LOX-mediated production of reactive oxygen species, culminating with the up-regulation of the pro-survival protein Bcl-2.

2. Materials and methods

2.1. *Mononuclear white blood cells isolation*

Peripheral blood mononucleated cells (PBMC) were isolated from the anti-coagulated peripheral blood of 22 healthy adult human donors (13 men and 9 women) using the standard Ficoll-Hypaque (Sigma) density separation method. After isolation, PBMC were washed, counted, resuspended at cell density of 1×10^6 cells/ml in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 IU/ml penicillin and streptomycin and kept in a controlled-atmosphere (5% CO_2) incubator at 37 °C.

All the experiments were performed on co-cultured mononuclear cells, i.e., lymphocytes+ monocytes, distinguished by differential labelling upon flow cytometric analysis (see below). Before each measurement, suspension cells (lymphocytes + undifferentiated monocytes) were removed; the remaining attached cells (differentiating monocytes) were scraped and mixed to the suspension cells; all analysis were performed by double labelling with the surface antigen plus the specific probe, and the parameters analyzed on the population of interest (monocytes) after gating the CD14+ population.

U937 cells were cultured as described [28]. All the experiments were performed in complete medium on log phase cells displaying viability ≥98% as results from exclusion of cell-impermeant dyes, at cell density of 7×10^5 cells/ml.

2.2. Surface staining

The expression of CD14–CD3/CD19 on PBMC was analyzed by adding 5 ul phycoerythrin (PE) conjugated anti-CD14, 5 ul FITC-conjugated anti-CD3 and 5 ul FITC-conjugated anti-CD19 (Becton Dickinson) to leukocyte pellets. The expression of CD14–CD11 on monocyte was analyzed by adding 5 ul phycoerythrin conjugated anti-CD14, 5 ul FITC-conjugated anti-CD11b (Becton Dickinson) to leukocyte pellets. The suspensions were incubated in ice for 20 min in the dark and then washed twice in PBS-EDTA. The cells were finally resuspended in 0.5 ml of PBS and processed in a DAKO Galaxy flow cytometer. Statistics were determined in 50,000 events/sample by FlowMax software. FlowMax software permitted to study on the same dot plot FSC vs. SSC quantization and counting of singular populations of monocytes and lymphocytes. Gating the area of labelled monocytes allowed getting information on the parameters of interest (abundance and the different fluorescence corresponding to GSH, ROS and Bcl-2 levels) of the specific area.

2.3. Cell treatments

Glutathione depletion was performed by inhibiting glutathione *de novo* synthesis with 1 mM buthionine sulfoximine (Sigma), which depletes PBMC and U937 of GSH in 18–24 h. Vitamin E analogue Trolox C (Fluka) was used as radical scavenger [29]; it was added to the ex-vivo monocytes at the final concentration of 500 μM (30 min prior to BSO when used together). CAPE, Caffeic Acid Phenethyl Ester (Sigma), was used as general inhibitor of lipoxygenase at the final concentration of 17 μM. The specific 5-lypoxygenase inhibitor, AA861, was added at the final concentration of 20 μM. Acivicin (Sigma) was used as inhibitor of GGT activity; it was added to the cell culture at the final concentration of 130 μM (45 min prior to BSO when used together). Methionine and cystathionine (Sigma) were used at the final concentration of 1 mM to inhibit glutathione efflux through sinusoidal glutathione channels.

2.4. Induction and analysis of apoptosis

VP16 (500 μM) and PMC (10 μg/ml) were used as apoptotic inducers. Monocytes were separated from the PBMC in order to consider the impact of the chemotherapeutic agents only in this population. Once the PBMC was isolated from the whole blood, 75×10^6 cells were resuspended in 15 ml of complete medium (RPMI 1640 supplemented with 10% Human Serum AB, 2 mM L-glutamine, 100 IU/ml penicillin and streptomycin) and incubated in flask for 2 h in incubator with a controlled-atmosphere (5% CO_2) at 37 °C. At the end of the incubation time, after three washing of the flask with warm RPMI 1640, the flask was incubated with a solution of RPMI/EDTA 10 mM for 10 min at 4 °C. Then, using a cell scraper, the isolated monocytes were removed, washed with RPMI and then resuspended in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 IU/ml penicillin and streptomycin and kept in a controlled-atmosphere (5% CO_2) incubator at 37 °C. For the chemosensitization experiments, BSO was added for 24 h as a pre-treatment before to treat the cells for further 24 h with VP16 (500 μM) and PMC (10 μg/ml). For detection of apoptosis, cells were stained with AnnexinV, as described in [30] and then with the DNA specific cell permeant dye Hoechst 33342 (Calbiochem, San Diego, CA, USA) at the concentration of 10 μg/ml. Apoptotic cells were recognized according to their nuclear morphology (different stages of nuclear fragmentation) and AnnexinV membrane exposure. The cells were counted using a fluorescence microscope (at least 300 cells in at least three independent fields). Propidium iodide (10 μg/ml) was added for detection of eventual cells in secondary necrosis.

2.5. Bcl-2 determination

Cells were fixed, permeabilized and stained with anti-Bcl-2 monoclonal antibody (Calbiochem Novabiochem Corp., San Diego, CA (#OP60)) according to the manufacturer's instruction [26]. Detection was done with FITC-conjugated antibodies and processed in a DAKO Galaxy flow cytometer. Statistics were elaborated in 50,000 events/sample by FlowMax software. Mean values given by this analysis were used for further elaboration. For comparison between different experiments, the value of each treated cell sample was compared with the value of the control cell sample (untreated cells after separation protocol), which was considered equal 1. The values are then given as fold increase with respect to control.

