Functional Alteration of the Lymphoma Stromal Cell Niche by the Cytokine Context: Role of Indoleamine-2,3 Dioxygenase

Hélène Maby-El Hajjami, Patricia Amé-Thomas, Céline Pangault, Olivier Tribut, John Devos, Rachel Jean, Nadège Bescher, Céline Monvoisin, Joëlle Dulong, Thierry Lamy, Thierry Fest, and Karin Tarte.

1Institut National de la Santé et de la Recherche Médicale U917, IFR140, Université Rennes 1, France; 2Pôle Cellules et Tissus, Laboratoire de Pharmacologie, and Service d’Hématologie Clinique, CHU Rennes, France; 3Institut National de la Santé et de la Recherche Médicale U847, CHU Montpellier, France; and 4EFS Bretagne, Rennes, France

Abstract

Human mesenchymal stem cells (MSC) strongly repress activated T-cell proliferation through the production of a complex set of soluble factors, including the tryptophan-catabolizing enzyme indoleamine 2,3-dioxygenase (IDO), which is induced by IFN-γ. Conversely, MSCs support survival of follicular lymphoma (FL) B cells, in particular after exposure to tumor necrosis factor-α (TNF) and lymphotxin-α1/2 (LT). The role of MSCs on normal and malignant B-cell growth in steady-state and inflammatory conditions remains to be fully explored. We show here that resting MSCs sustain activated normal B-cell proliferation and survival, whereas IFN-γ-conditioned MSCs mediate IDO-dependent B-cell growth arrest and apoptosis. IFN-γ, TNF, and LT are significantly overexpressed by the microenvironment of invaded FL lymph nodes, but their relative expression patterns are highly heterogeneous between samples. In vitro, IFN-γ abrogates the B-cell supportive phenotype induced by TNF and LT on MSCs. Moreover, IFN-γ overrules the growth promoting effect of MSCs on primary purified FL B cells. Altogether, these results underline the crucial role of the cytokine context in the local crosstalk between malignant cells and their microenvironment and provide new insights into our knowledge of the FL cell niche that emerges as a new promising target for innovative therapeutic strategies.

Introduction

Multipotent mesenchymal stromal cells, also referred as mesenchymal stem cells (MSC), represent a rare and heterogeneous population of nonhematopoietic cells without specific phenotypic marker but sharing the functional capacities of self-renewal and differentiation along multiple mesodermal lineages including bone, fat, and cartilage. Although MSCs were originally identified in the bone marrow, where they contribute to the formation of the hematopoietic stem cell niche, they have also been isolated from other tissues, including adipose tissue, amniotic fluid, fetal tissues, or umbilical cord blood. Several studies have recently suggested that adoptively transferred MSCs could favor tumor engraftment and progression in vivo. This deleterious effect could be associated with at least three major characteristics. First, MSCs specifically migrate toward sites of active tumorigenesis and genetically manipulated MSCs have been successfully exploited to deliver antitumor therapeutic molecules. Second, MSCs could integrate the specialized tumor niche where they contribute to the development of tumor-associated fibroblasts, stimulate angiogenesis, and promote malignant cell growth, cancer metastasis, and drug resistance. Finally, an emerging body of data indicates that human MSCs elicit pleiotropic immunoregulatory properties both in vitro and in vivo. In particular, they suppress CD4+ and CD8+–activated T-cell expansion through the release of soluble factors produced after a dynamic crosstalk between MSCs and T-lymphocytes. As yet, the mechanisms underlying MSC-mediated T-cell suppression remain a highly controversial issue but IFN-γ is proposed as a prime candidate for the initiation of MSC-mediated immunoregulation. Upon stimulation with IFN-γ, MSCs express the tryptophan-catabolizing enzyme indoleamine 2,3-dioxygenase (IDO; ref. 13), a key immunosuppressive effector pathway in peripheral T-cell tolerance. Conversely, transforming growth factor-β, hepatocyte growth factor, HLA-G, and prostaglandin E2 have all been proposed as potential mediators of MSC-related inhibition of T-cell proliferation.

