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CD30L up-regulates CD30 and IL-4 expression by T cells

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Abstract CD30L is frequently expressed on acute myeloid leukemia (AML) blasts. Its presence is associated with the co-expression of interleukin-4 (IL-4) receptor and with the expansion of specific T-helper 2 (Th2) cell subsets producing IL-4 and expressing CD30. Recombinant CD30L-bearing cells up-regulated the expression of surface CD30 and increased the production of IL-4 and soluble (s) CD30 by co-cultured T cells. These findings were confirmed with AML blasts expressing surface CD30L, where blocking anti-CD30 antibodies completely abolished the release of sCD30 and reduced the production of IL-4. Our data indicates a direct role of CD30L$^+$ neoplastic cells in driving the immune response toward a Th2-polarized non-protective state.

Key words: CD30 ligand; CD30; Interleukin-4; T-helper 2 cells

1. Introduction

The tumor necrosis factor (TNF)/TNF receptor superfamilies have been shown to play a pivotal role in the regulation of cellular growth, differentiation and programmed cell death in several biologic systems, including the immune compartment [1,2]. In addition to well known members, TNFRs, CD40, CD30, CD95, OX40, 4-1BB and their respective ligands [1,2], new related receptors and ligands have been more recently identified, such as RANK and its specific ligand, RANKL, and TRAIL with its three related receptors, TRAIL-R1, 2, 3 [1,2].

We have previously shown that CD30L [3] is frequently expressed in human hemopoietic malignancies of myeloid origin, being detectable on tumor cells from about 60% of patients with acute myeloid leukemia (AML) [4]. Expression of CD30L on AML blasts is statistically associated with some unfavorable clinical features, including hyperleukocytosis, high absolute numbers of circulating blasts and thrombocytopenia [4], thus suggesting the involvement of CD30L in determining a selective enhancement of leukemic blasts growth. Subsequently, we found [5,6] that leukemic blasts presenting surface CD30L possess a characteristic cytokine receptor pattern, that makes them ideal targets for T-helper 2 (Th2)-type cytokines [7–9]. Moreover, CD30L$^+$ AMLs are associated with an expansion of specific Th2 cell subsets producing interleukin-4 (IL-4) and expressing the CD30L counter-receptor, CD30 [5,6]. Since IL-4 enhances in vitro proliferation of CD30L$^+$ leukemic blasts [5,6], our data indicates that the selective interaction of CD30$^+$ Th2 cells with leukemic progenitors may contribute to the progression of this peculiar subset of AMLs.

In the present study, we demonstrate the capability of CD30L to up-regulate expression of surface CD30, release of soluble (s) CD30 and production of IL-4 in pre-activated T cells upon co-culture. This data demonstrates a direct role of CD30L in favoring the expansion of Th2 cell subsets.

2. Materials and methods

2.1. Cell lines and culture conditions

The CD30L-expressing Burkitt lymphoma cell line DG-75 was obtained through the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany); the QT6 quail fibroblast cell line was kindly provided by G. Manfioletti (University of Trieste, Italy). Cell lines were kept in Iscove's modified Dulbecco's medium (Biochrom KG, Germany) supplemented with 20% fetal calf serum (Biochrom KG).

2.2. Monoclonal antibodies (mAbs) and flow cytometry

Expression of CD3, CD30 and CD30L in transfected QT6 fibroblasts, resting or activated T cell and AML samples were analyzed by single or two-color immunofluorescence methods, essentially as reported [4]. Sources and specificities of mAbs and control immunoglobulins have been reported in detail previously [4,10]. Viable, antibody-labeled cells were identified according to their forward and side scatter, electronically gated and assayed for surface fluorescence on a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA, USA).

2.3. RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA (1 μg) was extracted by the guanidium thiocyanate method [11] and reverse-transcribed by avian myeloblastosis virus RT (Promega Co., Madison, WI, USA), as described [4]. A correct evaluation of the amount of cDNA was guaranteed by a careful check of first-strand synthesis through a competitive PCR for the housekeeping gene β_2-microglobulin [12]. Two microliters of cDNA were amplified in a 50-μl volume of final reaction mix in a PTC150 thermal cycler (MJ Research, Watertown, MA, USA) with 25 pmol of primer pairs specific for CD30 and IL-4. PCR conditions were as follows: 3 min at 94°C followed by 40 cycles of 45 s at 94°C, 45 s at 60°C (62°C for CD30), 1.5 min (1.0 for CD30) at 72°C, and a final extension of 5 min at 72°C. Ten-microliter aliquots of amplified products were removed after 30, 35 or 40 cycles, run on 1.5% ethidium bromide-stained agarose gels and separately analyzed under UV light.