2.6. Glutathione determination

GSH intracellular levels were detected with Ortophthalaldehyde (OPTA, Molecular Probes) [31] (exc. wavelength = 340 nm to em. wavelength = 445 nm). OPTA was added to the cell suspension at the final concentration of 50 μM. The suspensions were incubated at 37 °C for 20 min in the dark and then washed. The cells were finally resuspended in PBS and processed in a DAKO Galaxy flow cytometer. Statistics were elaborated in 50,000 events/sample by FlowMax software. Mean values from this analysis were used for further elaboration. For comparison between different experiments, the value of each treated cell sample was compared with the value of the control cell sample (untreated cells after separation protocol), as fold increase (control = 1).

2.7. Determination of ROS production

ROS levels were detected with 2-7-Dichloro-fluorescein-diacetate (DHCFDA) (exc. 490 nm to em. 520 nm), which was added to the cells at the final concentration of 10 μM. DHCFDA fluoresces only when oxidized. The suspensions were incubated at 37 °C in the dark for 20 min and then washed. Cells were finally resuspended in PBS and processed in a DAKO Galaxy flow cytometer. Statistics were elaborated in 50,000 events/sample by FlowMax software. Mean values given by this analysis were used for further elaboration. For comparison between different experiments, the value of each treated cell sample was compared with

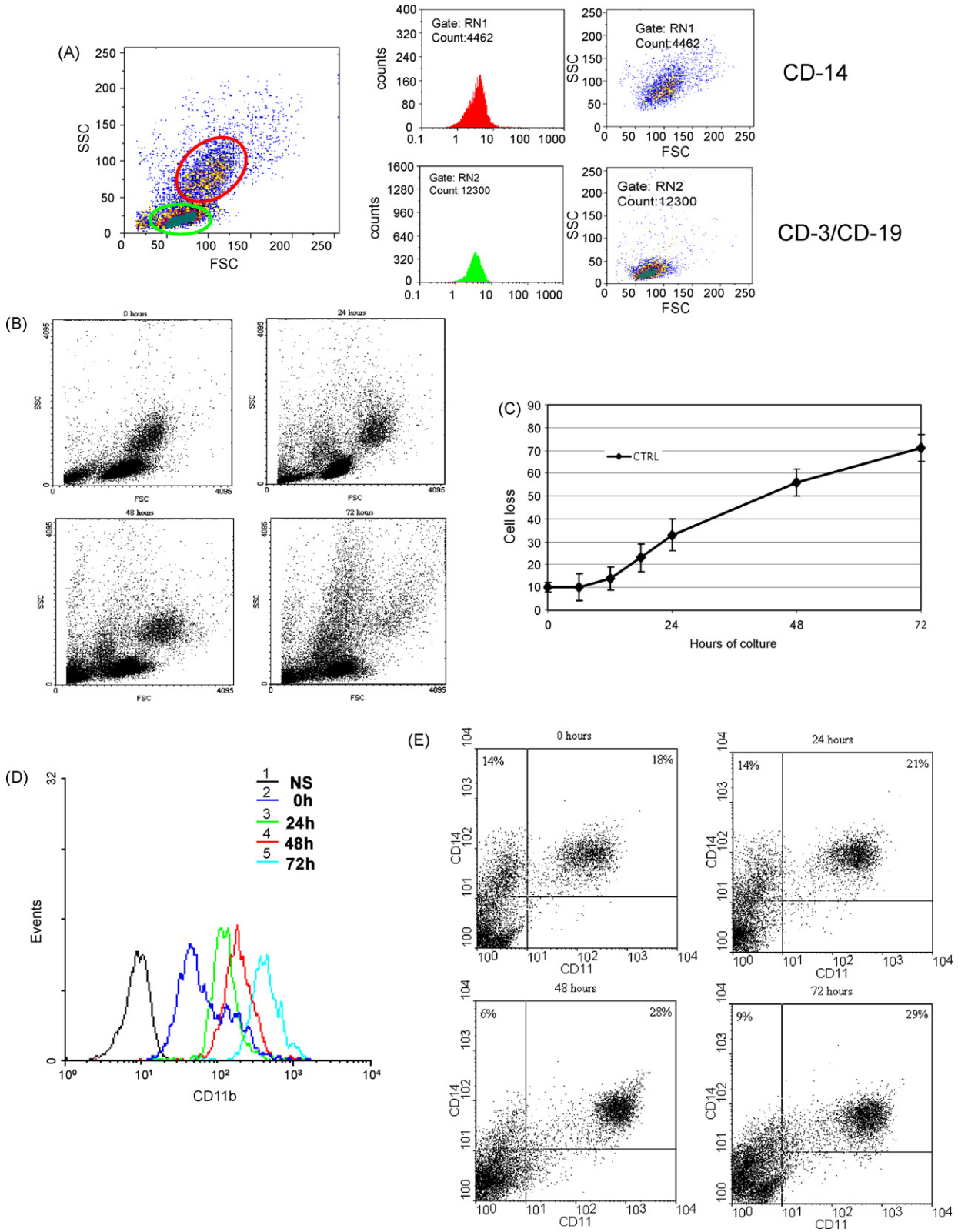

Fig. 1 – Survival and differentiation of cultured monocytes (A) flow cytometric dot plot analysis of FSC vs. SSC of mononuclear leukocytes isolated as described, at 0 h of culture (left panel). The cells in the areas gated in red (upper circle) and green (lower circle) are positive for CD14 and CD3/CD19, respectively, thus corresponding to monocytes (upper right panel) and B + T lymphocytes (lower right panel). (B) Time course dot plot analysis of FSC vs. SSC of control cells. (C) CD14+ cells loss: relative monocytes loss with time was estimated according to the ratio of CD14+ cells over total cells (regular + deteriorated). Results are the mean of 22 experiments performed on blood samples of different individuals, ±S.D. Flow

the value of the control cell sample (untreated cells after separation protocol), as fold increase (control = 1).