Regarding interaction of human MSCs and normal B cells, data are scarce and confusing, with some studies suggesting that B-cell proliferation and functions are dampened by MSCs per se, whereas others report that MSCs only affect activated B-cell growth in the presence of IFN-γ. In contrast, MSCs have also been described as promoting normal B-cell survival and differentiation. These discrepancies may be explained, at least in part, by MSC plasticity and/or by the presence of exogeneous factors in culture, such as IFN-γ or tumor necrosis factor-α (TNF). How MSCs alter B-cell behavior remains completely unknown.

Within secondary lymphoid organs, two mesenchymal-derived stromal cell subsets collaborate to drive mature B-cell migration, proliferation, selection, and differentiation: fibroblastic reticular cells in the paracortex, and follicular dendritic cells in germinal centers. We recently showed that bone marrow–derived MSCs, as well as resident MSCs isolated from SLO, give rise, in the presence of TNF and lymphotxin-α1/2 (LT), to fully competent fibroblastic reticular cells able to recruit non–Hodgkin’s lymphoma B-cells and to sustain their survival. This stromal cell–dependent antiapoptotic effect has been further extended to chemotherapy induced malignant B-cell death. Furthermore, animal and human studies corroborate the migration of MSCs into SLO in inflammatory conditions. Collectively, these data suggest that MSCs and their medullar and
SLO-related progeny could variably modulate normal and lymphoma B-cell survival and growth, depending on the environmental context. This could be of particular importance in follicular lymphoma (FL), the most frequent indolent non–Hodgkin’s lymphoma, which is generally a disseminated disease with initial involvement of lymph nodes and bone marrow (24, 25). IFN-γ and TNF have been detected in FL-invaded lymph nodes using in situ hybridization or PCR experiments (26, 27), but the reciprocal role of these two cytokines on the bidirectional crosstalk between malignant cells and their stromal microenvironment has not been considered.

The focus of this study is to fully clarify the role of MSCs both on normal and malignant B-cell survival and proliferation. In particular, we aim to identify the molecular signals involved in the interactions between MSCs and lymphoma B cells either in steady-state condition, or in the presence of the cytokines commonly found within SLO during inflammation and lymphomagenesis.

Materials and Methods

Cell samples. Subject recruitment followed institutional review board approval and written informed consent process according to the Declaration of Helsinki.

MSCs were obtained and characterized as previously described starting from human adult bone marrow aspirates (20). Primary B cells were isolated from human tonsils collected from children undergoing routine tonsillectomy and from lymph node biopsies collected from patients with FL without any feature of transformation. B cells were purified as the unbound fraction of magnetic cell sorting using the B-cell isolation kit II (Miltenyi Biotech). Purity was >99% CD19<sup>+</sup> B cells. In addition, B cells purified from FL samples were further depleted of cells expressing the nontumor light chain isotype, using purified from FL samples were further depleted of cells expressing the nontumor light chain isotype, using λ (Beckman Coulter) or λ (Becton Dickinson) light chain monoclonal antibodies (mAb) and goat anti-mouse IgG microbeads (Miltenyi Biotech). Purity was consistently >99% CD19<sup>+</sup> B cells expressing the malignant isotype. BL2 and RL lymphoma cell lines were grown in RPMI1640 with 10% fetal bovine serum (FBS).

MSC stimulation. Confluent monolayers of MSCs were treated or not for 3 d with increasing concentrations of IFN-γ (R&D Systems) or IFN-α2a (Roferon-A). In some experiments, a cotreatment of MSCs with IFN-γ and TNF-α (10 ng/mL), or with TNF-α and LT-α1/2 (100 ng/mL; R&D Systems), was performed. For coculture experiments, MSCs were extensively washed before addition of B cells.