2.4. Preparation of QT6/CD30L transfectant

cDNA encoding full-length sequence of CD30L was obtained from

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the DG-75 cell line [14] upon amplification with the following primer pairs: a sense primer (5′-CTC GAG AAG CTT ACC ACC ATG GAC CCA GGG CTG CA-3′) annealing to the start codon of CD30L and tagged with the *Hin*dIII site (sequence underlined), and an antisense primer (5′-ACC GGT GGA TCC CTA AAG GCC AAG AGA AAC TGT-3′) recognizing the stop codon of CD30L and tagged with the *Bam*HI site (sequence underlined). PCR conditions were 3 min at 94°C followed by 35 cycles of 30 s at 94°C, 45 s at 57°C, 1.5 min at 72°C, and a final extension of 5 min at 72°C. The amplification product, following electrophoresis in 1.5% agarose gel, was excised from gel, purified using Qiaex II resin (Qiagen GmbH, Hilden, Germany) and cloned in TA-vectors (Invitrogen Corporation, San Diego, CA, USA) according to the manufacturers' guidelines. DNA from the resulting clone was digested with *Hin*dIII–*Bam*HI, purified and subcloned into a *Hin*dIII–*Bam*HI-cut pREP7 (Invitrogen Corporation, San Diego, CA, USA) plasmid. The resulting expression vector was employed to transfect QT6 quail fibroblasts (QT6/CD30L) by using a blend of non-liposomal lipids as transfection-enhancing reagent (FuGENE 6, Roche Bioscience, Palo Alto, CA, USA). The same cells transfected with the vector alone (QT6/pREP7) were prepared and utilized as controls. Expression levels of CD30L were measured by Northern blotting and/or flow cytometry. QT6 transfectants were fixed at day 2 post-transfection with 1% paraformaldehyde for 15 min, and utilized for co-culture experiments.

2.5. Northern blotting

Twenty micrograms per lane of total RNA, extracted from transfected QT6 and DG-75 cells, were size-fractionated on 1% agarose gels containing 6.7% formaldehyde, and subsequently blotted onto nylon membranes (Boehringer Mannheim, Mannheim, Germany) [10]. Filters were hybridized as described [10] with 1.0×10^6 cpm/ml of random primed-labeled CD30L probe (0.7-kb *Not*I cDNA fragment containing the entire coding region of human CD30L) [3]. After washings to a final stringency of 0.2× standard sodium cytrate and 0.1% SDS at 65°C, filters were exposed to XAR-5 films (Eastman Kodak, Rochester, NY, USA) at −80°C.

2.6. Co-culture experiments of $CD3^+$ T cells with QT6 transfectants

$CD3^+$ T cells ($0.5–1.0\times10^6$/ml) were purified from leukapheretic products of healthy donors by immunomagnetic beads conjugated with anti-CD3 mAbs [4,10] (Miltenyi Biotec, Italy), and co-cultured in 35-mm 6-well plates along with paraformaldehyde-fixed QT6/CD30L or QT6/pREP7 cells, in the presence of 1% PHA. After 48 h of incubation, T cells were collected and utilized for RNA studies or for two-color analysis in flow cytometry. Supernatants were harvested and utilized for determinations of IL-4 and sCD30 levels by specific sandwich ELISA (R&D System, Minneapolis, MN, USA, IL-4; Bender MedSystems Diagnostics GmbH, Wien, Austria, sCD30). To investigate the involvement of the CD30–CD30L pair in cell–cell interactions, in some cases, co-culture experiments were performed in the presence of an excess (100 μg/ml) of blocking anti-CD30 BerH2 mAb [13]. This mAb was chosen because it did not interfere with ELISA-specific mAbs, as demonstrated by almost superimposable standard curves for sCD30 ELISA assays (Fig. 1).

2.7. Co-culture experiments of autologous $CD3^+$ T cells with AML blasts

Blast cells from selected AML cases (three $CD30L^+$ and two $CD30L^-$ AMLs), diagnosed according to the French–American–British criteria [14], were purified by centrifugation on a Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) gradient, and separated from the autologous T-cell fraction as described above. Immunomagnetically purified autologous $CD3^+$ T cells were co-cultured as above, together with autologous paraformaldehyde-fixed $CD30L^+$ or $CD30L^-$ AML blasts, in the presence or in the absence of an excess of blocking anti-CD30 BerH2 mAb [13].