2.8. *Indirect immunofluorescence*

After treatments, U937 cell were fixed and permeabilized with BD Cytofix/Cytoperm Kit according to the manufacturer's instruction (BD, Becton Dickinson, San Jose, CA, USA). Primary antibody incubation was performed in BD Perm/Wash solution (BD) for 1 h at room temperature with 10 μg/ml of following antibodies diluted in BD Perm/Wash solution: anti-Bcl-2 mouse monoclonal (Calbiochem). After washing two times with PBS, the cells were incubated for 30 min at room temperature with 8 μg/ml anti-mouse secondary Alexafluor 488 (Molecular Probes, Junction City, OR, USA). After washing again two times with PBS, the cells were counterstained with Hoechst 33342 and observed with a Leica (Wetzlar, Germany) DM IRB microscope, and the images were analyzed with Image J software.

2.9. *Statistical analysis*

Statistical analyses were performed using Student's *t*-test for unpaired data, and p values <0.05 were considered significant. Data are presented as fold increase vs. 0 h of treatment cells ±S.D.

3. Results

3.1. *Survival and differentiation of cultured monocytes*

Soon after separation, peripheral blood mononuclear leukocytes (PBML) were analyzed by flow cytometry. They display two populations according to the FSC vs. SSC (Fig. 1A) analysis, which show two homogeneous populations of monocytes and lymphocytes after labelling with surface antigens (Fig. 1A, right panels), labelled with CD14 (upper panel) and CD3/19 (lower panel). These cells were cultured for 4 days, and the evolution of FSC vs. SSC pattern in shown in panel B. CD14+ cells becomes larger (FSC increases) and their abundance decreases: the accumulation of cells outside the lymphocyte or monocyte region is a sign of cell deterioration; relative monocytes loss with time was estimated according to the ratio of CD14+ cells over total cells (regular + deteriorated), and its extent with time is shown in panel C. It is worth noting that the CD14+ cells (red) are lost before the CD19+ cells (green area), supporting the longer survival times of lymphocytes in culture.

Monocytes attached to the flask in function of time; at 24 and 48 h monocytes increasingly adhere displaying a round shape; at 72 h some cells acquire an elongated morphology, and by 96 h most monocytes are elongated (not shown), thus indicating that a process reminiscent of activation/maturation is occurring. Accordingly, the level of integrin CD11b expression increases with time (panels D, E) in the CD11+ cells, and

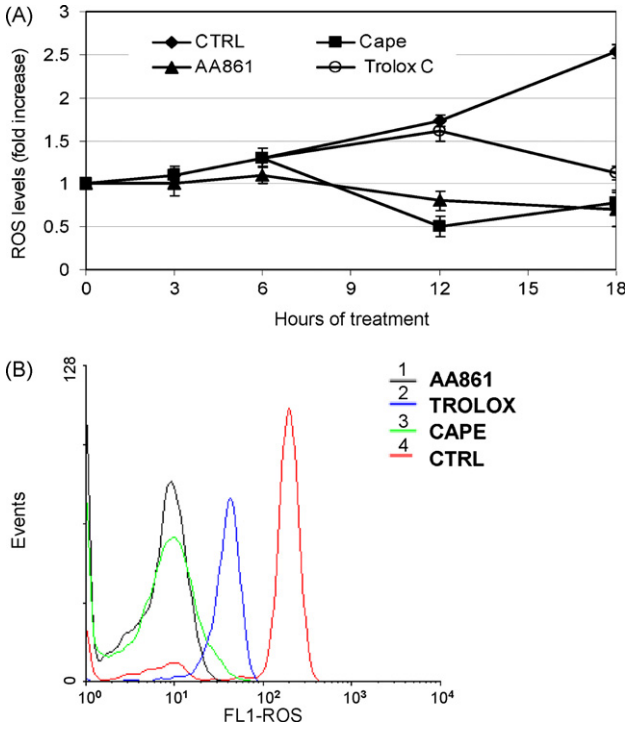

Fig. 2 – Time-dependent lipoxygenase-mediated oxidation of cultured monocytes. (A) Time course of ROS levels, measured by DCFHDA staining as described in Section 2, among the gated CD14+ cells population in the presence/absence of Trolox, CAPE and AA861. Values are expressed as fold increase with respect to control cell sample at 0 h of culture, which was considered = 1. Results are the mean of 10 experiments performed on blood samples of different individuals, ±S.D. (B) Overlay of the flow cytometric profiles of DCFHDA-stained CD14+ cells at 24 h of culture: one representative experiment of 10 performed with similar results is shown. The decrease of ROS by the three compounds is statistically significant ($p < 0.05$).

the fraction of CD11b+ cells among the total CD14+ cells increases too (panel E).