B-cell growth assays. Tonsil B cells (750 × 10<sup>3</sup> cells/mL) were activated by a T-cell–dependent stimulation cocktail including 50 ng/mL CD40L, 5 µg/mL enhancer polyhistidine mAb, 50 ng/mL interleukin (IL)-4 (R&D Systems), and 20 IU/mL IL-2 (Proleukin), or by a T-cell–independent stimulation cocktail comprising 10 ng/mL IL-15 (R&D Systems), 2.5 µg/mL CpG oligodeoxynucleotide 2006 (InVivoGen), and 2 µg/mL F(ab')<sub>2</sub> anti-human IgM/IgA/IgG (Jackson ImmunoResearch). After a 3-d activation was performed. For coculture experiments, MSCs were extensively washed (Roferon-A). In some experiments, a cotreatment of MSCs with IFN-γ (Invitrogen) before activation by the T-cell–dependent or T-cell–independent stimulation cocktail comprising 10 ng/mL IL-15 (R&D Systems), 2.5 µg/mL F(ab')<sub>2</sub> anti-human IgM/IgA/IgG microbeads (Miltenyi Biotech). Purity was consistently >99% CD19<sup>+</sup> B cells. After 3 d, cells were pulsed with tritiated thymidine (3H-TdR; Sigma) and the ModFit software (Verity Software). MSCs cultured alone always showed 3H-TdR incorporation of <500 cpm.

B-cell proliferation and apoptosis. BL2 (25 × 10<sup>3</sup> cells/mL) were cultured alone or in the presence of MSCs pretreated or not with 500 IU/mL IFN-γ. After 3 d, cell cycle was studied on CD45<sup>+</sup> B cells using propidium iodide staining (Sigma) and the ModFit software (Verity Software).

Normal tonsil and FL B cells were stained with 1 µmol/L CFSE (Invitrogen) before activation by the T-cell–dependent or T-cell–independent stimulation cocktail, in the presence or not of MSCs pretreated or not with IFN-γ. After 3 d, CFSE<sup>+TOPRO-3</sup> viable B cells were analyzed. For tonsil B cells, six individual peaks and corresponding generations (G0-G5) were identified using the ModFit software. For FL B cells, the absolute number of CFSE<sup>+</sup> proliferating cells was evaluated using FlowCount beads.

B-cell apoptosis was analyzed using the phycoerythrin-conjugated active caspase-3 apoptosis kit (Becton Dickinson) on CD45<sup>+</sup> B cells.

IDO expression and activity assessment. RNA was extracted from MSCs using RNeasy kit (Qiagen) and cDNA generated using Superscript II reverse-transcriptase (Invitrogen). For quantitative-PCR, we used assay-on-demand primers and probes, and the Taqman Universal MasterMix from Applied Biosystems. Gene expression was measured using the ABI Prism 7700 (Applied Biosystems). For each sample, Ct values for INDO and GAPDH were determined, normalized to their respective value for the internal standard gene 18S, and the resulting ΔCt were compared with that obtained for unstimulated cells.

For Western blot analysis, after lysis in radioimmunoprecipitation assay lysis buffer, 10 µg of proteins were loaded on 12% to 15% SDS-polyacrylamide gels and transferred onto a polyvinylidene difluoride membrane (Millipore). Membranes were blocked with 5% nonfat dry milk in PBS-Tween, and incubated with rabbit anti-human IDO ( Alexis) or glyceraldehyde-3-phosphate dehydrogenase (Cell signaling) mAb, followed by horseradish peroxidase–conjugated goat anti-rabbit secondary Ab (Bio-Rad). Visualization was performed by a chemiluminescence protocol (ECL plus; GE Healthcare).

Final IDO activity was evaluated by measuring kynurenine concentration in culture supernatants by high performance liquid chromatography (28) using 3-nitro-l-tyrosine as an internal standard. Kynurenine and 3-nitro-l-tyrosine were detected by UV absorption at 360 nm.