3. Results

3.1. Expression of CD30L by QT6 transfectants

Levels of recombinant CD30L in QT6/CD30L cells, as detected by flow cytometry (Fig. 2A) and Northern blot analysis (Fig. 2B), were fully comparable to those expressed by the $CD30L^+$ cell line DG-75 [4]. Conversely, no expression of CD30L was found in QT6 cells transfected with the empty vector (QT6/pREP7) (Fig. 2A,B).

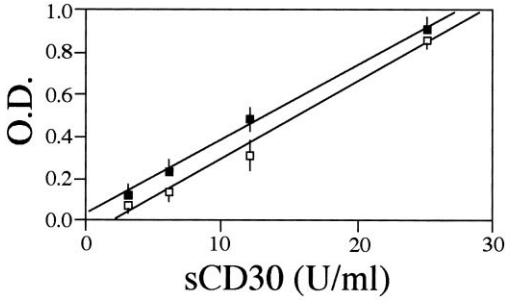

Fig. 1. Standard curves for sCD30 ELISA assays. Recombinant sCD30, provided by the manufacturer (Bender MedSystems Diagnostics), was diluted in serial two-fold steps starting from a concentration of 25 U/ml. Optical density (O.D.) values were obtained by reading absorbances on a spectrophotometer at a wavelength of 450 nm. Closed and open squares refer to curves carried out in the absence or in the presence of an excess (100 μg/ml) of the anti-CD30 mAb BerH2, respectively. Each experimental point represents the mean ± S.E.M. of triplicate measurements.

3.2. Recombinant CD30L up-regulates the expression of CD30 and IL-4 transcripts by T cells

To evaluate a direct role of CD30L in the development of Th2 cell subsets, paraformaldehyde-fixed QT6/pREP7 or QT6/CD30L cells were co-cultured for 48 h with purified T cells from healthy donors in the presence or in the absence of 1% PHA. As shown in Fig. 3, recombinant CD30L was able to modulate the expression of CD30- and IL-4-specific transcripts, as assessed by semi-quantitative RT-PCR. In this regard, while in unstimulated T cells CD30-specific mRNA was detectable only following 40 amplification rounds (lane 1), activation of PBMC by PHA yielded a significant increase of CD30 transcripts. In particular, CD30-specific amplified products became clearly detectable after 35 cycles in samples deriving from activated T cells co-cultured with QT6/pREP7 rounds (lane 2), or as low as 30 cycles when QT6/CD30L cells were employed (lane 4). A similar behavior was observed when the modulation of IL-4-specific transcripts was investigated (Fig. 3, lanes 5–8). Again, the highest levels of IL-4 mRNA were seen when PHA-activated T cells were co-cultured with CD30L-expressing QT6 cells (Fig. 3, lane 8).

3.3. Recombinant CD30L up-regulates the expression of surface CD30 protein by pre-activated T cells

The up-regulation of the expression of CD30 in activated T cells co-cultured with CD30L-bearing cells was confirmed at a protein level. As shown in Fig. 4, a 48-h co-culture of PHA-activated T cells with QT6/CD30L cells resulted in the up-regulation of surface CD30 (37% of double-stained cells), as compared to the same cells co-cultured in the presence of QT6 cells (QT6/pREP7) transfected with the empty vector (16% of double-stained cells). Conversely, no expression of surface CD30 was observed on $CD3^+$ T cells co-cultured with QT6 cells in the absence of PHA (data not shown).

3.4. Recombinant CD30L up-regulates the production of sCD30 and IL-4 in supernatants from $CD3^+$ T cells

In a third series of experiments, supernatants from 48-h co-cultures were collected and analyzed for IL-4 and sCD30 pro-