3.2. *Time-dependent lipoxygenase-mediated oxidation of cultured monocytes*

The amount of reactive oxygen species was monitored during the first day of monocytes culture by means of flow cytometric analysis of the gated CD14+ cell population stained with the ROS-sensitive, cell permeant dye dichlorofluorescein-diacetate (DCFHDA), which fluoresces when oxidized (DCF); stained cells were analyzed at the flow cytometer, thus getting a quantitative measure of intracellular oxidations. Fig. 2A shows that DCF fluorescence increases as a function of time, and reaches a 2.5 fold increase at 24 h. This ROS increase could be effectively buffered by the antioxidant Trolox (Fig. 2A). In the search for the mechanism responsible for ROS production, we

cytometric analysis of the time course of CD11b exposure: (D) CD11b among gated CD14+ cells: the overlay of the profiles of one representative experiment is shown. (E) Dot plot analysis of CD14 vs. CD11.

discovered that ROS could be effectively prevented by two inhibitors of lipoxygenases, namely the generic CAPE and the 5-LOX quasi-specific AA861. This indicates that monocyte ROS are produced by LOX.

3.3. *Oxidations play a pro-survival and activation role in cultured monocytes*

To understand whether LOX-derived ROS play a role in monocytes survival/activation, we challenged these parameters with the treatments that prevent/scavenge spontaneous ROS production. As shown in Fig. 3A, CAPE, AA861 and Trolox consistently and reproducibly accelerate the rate of cell loss, suggesting a cause–effect relationship between ROS production and survival.

In addition to decreasing survival, CAPE, AA861 and Trolox prevented monocyte attachment to substrate (not shown). To understand if this was due to an interference with the activation process, the extent of CD11 expression among the CD14+ cells was measured at 48 h of culture in the presence/absence

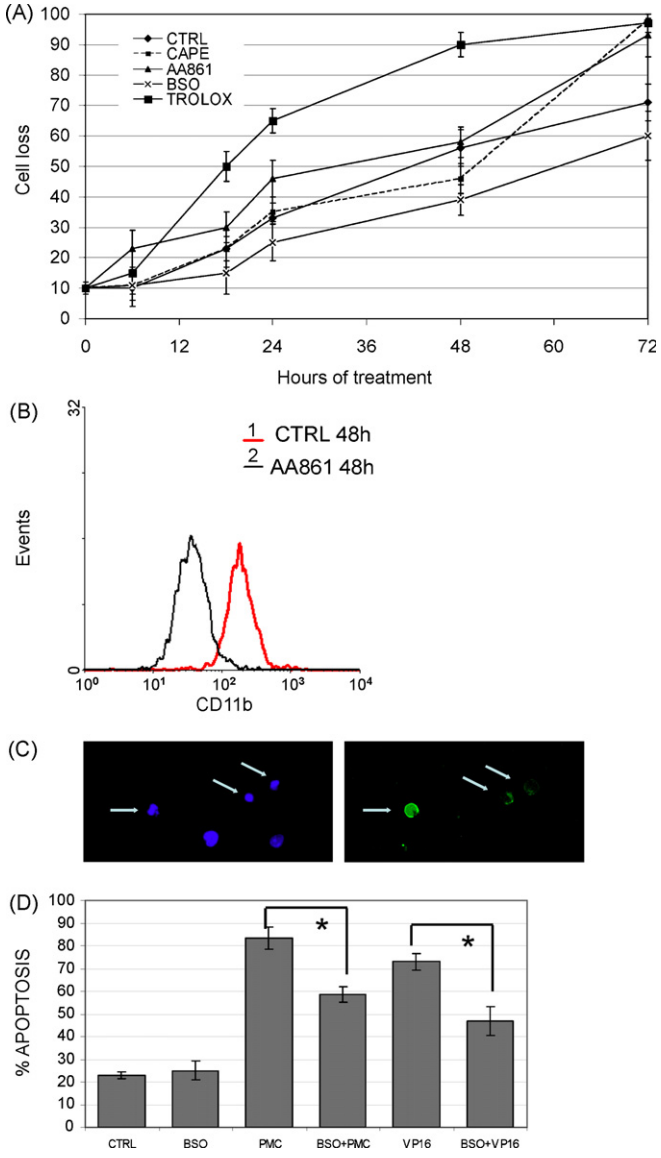

Fig. 3 – Oxidations play a pro-survival and activation role in cultured monocytes. (A) CD14+ cells loss: relative monocytes loss with time was estimated according to the ratio of CD14+ cells over total cells (regular + deteriorated, see also legend to Fig. 1). Results are the mean of four experiments performed on blood samples of different individuals, ±S.D. (B) flow cytometer profile of CD11b exposure at 48 h of culture in the presence/absence of AA861 (one representative experiment of four performed with similar results). (C) analysis of apoptosis by fluorescence microscopy analysis of monocytes isolated for differential adherence as described in Section 2, treated with puromycin. The same microscopic field is stained with Hoechst (left panel) to assess nuclear morphology, and FITC-conjugated AnnexinV (right panel) to detect PS exposure. Arrows indicate apoptotic cells, which display fragmented or shrunken nuclei and PS exposure. (D) BSO was added for 24 h as a pre-treatment before treating cells for further 24 h with VP16 (500 μM) and PMC (10 μg/ml). Apoptotic cells were counted as described in Section 2; the decrease of apoptosis in the presence of BSO is significant for both VP16 and PMC treatment ($p < 0.05$).

of AA861. As shown in Fig. 3B, AA861 prevents the increase of CD11b staining observed in cultured monocytes (see Fig. 1E); in fact, CD11b results even down-regulated.