Inhibition of IDO. MSCs were electroporated with 40 nmol/L IDO-1 or negative control siRNA (Ambion) using the Human MSC kit (Ambaxa) and cultured for 24 h in expansion medium before treatment with IFN-γ. MSCs supernatants were then collected for kynurenine quantification, and MSCs were extensively washed before addition of BL2. In addition, BL2 cell growth and kynurenine level were evaluated in the presence of 1 µmol/L of l- or D-1 methyltryptophan (Sigma).

Microarray experiments. Lymph node biopsies from 17 FL patients at primary diagnosis (8 grade I and 9 grade II) and 5 nonmalignant inflamed lymph nodes were collected. CD19<sup>+</sup> B cells were obtained by positive selection using anti-CD19 microbeads (Stem Cell Technologies; purity=94.5%). CD19<sup>+</sup>CD22<sup>+</sup> non-B cells were further enriched from the unbound fraction by a second round of negative selection using anti-CD22 microbeads (Miltenyi). RNA were extracted, quality controlled on a BioAnalyzer 2100 (Agilent Technologies), and biotinylated cDNA were amplified according to the Affymetrix small sample labeling protocol and hybridized to HGU133 Plus 2.0 arrays (Affymetrix). Raw data were analyzed using ArrayAssist (Stratagene). The list of the probesets differentially expressed between FL and normal non–B cells is available at the Resmed Web site<sup>7</sup> (FL microenvironment signature). In addition, the Top 50 genes up-regulated in FL microenvironment compared with normal microenvironment were underlined in Supplementary Table. Expression of IFNγ, TNF, and LTA was validated by quantitative-PCR using appropriate assay-on-demand and ABL as internal standard gene.

Statistical analyses. Statistical analyses were performed with Prism software (GraphPad software) using the Wilcoxon or Student's t test for matched pairs or using Mann-Whitney nonparametric U test (*, P < 0.05; **, P < 0.01; ***, P < 0.001).

Results

MSCs favor normal B-cell proliferation and survival but this supportive effect is abolished by IFN-γ. As previously established for malignant B cells (20, 21), spontaneous apoptosis of

<sup>7</sup>http://resmed.univ-rennes1.fr/micareviewers/
normal purified tonsil B cells was delayed by coculture with MSCs (data not shown). To further explore the role of MSCs on B-cell behavior, we took advantage of two well-defined in vitro stimulation systems matching our current knowledge of normal B-cell biology and mimicking, respectively, a T-cell–dependent and a T-cell–independent B-cell activation.

Normal B cells activated through CD40 engagement exhibited a strong proliferation as evaluated by dilution of the proliferation marker CFSE (Fig. 1A). In the presence of MSCs, T-cell–dependent B-cell proliferation was enhanced, and a significant increase of the percentage of viable B cells that have undergone two or more divisions was detected. Concomitantly, MSCs significantly reduced activated B-cell apoptosis, as assessed by active caspase-3 staining (8.7% ± 2.9% without MSCs versus 4.3% ± 2.9% with MSCs; P < 0.01; Fig. 1B). Similarly, MSCs amplified the proliferation of normal B cells stimulated with a combination of TLR9- and...
Importantly, after washing of IFN-γ-CD45posCaspase-3pos cells with IFN-γ-enhanced activated B-cell apoptosis, in particular, in the context to 100 IU/mL (Supplementary Fig. S1). The IFN-γ had no direct effect on normal B-cell proliferation and survival up to 500 IU/mL for BL2 and 500 IU/mL for RL (data not shown), thus excluding a direct cytotoxic effect of this cytokine. Given the well-known interest of IFN-α for the maintenance therapy of patients with lymphomas (29), we investigated the action of IFN-α on the B-cell supportive properties of MSCs. In our culture conditions, IFN-α pretreated MSCs had no suppressive effect on BL2 cell growth, whereas they only faintly inhibited RL proliferation (14.5% ± 2.6%; P < 0.05) after treatment with the highest concentration of IFN-α (Fig. 1B). This is in agreement with the sensitivity of RL to the direct cytotoxic effect of very low doses of IFN-α that could persist after MSCs washing (inhibition of RL thymidine incorporation at 4 IU/mL of IFN-α: 18.3% ± 4.7%; P < 0.05; data not shown). In conclusion, IFN-γ specifically induced a lymphoma B-cell inhibitory phenotype on MSCs and this indirect antitumor effect is not shared by IFN-α.