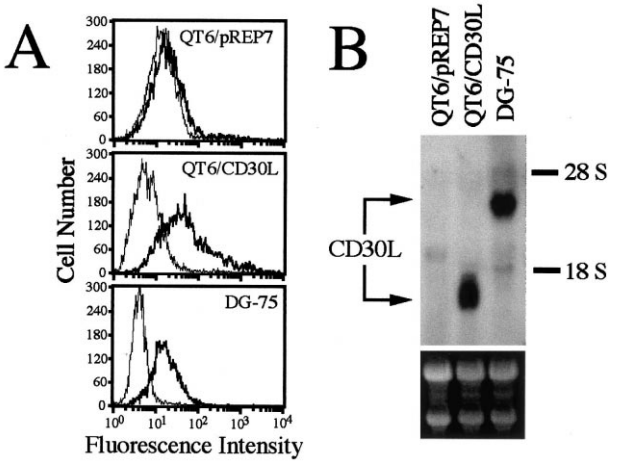

Fig. 2. Expression of recombinant CD30L by QT6 transfectants. A: Surface expression of CD30L on QT6 cells transfected with the vector alone (QT6/pREP7, upper panel), or containing the human full-length CD30L cDNA (QT6/CD30L, intermediate panel) and the CD30L$^+$ DG-75 cell line (DG-75, lower panel), as detected in flow cytometry by anti-CD30L mAbs (thick lines). Background fluorescence (thin lines) was determined by staining cells with an irrelevant isotype-matched (IgG2b) control mAb. B: Expression of CD30L gene in the same cells as above. A band corresponding to the entire open reading frame of the CD30L cDNA (726 bp) was documented in QT6/CD30L but not in QT6/pREP7 cells, while in the control cell line DG-75, a single specific transcript of about 3.0 kb, was observed.

duction (Fig. 5). No modulation was observed in the absence of PHA (Fig. 5). In supernatants deriving from PHA-treated co-cultures, a slight increase in the concentration of both sCD30 and IL-4 was observed. However, co-cultures of activated T cells with CD30L-transfected QT6 cells produced the

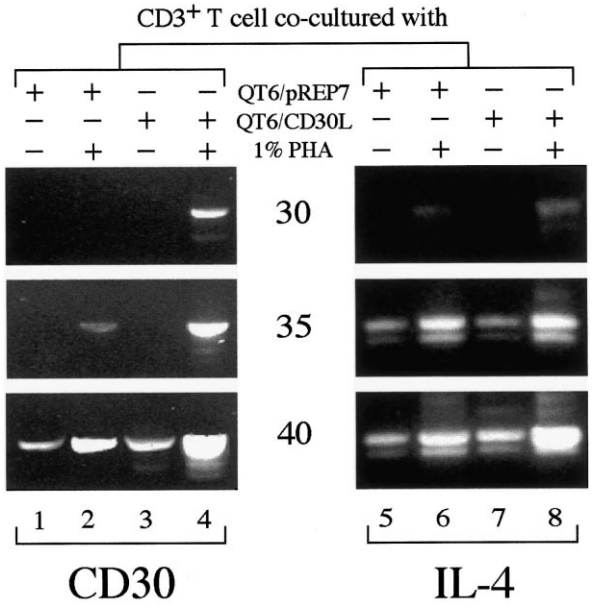

Fig. 3. Recombinant CD30L up-regulates the expression of CD30 and IL-4 mRNAs by purified CD3$^+$ T cells. Expression of CD30 (lanes 1–4) and IL-4 (lanes 5–8) mRNAs by CD3$^+$ T cells of healthy donors following co-culture with QT6/pREP7 or QT6/CD30L cells. T cells were co-cultured with paraformaldehyde-fixed QT6/pREP7 or QT6/CD30L cells, in the presence or in the absence of 1% PHA for 48 h of incubation. Amplifications were carried out as in Section 2.3.

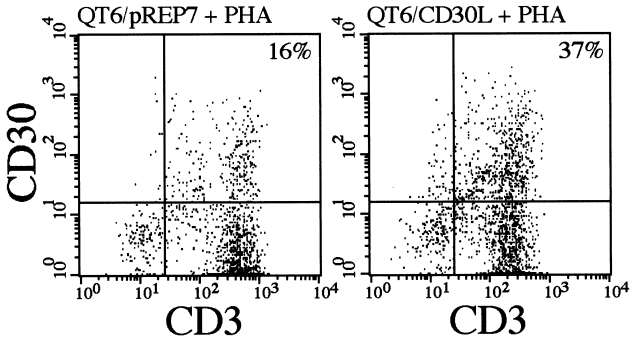

Fig. 4. Recombinant CD30L up-regulates the expression of surface CD30 protein by purified CD3$^+$ T cells. Two-color immunofluorescence showing the simultaneous expression of CD3 and CD30 on T cells co-cultured for 48 h with paraformaldehyde-fixed QT6/pREP7 or QT6/CD30L cells, in the presence of 1% PHA. Cells were stained by the anti-CD30 mAb BerH2 (*y*-axes, red fluorescence) and by anti-CD3 (*x*-axes, green fluorescence).