The role of ROS production in monocyte survival was probed by performing a pro-radical treatment, i.e., the opposite experiment with respect to radical scavenging. To this purpose, cells were treated with BSO that depletes GSH thus producing oxidative stress. BSO accelerates the increase in ROS production over the control monocytes (not shown); in support of the link between oxidations and survival, this is accompanied by a delay of monocyte loss (Fig. 3A). In order to further test BSO promotion of survival, monocytes cultured in the presence/absence of BSO were treated with two different apoptotic inducers, i.e., the protein synthesis inhibitor puromycin and the topoisomerase II inhibitor etoposide, a largely used anti-tumor agent. BSO-treated monocytes are protected from both agents, thus indicating that BSO induces chemo-resistance (Fig. 3C–D).

3.4. *Oxidation-dependent survival implies the up-regulation of Bcl-2*

To explore the mechanism through which oxidations facilitate cell survival, we analyzed the levels of the anti-apoptotic protein Bcl-2, which was found to increase and provide survival, in response to oxidative stress in the monocytic cell line U937 [26]. To this purpose, we performed a flow cytometric analysis of gated monocytes labeled with FITC-conjugated anti-Bcl-2 antibodies. The time course of untreated monocytes shows that a 1.5 fold-increase occurs at 24 h of culture (Fig. 4A). The anti-ROS agents Trolox, CAPE and AA861 prevent Bcl-2 over-expression, in fact causing a down-regulation (Fig. 4B). Accordingly, the pro-oxidant BSO strongly enhances Bcl-2 over-expression. Thus, Bcl-2 levels seem to respond to the oxidative status of the cultured monocytes.

3.5. *Dynamics of GSH and implications for Bcl-2 regulation in monocytes*

We next investigated the dynamics of GSH in the cultured monocytes. Intracellular GSH levels were analyzed by performing single-cell flow cytometric analysis; the choice was obliged, since bulk analysis of GSH by means of standard cell lysis, thiol derivatization and HPLC protocols cannot be performed in mixed-cell populations such as the monocytes + lymphocytes here analyzed. To this purpose, cells were labeled with the fluorescent dye ortophthalaldehyde [17], which derivatizes thus detecting intracellular free thiols; in U937 cells, where GSH constitutes >90% of the non-protein thiols pool, this is a bona fide measure of intracellular GSH level [32]. Fig. 5A shows that GSH is lost by the cultured monocytes as a function of time; this decrease is due to oxidation, since it is prevented by Trolox. Treatment with BSO increases GSH loss, as expected. We explored whether the

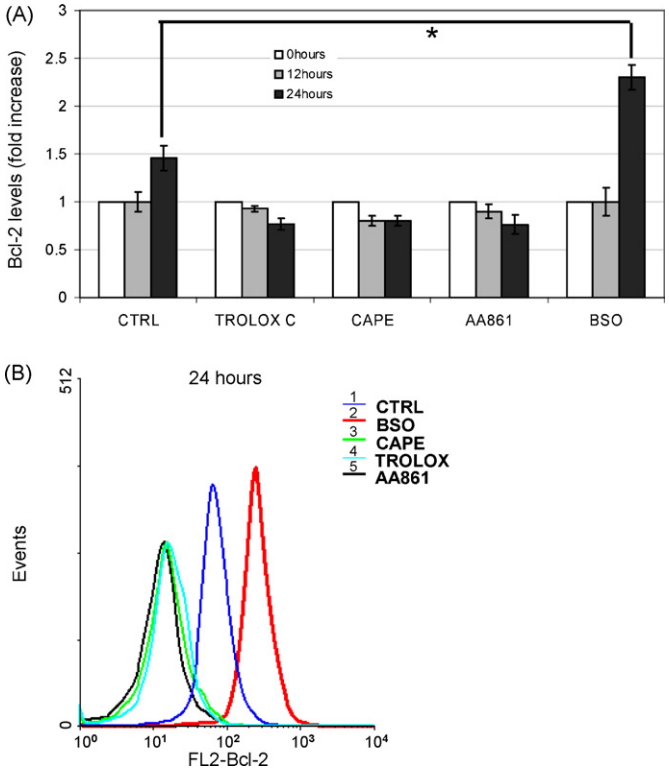

Fig. 4 – Oxidation-dependent survival implies the up-regulation of Bcl-2. (A) Levels of Bcl-2 in monocytes (gated CD14+ population) treated as described. Values are expressed as the mean values got from the flow cytometric analysis. Bcl-2 increase in cells treated with BSO with respect to untreated is statistically significant ($p < 0.05$). All values are expressed as fold increase with respect to control cell sample at O hours of cultures, which is posed = 1. Results are the mean of 10 experiments performed on blood samples of different individuals, ±S.D. (B) Flow cytometric profile of Bcl-2 levels at 24 h of culture; one representative experiment among 10 performed with similar results is shown.