IDO is involved in the IFN-γ–mediated B-cell suppressive phenotype of MSCs. Several reports have suggested that human MSCs could produce functional IDO in response to IFN-γ stimulation (13), but these data remain controversial (15). Because tryptophan catabolism was associated with an inhibition of T- and natural killer (NK) cell proliferation, we investigated IDO status in our MSCs. Whereas IDO was not constitutively produced by MSCs, its expression and its enzymatic activity were simultaneously induced by IFN-γ, unlike IFN-α, in a dose-dependent manner (Fig. 3A). IDO siRNA strongly abrogated inducible IDO expression both at transcriptional and protein levels (Fig. 3B–C), without modulating the mRNA level of GAPDH (data not shown). Of note, neither IDO nor negative control siRNA electroporation significantly altered the survival and growth of MSCs.

We then investigated whether IDO blockade could reverse the B-cell inhibitory effect of IFN-γ–treated MSCs. Indeed, IDO-specific down-regulation resulted in a marked reduction of kynurenine production by MSCs in response to IFN-γ that was correlated to a restoration of BL2 cell growth (Fig. 3D). In addition, L-1 methyltryptophan, a specific IDO-1 inhibitor, could also reverse both the inhibition of BL2 cell growth (84.8% ± 34.6% of reversion; P < 0.001) and the induction of kynurenine release (72.6% ± 7.4% of reversion; P < 0.0001) mediated by IFN-γ–conditioned MSCs. Conversely, D-1 methyltryptophan, the preferred IDO-2 inhibitor (30), had no significant effect on IDO activity or B-cell suppression in this context. Although proliferation arrest mediated by tryptophan depletion represents a critical component of the tolerogenic function of IDO, tryptophan metabolites could also promote T-cell death (31). As depicted for tonsil B cells, IFN-γ treatment of MSCs simultaneously induced a blockade of cell cycle and a strong apoptosis of BL2 cell line (Fig. 4). Interestingly, specific extinction of IDO-1 restored BL2 proliferation and survival.
Collectively, these results suggested that the inhibition of lymphoma cell growth associated with IFN-γ treatment of mesenchymal cells was due, at least in part, to the induction of functional IDO-1.

**IFN-γ abrogates the B-cell supportive phenotype induced by TNF and LT on MSCs.** Reciprocal expression of TNF, LT, and IFN-γ within lymphoma biopsies has not been reliably assessed. Our microarray experiment showed that IFNG, TNF, and LTA were highly overexpressed in FL microenvironment, whereas only TNF was significantly up-regulated in malignant B cells (Fig. 5A). TNF and LTA expression levels were correlated among the whole data set ($r = 0.69; P < 0.0001$), suggesting common regulatory mechanisms. Conversely, IFNG expression was not correlated to that of TNF or LTA and the ratio TNF/IFNG varied from 0.7 to 10.8 in FL microenvironment (median, 3.9; $n = 9$). These results were confirmed by quantitative-PCR (Fig. 5A). Because the three cytokines were variably present within FL tumors, we next sought to explore their combined effect on the crosstalk between malignant B cells and MSCs. TNF and LT strongly reinforced the B-cell inhibitory effect of IFN-γ pretreated MSCs in optimal B-cell