highest levels of sCD30 (7.21 ± 1.66 U/10^6 T cells) and IL-4 (51.75 ± 15.5 pg/10^6 T cells), as compared to supernatants deriving from co-cultures of activated T cells and QT6 cells transfected with an empty vector (sCD30 = 2.1 ± 1.28 U/10^6 T cells; IL-4 = 10.5 ± 2.9 pg/10^6 T cells). By blocking CD30/CD30L interactions by means of the anti-CD30 mAb BerH2, a striking down-regulation of both sCD30 (2.7 ± 0.8 U/10^6 T cells) and IL-4 (23.5 ± 7.0 pg/10^6 T cells) was observed in supernatants from co-cultures of QT6/CD30L and activated T cells (Fig. 5).

3.5. *CD30L$^+$ AML blasts up-regulate the production of sCD30 and IL-4 in supernatants from autologous CD3$^+$ T cells*

The production of sCD30 and IL-4 in supernatants derived from co-cultures of autologous pre-activated CD3$^+$ T cells with blast cells isolated from primary AMLs either positive (Fig. 6A) or negative for surface CD30L [5,6] was investigated. In agreement with data obtained with recombinant CD30L, a strong increase of both sCD30 (range: 15.4–25.0 U/10^6 T cells) and IL-4 (range: 79.7–98.6 pg/10^6 T cells) was documented in supernatants from co-cultures of PHA-treated autologous T cells and paraformaldehyde-fixed blasts from three cases of CD30L$^+$ monoblastic leukemia, but not from two CD30L$^-$ AML cases (Fig. 6B). Again, addition to the

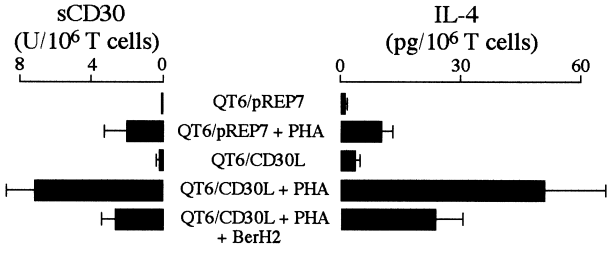

Fig. 5. Recombinant CD30L up-regulates the production of sCD30 and IL-4 by purified CD3$^+$ T cells. Detection of soluble sCD30 and IL-4 in supernatants of 48-h-old co-cultures by specific ELISA assays. CD3$^+$ T cells from healthy donors were co-cultured with paraformaldehyde-fixed QT6/pREP7 or QT6/CD30L, in the presence or in the absence of 1% PHA for 48 h of incubation. In some cases, co-cultures of QT6/CD30L and activated T cells were carried out in the presence of an excess (100 μg/ml) of the blocking anti-CD30 mAb BerH2 (QT6/CD30L+PHA+BerH2).

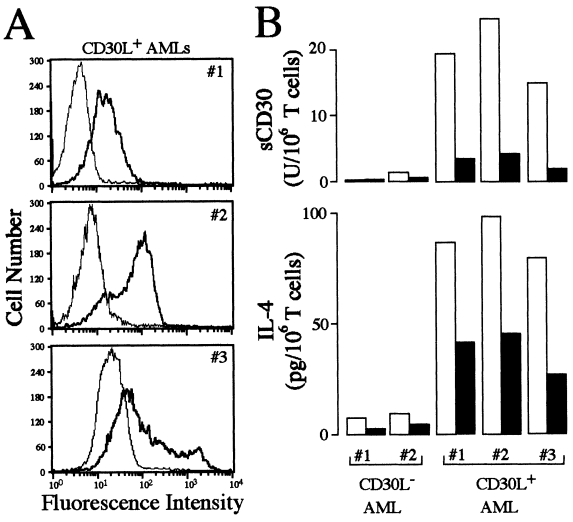

Fig. 6. CD30L$^+$ AML blasts up-regulate the production of sCD30 and IL-4 by purified CD3$^+$ T cells. A: Expression of CD30L on the surface of blast cells from three cases of CD30L$^+$ AMLs (#1, #2 and #3) of monocytic phenotype (FAB-M5) employed in autologous co-culture experiments, as detected in flow cytometry (thick lines). Background fluorescence (thin lines) was determined by staining cells with an irrelevant isotype-matched (IgG2b) control mAb. B: Autologous CD3$^+$ T cells, immunomagnetically purified from five AML samples (two CD30L$^-$ and three CD30L$^+$ AML cases), were co-cultured with their homologous paraformaldehyde-fixed AML blasts for 48 h in the presence of 1% PHA (open bars). The involvement of the CD30–CD30L pair in cell–cell interactions was investigated by performing co-cultures in the presence of an excess of blocking anti-CD30 BerH2 mAb (closed bars).

cultures of an excess of the blocking anti-CD30 BerH2 mAb [13] completely abolished the release of sCD30 (range: 1.8–4.1 U/10^6 T cells) and reduced the production of IL-4 (range: 27.0–45.9 pg/10^6 T cells) at about 50% (Fig. 6B).