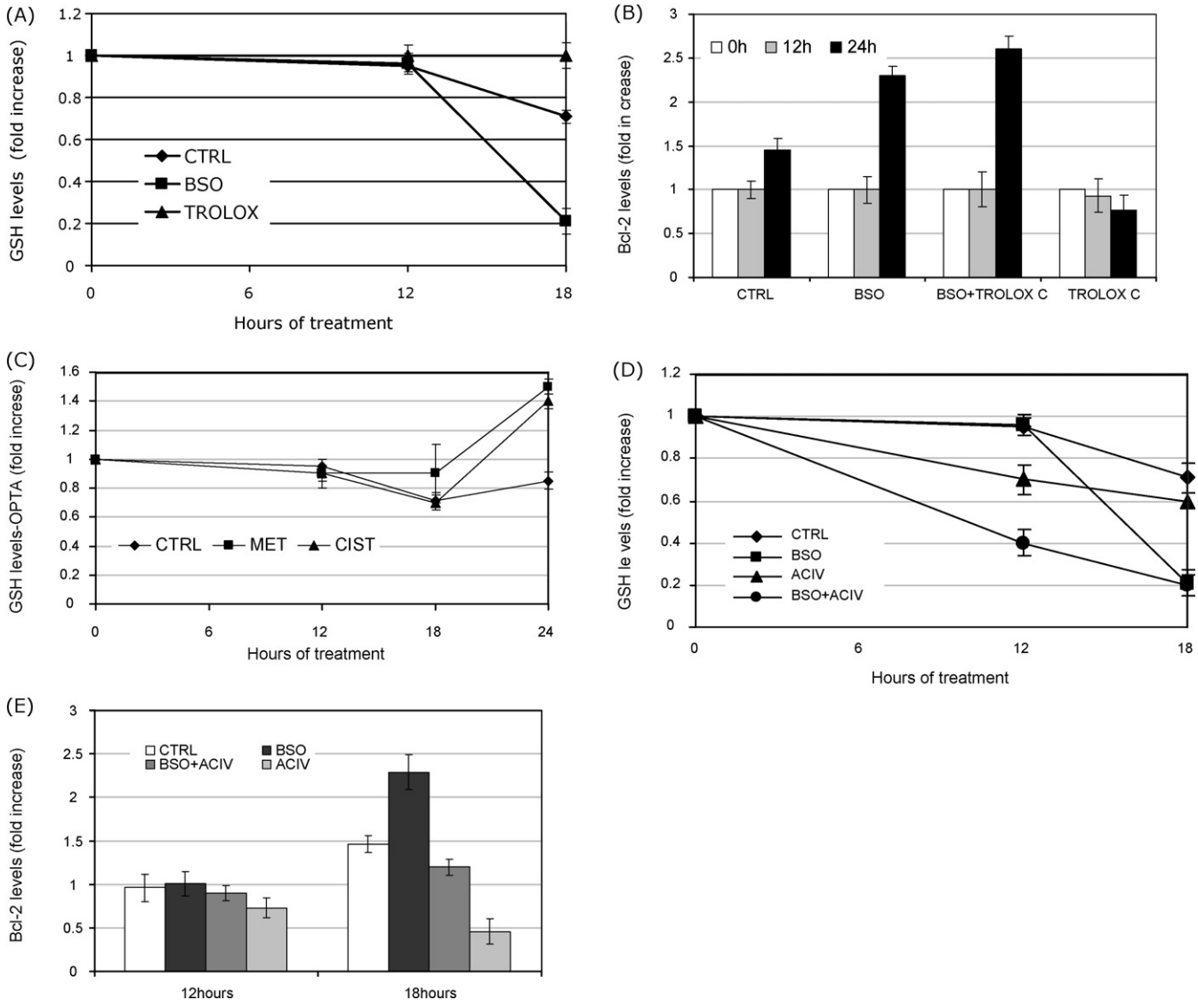

Fig. 5 – Dynamics of GSH and implications for Bcl-2 regulation in monocytes. (A) GSH levels in monocytes treated as described were measured at the single cell level with OPTA as described in Section 2 among the CD14+ cell population. Values are expressed as fold increase with respect to control cell sample at 0 h of culture, which was posed = 1. Results are the mean of 10 experiments performed on blood samples of different individuals, ±S.D. (B) Levels of Bcl-2 in monocytes (gated CD14+ population). Trolox pre-treatment does not affect BSO-induced Bcl-2 up-regulation. Values are expressed as fold increase with respect to control cell sample at 0 h of cultures, which was posed = 1. Results are the mean of 10 experiments performed on blood samples of different individuals, ±S.D. (C) GSH levels in monocytes (gated CD14+ cell population) were measured as described. Values are expressed as fold increase with respect to control cell sample at 0 h of culture, which was posed = 1. Results are the mean of five experiments performed on blood samples of different individuals, ±S.D. (D) and (E): GSH and Bcl-2 levels, respectively, in monocytes (gated CD14+ cells) were measured as described in Section 2. Values are expressed as fold increase with respect to control cells at 0 h of culture, which was posed = 1. Results are the mean of 5 and 10 (respectively) experiments performed on blood samples of different individuals, ± cultures, which was considered = 1.

strong up-regulation of Bcl-2 due to BSO treatment was due to GSH decrease or ROS increase, by analyzing Bcl-2 levels in BSO-treated cells in the presence/absence of Trolox. As shown in Fig. 5B, Trolox did not alter Bcl-2 levels in BSO-treated monocytes, indicating that the depletion of GSH is enough of a signal to promote Bcl-2 increase. We also investigated the possible role of other GSH dynamics. We inhibited GSH carriers with cystathionine or methionine, and found that these two compounds do not affect intracellular GSH levels until 18 h, producing a strong increase at 24 h (Fig. 5C). This seems not due to their effect as antioxidant, since their effect is quite different than Trolox (see Fig. 5A), suggesting that it is instead due to the intracellular accumulation of GSH destined to be exported.