**Figure 3.** IFN-γ–dependent malignant B-cell inhibition is associated to the induction of functional IDO in MSCs. A, induction of IDO in MSCs. MSCs were cultured with increasing concentrations of IFN-γ or IFN-α before analysis of INDO expression by quantitative-PCR (left) and IDO activity by measurement of kynurenine concentration in MSCs supernatants (right). For PCR experiments, results were compared with expression levels in untreated (UT) MSCs. The arbitrary value of 1 was assigned to UT MSCs. INDO was not detected in UT MSCs and the ΔΔCt was calculated with an arbitrary Ct value of 40. Statistical analyses compared INDO expression in IFN-γ–conditioned MSCs to INDO expression in UT MSCs ($n = 3$), and kynurenine concentrations in supernatants obtained from MSCs stimulated with IFN-γ or IFN-α to kynurenine concentration obtained from UT MSCs (IFN-γ, $n = 4$; IFN-α, $n = 3$). B, PCR quantification of INDO expression after gene silencing. MSCs were transfected with negative control (NEG) or IDO-1 siRNA and thereafter treated or not with 100 or 500 IU/mL IFN-γ. To assess the efficiency of IDO silencing, INDO expression was analyzed by quantitative-PCR. Each result was compared with expression levels in UT MSCs; one representative experiment of three. C, expression of IDO protein. MSCs were transfected with NEG or IDO-1 siRNA and thereafter treated or not with 500 IU/mL IFN-γ. Visualization of IDO protein was performed by Western blot using GAPDH as a loading control (MSC-1 and MSC-2 represent MSCs from two independent donors). D, relationship between IDO activity and BL2 cell growth. MSCs were transfected with NEG or IDO-1 siRNA and treated or not with 100 or 500 IU/mL IFN-γ (left) or were treated with L-1 methyltryptophan or D-1 methyltryptophan in the presence or not of 500 IU/mL IFN-γ (right). Kynurenine concentration was evaluated after 3 d (dotted line). BL2 cell line was then added to the MSC monolayer and cell growth was quantified by 3H-TdR incorporation. Data are expressed relatively to 3H-TdR incorporation of BL2 cocultured with MSCs not treated by IFN-γ and either transfected with NEG siRNA or cultured without 1MT (right; assigned to 100%). Results are those of one representative experiment of three (left) or eight (right).
culture conditions, i.e., 10% FBS (Fig. 5B). In agreement, whereas TNF alone was unable to promote IDO activity, stimulation of MSCs by TNF strongly potentiated the inducer effect of low dose of IFN-γ (Fig. 5C). As previously reported, serum deprivation-induced growth arrest of BL2 was significantly reversed by coculture with untreated MSCs and preliminary treatment of MSCs by TNF/LT further improved neoplastic B-cell growth (20). In these suboptimal culture conditions, IFN-γ abolished the growth-promoting capacity of MSCs mediated by TNF/LT in a dose-dependent manner (Fig. 5D). Overall, these data showed that the B-cell inhibitory phenotype of MSCs induced by IFN-γ was reinforced by TNF/LT that, conversely, promoted a B-cell feeder phenotype on MSCs only in the absence of high levels of IFN-γ.

**IFN-γ overrules the positive effect of MSCs on primary FL B-cell proliferation.** Primary FL B cells are poorly proliferating in vitro. Therefore, we decided to undertake proliferation assay on purified FL B cells activated through our T-cell–dependent stimulation cocktail comprising CD40L and IL-4, two mitogenic
factors for neoplastic mature B cells (Fig. 6; refs. 32, 33). Under these conditions, a small but significant fraction of FL cells were committed to cell division (2.5% ± 1.5% with stimulation versus 1.3% ± 0.7% without stimulation; P < 0.05). In addition, MSCs significantly enhanced the proliferation of activated B cells and the percentage of CFSElow viable malignant B cells was increased to a mean of 4.5% with MSCs (range, 0.8–8.4%). Interestingly, pretreatment of MSCs with IFN-γ abrogated this proliferative effect (2.8% ± 2% for FL cells cocultured with IFN-γ–conditioned MSCs).

To further compare the effect of TNF/LT versus IFN-γ on the crosstalk between malignant B cells and MSCs, we selected cells from 5 FL patients that displayed a more pronounced CD40L-dependent B-cell growth (Fig. 6B). We first confirmed the additional stimulatory effect of T-cell–dependent stimulation and MSCs (P < 0.05), whereas TNF/LT pretreatment of MSCs had no supplemental effect on these fully activated malignant B cells. More importantly, TNF/LT potentiated the B-cell inhibitory phenotype induced by IFN-γ on MSCs (P < 0.05 for MSCs versus IFN-γ–pretreated MSCs, and for IFN-γ–pretreated MSCs versus IFN-γ/TNF/LT–pretreated MSCs).