4. Discussion

Leukemic blasts presenting surface CD30L [4] display a characteristic cytokine receptors pattern, including the exclusive expression of IL-4R in the absence of its cognate cytokine, that makes them ideal targets for those cytokines usually produced by Th2-type cell subsets [5,6]. Furthermore, the expression of the Th2-associated markers CD30, IL-4 and GATA-3 [7–9,15] was detected in residual T cells derived from CD30L$^+$ AMLs, but not from CD30L$^-$ AMLs [5,6].

Within the spectrum of hematological malignancies, a Th2-dominated phenotype by residual normal T cells has been recently reported in B-cell precursor acute lymphoblastic leukemia [16,17]. The expansion of T-cell clones expressing surface CD30, but producing both IL-4 and interferon-γ, was described also in the residual non-B-cell compartment of B-cell chronic lymphocytic leukemia (B-CLL) [18]. Interestingly, CD30L is expressed, although at different levels, in acute leukemias of lymphoid and myeloid origin, as well as in B-CLL [4–6]. Since the development of a polarized T-cell response largely depends on the characteristics of the antigen presenting cells [7–9,15], it is conceivable that the expression of CD30L on neoplastic cells may contribute to determine the skewing of the immune response toward a Th2 phenotype. In fact, transfectants expressing recombinant CD30L at a high surface density, comparable to that expressed by DG-75 cells [4] or found in CD30L^{high} AMLs [4–6], were able to drive the differentiation of purified CD3$^+$ T lymphocytes toward a Th2 phenotype. Consistent results were obtained in experiments carried out with fixed AML blasts expressing surface CD30L. The specificity of these findings were confirmed by the observation that addition of neutralizing anti-CD30 mAb [13] completely abolished the release of sCD30 and significantly reduced the production of IL-4.

Experiments with QT6 transfectants and autologous AML blasts, however, suggest that the expression of CD30L, although necessary, is not sufficient to determine a Th2 polarization. In fact, the presence of a T-cell mitogenic factor was required to attain a significant up-regulation of CD30 on CD3$^+$ T cells or to increase the release of sCD30 and IL-4. A number of soluble and/or membrane-bound factors, including IL-6, MCP-1 and CD86/B7-2, have been proposed to influence T-cell subsets commitment specifically tilting their development toward a Th2 phenotype [19]. Interestingly, MCP-1 is expressed, as CD30L [4], mainly by monocytic-oriented leukemic cells [20], CD86/B7-2 has been described on the surface of blast cells in almost one-third of AML cases [21], and CD40 triggering on CD30L$^+$ AMLs results in an increased production of endogenous cytokines, including IL-6 [22]. A model could be therefore depicted in which blast cell-derived cytokines (e.g. IL-6 or MCP-1) along with engagement of surface co-stimulatory molecules, such as CD86/B7-2, may contribute to the initial priming of T cells toward a Th2 phenotype, including expression of CD30. The polarized Th2 response could then be amplified and maintained through specific interactions of CD30$^+$ T cells with CD30L molecules expressed on AML blasts.

The selective production of IL-4 by CD30$^+$ T-cells may contribute to determine the proliferative and phenotypic features of IL-4R$^+$/CD30L$^+$ AMLs. In agreement with this line of reasoning, exogenous IL-4 is able to enhance the proliferation of myeloid leukemic blasts [6,23] and to modulate the membrane expression of some adhesion structures, such as β_2 integrins and CD54 (D. Aldinucci et al., unpublished data), whose presence is significantly associated with the expression of CD30L on leukemic cells [4].

Current paradigms indicate that AML cells evade the immune response mainly by lacking essential co-stimulatory signals for T-cell activation [24,25]. Data presented here implies that CD30L$^+$ neoplastic cells may have a direct role in driving the immune response toward a non-protective state, including a Th2 polarization. A better understanding of the mechanisms by which leukemic cells are able to drive the immune system may eventually lead to alternative therapeutic approaches aimed to favor the development of a protective anti-leukemic response.

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