Then we tested the role of GGT in GSH and Bcl-2 levels by treating monocytes with the GGT inhibitor acivicin. Acivicin produces an early decline in intracellular GSH levels at 12 h; afterwards, the rate of decline is similar to control cells. In BSO-depleted cells, acivicin accelerates early GSH loss, whereas at 24 h it does not induce any extra decrease with respect to BSO alone (Fig. 5D). This suggests that at 12 h much of GSH levels is guaranteed by the extracellular GSH metabolism (i.e., GSH is exported, cleaved and the building blocks re-imported), whereas at 24 h some of these steps are missing. Up-regulation of Bcl-2 by BSO occurs in spite of the presence of acivicin (compare CTRL vs. BSO and acivicin vs. acivicin + BSO); however, acivicin by itself strongly reduces the ability of monocytes to up-regulate Bcl-2 in spite of promoting GSH loss (Fig. 5E).

3.6. *GSH and Bcl-2 in U937 cells*

We have deeply analyzed the relationship between GSH and Bcl-2 in the human tumor monocytic cells U937. U937 extrude a substantial amount of GSH through methionine/cystathionine-sensitive carriers [33]; here, we analyze whether acivivin may reveal a possible role for GSH extracellular metabolism. Fig. 6A shows that basal levels of GSH slightly increase with time in control cells due to the presence of fresh medium; acivicin does not alter this profile. The same was found when cells were treated with BSO: acivicin only slightly accelerates the rate of GSH depletion of BSO, without substantial difference. Thus, the GSH exported outside the cells is not used by U937 cells for re-synthesis of intracellular GSH.

Next, we analyzed the role of acivicin in the BSO-induced Bcl-2 up-regulation. To this purpose, we performed an immuno-fluorescence analysis to detect Bcl-2 in U937 at the fluorescence microscopy. The results are shown in Fig. 6B. In these cells, acivicin lowers Bcl-2 levels and hampers BSO ability to up-regulate it.

4. Discussion

With this study we begun to explore the survival mechanisms of monocytes isolated from the blood of healthy individuals. The experiments were carried on by co-culturing monocytes with lymphocytes, thus providing naturally occurring extra-growth factors that may help monocyte survival. Indeed, in experiments where lymphocytes were eliminated, monocytes were lost earlier; however, the dynamics of ROS, GSH and Bcl-2 were unaffected by the presence of lymphocytes up to 24 h of culture (not shown).

The production of reactive oxygen species by monocytes may be due to a cell reaction to the harsh protocols of mononuclear leukocyte isolation; alternatively, they may mimic a regular activation process. The development of a survival pathway is in line with both hypotheses: on the one side, oxidative stress triggers the development of survival pathways to enable cells to cope with stress conditions; on the other hand, activated monocytes are known to lose the proneness to apoptosis that characterizes circulating monocytes. We provide evidences that suggest that we are dealing with a similar-activation process. First, ROS are produced by LOXs, a set of

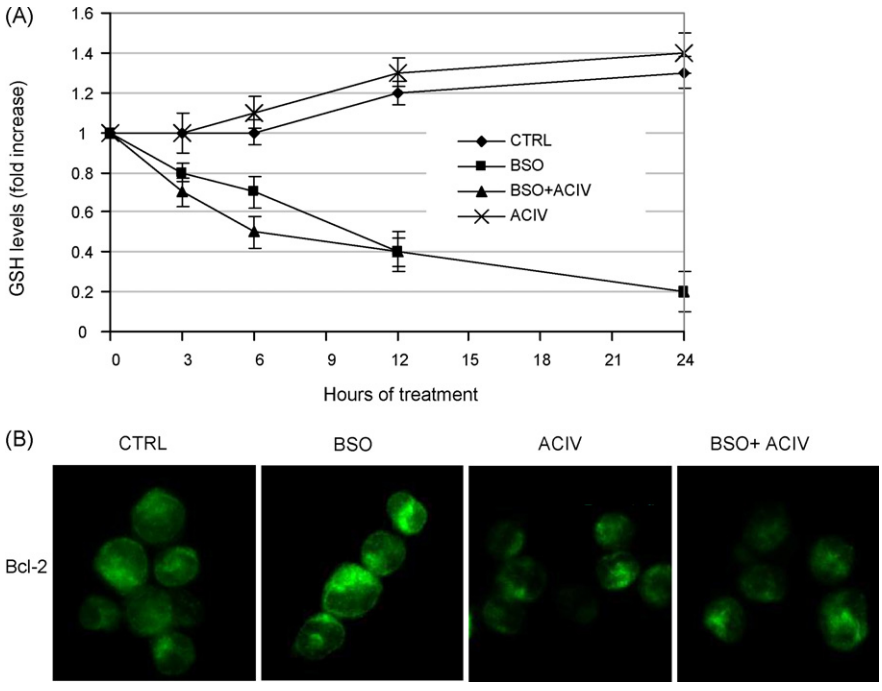

Fig. 6 – GSH and Bcl-2 in U937 cells. (A) Time course of GSH levels in U937; GSH was measured by OPTA staining as described in Section 2. Inhibition of GGT does not alter control cells GSH levels, nor significantly increase the speed of depletion of GSH induced by BSO. All values are expressed as fold increase with respect to control cell sample at 0 h of culture, which was posed = 1. Results are the mean of five experiments ±S.D. (B) U937 were treated with BSO and/or acivicin for 24 h; Bcl-2 was detected by staining with anti-mouse Bcl-2 antibodies and Alexa-488-conjugated anti-mouse IgG as described in Section 2. The images represent a typical picture of five experiments performed with similar results.

enzymes that play important roles in monocytes functions. Second, the increased adhesion and enhanced expression of the integrin CD11b, which are markers of activation, depend on the LOX–ROS chain of events. Our data are in line with the hypothesis that circulating monocytes taken out of the vessel ''sense'' the different environment as a pro-activating event resembling translocation to the inflamed tissue. Thus, activation per se stimulates ROS, and ROS stimulate activation. This cycle provides a reciprocal cause–effect relationship, maybe rendering the issue semantic rather than substantial.

Our data are consistent with the hypothesis that the trigger for Bcl-2 up-regulation is the oxidative stress produced by LOX activation. Indeed, the toxic effect of LOX inhibitors is mimicked by the antioxidant Trolox, suggesting that the products of LOX (leukotrienes or HETE) are not involved in survival. In fact, they may even exert a pro-apoptotic role, since Trolox is more toxic than AA861 or CAPE, even though the three compounds equally prevent ROS production; thus, eliminating LOX products would partially compensate for the lack of induction of the survival pathway. LOX effect on survival is an important issue; HETE, especially 5-HETE, have been demonstrated to be required for tumor cells survival [34]; on the other side, leukotrienes have been implicated in the induction of apoptosis [35].

Bcl-2 and GSH are two pro-survival molecules; their levels are often coordinated, with parallel decrease or increase in their levels, as though acting to reinforce and finalize a pro-apoptotic or pro-survival response, respectively. The molecular mechanisms at the basis of such coordination were explored: on the one side, Bcl-2 may act as a blocker of GSH extrusion carriers, thus favoring the accumulation of GSH inside the cells [36]; on the other side, Bcl-2 half-life was reported to be decreased in an oxidant environment, thus lowering Bcl-2 levels in the absence of GSH [37]. We described that in U937 the coordination between GSH and Bcl-2 exists, but has an opposite nature, i.e., Bcl-2 increases when GSH is depleted. U937 are part of a set of ''responders'' cells that are able to build up a NF-kappaB non-canonical survival pathway that culminates with Bcl-2 up-regulation [26] (see below). When we first described such phenomenon, we proposed that the inverse coordination found in U937 and other tumor cells may be the result of tumor transformation [26]. However, we show here that also primary monocytes display the same behavior as U937; this suggests that it is a matter of tissue specificity rather that tumor transformation. Oxidative stress may cause DNA breaks, increasing the risk of mutation and transformation if cells try to repair DNA damage and survive: this may have favored the evolution of the coordinated response between GSH and Bcl-2, with the aim of hampering survival of possibly mutated oxidative stressed cells. Circulating leukocytes are generally speaking ''disposable'' cells, which have to perform a short-term job in an oxidative environment. The organism may acquire advantages in prolonging monocyte life-time while performing their functions, considering that at the resolution of inflammation they will be disposed of in any case.

An intriguing issue is the mechanisms through which monocytes sense oxidation and promotes Bcl-2 up-regulation. We have recently shown that in U937 cells BSO causes the up-regulation of Bcl-2 by triggering a set of events that lead to the transcriptional activation of the non-canonical pathway of NF-kappaB [27]. This is achieved by promotion/recruitment of the p50–p50 homodimer by a cytosolic Bcl-3, a known partner of the p50 subunit of NF-kappaB. The recruitment occurs via disulfide formation between Bcl-3 and p50-p50, producing a trimer that migrates to the nucleus. The promotion of the disulfide occurs at early time points, i.e., after substantial GSH loss, but before the increase of ROS; more importantly, it occurs even in the presence of radical scavenging by Trolox. This indicates that it is the redox imbalance, rather than the oxidative stress, the molecular inducer of the response. In primary monocytes we have a similar scenario. Indeed, Trolox prevent Bcl-2 up-regulation of untreated cells, but is ineffective in preventing the extra-increase due to BSO. The two types of GSH loss are different; cultured monocytes lose GSH as a result of the oxidation due to LOX activation, with the consequence that ROS scavenging is effective in preventing GSH loss. Instead, BSO induces GSH depletion by inhibiting the turn-over of GSH molecules, event that cannot be affected by Trolox. This would indicate that also in primary monocytes the event that triggers Bcl-2 up-regulation is the redox imbalance following GSH loss, rather than on oxidation. The similar requirement for U937 and primary monocytes suggests that Bcl-2 up-regulation may occur through similar mechanisms.

A last consideration about the dynamics of GSH. The finding that BSO is still able to up-regulate Bcl-2 in the presence of acivicin suggests that acivicin's target acts as a co-factor to help the formation of the disulfide. It is conceivable that the redox imbalance, necessary to promote the formation of disulfide such as those promoted by BSO, requires the help of tutoring factors such as disulfide isomerase or thioredoxin; in this view, acivicin, or the inhibition of GGT action, may impair, without completely inhibit, the tutoring of protein disulfides. However U937, which do not use the extracellular GSH metabolism for maintaining intracellular GSH level, become insensitive to BSO in the presence of acivicin; in fact acivicin per se seems to lower intracellular Bcl-2 levels, perhaps suggesting an extra, GGT independent effect of acivicin; indeed, it was reported that acivicin may have additional intracellular targets [38].

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