**Discussion**

Accumulating evidence has suggested that human MSCs are potent modulators of T-cell activation and proliferation through the production of poorly defined factors including the tryptophan-degrading enzyme IDO (8, 9). However, the influence of MSCs on resting and activated normal B cells seems exceedingly more complex, probably depending on the microenvironment in which the interaction takes place. In addition, the role of IDO in this context has never been explored. We first confirm, as recently recorded for naive and memory peripheral blood B cells (18), that MSCs favor tonsil B-cell growth by promoting cell proliferation and survival. MSCs were reported to similarly protect lymphoma B-cell lines from cell death induced by serum

Figure 5. IFN-γ abolishes the malignant B-cell supportive effect of MSCs stimulated with TNF-α and LT-α. A, relative expression of IFNG, TNF, and LTA in CD19pos B cells and CD19negCD22neg non–B cells isolated from FL and nonmalignant lymph nodes. Samples were analyzed on Affymetrix microarrays (top). FL_Env and NEG_Env: CD19negCD22neg fractions from FL and nonmalignant lymph nodes, respectively. FL_B and NEG_B: CD19pos fractions from FL and nonmalignant lymph nodes, respectively. Results obtained for FL_Env and NEG_Env were validated by quantitative-PCR (bottom). For each sample, the ΔCt value for the gene of interest was compared with the mean value obtained for NEG_Env. B, effect of MSCs on BL2 cell growth in optimal culture conditions. MSCs were costimulated with increasing concentrations of IFN-γ with or without TNF-α + LT-α (TNF+LT). MSCs were then washed and BL2 were added in RPMI1640/10% FBS. Three days later, cells were pulsed with 3H-TdR (n = 4). Data are expressed relatively to the value of 3H-TdR incorporation obtained with BL2 cells cocultured with unstimulated MSCs (assigned to 100%). C, synergy of IFN-γ and TNF-α in induction of functional IDO. Kynurenine concentrations were measured in supernatants of MSCs treated or not for 3 d with 20 IU/mL IFN-γ and/or 10 ng/mL TNF-α (n = 5). D, effect of MSCs on BL2 cell growth in suboptimal culture conditions. MSCs were stimulated with or not with TNF+LT and increasing concentrations of IFN-γ. MSCs were then washed and BL2 cells were added in RPMI 1640/1% FBS. Three days later, cells were pulsed with 3H-TdR (n = 4). Data are expressed relatively to the value of 3H-TdR incorporation obtained with BL2 cells cocultured with MSCs stimulated only with TNF+LT (assigned to 100%).
deprivation or drugs and to improve survival of primary FL B-cells in vitro (20, 21). Several mechanisms such as activation of nuclear factor-κB or hedgehog signaling pathways have been proposed to account for the antiproliferative effect of stromal cells on neoplastic B cells (21, 34). Conflicting results were previously advanced to support the opposite assertion that MSCs are intrinsically able to suppress normal B-cell proliferation and differentiation (16). However, this contradiction could be linked to the unexpected detection by the investigators of a high proportion of IFN-γ-secreting B cells, whereas we never detected IFN-γ synthesis by highly purified tonsil B-cells. The significance of this discrepancy becomes obvious when considering that MSCs pretreated by IFN-γ became powerful inhibitors of activated B-cell growth. Interestingly, dendritic cells exposed to IFN-γ before injection acquire therapeutic potential in an experimental autoimmune myasthenia gravis rat model, in association with an IDO-dependent inhibition of B-cell function in vivo (35).

In agreement, our results provide the first direct demonstration that IDO activity is critical for B-cell suppression induced by IFN-γ–conditioned MSCs. In a wide range of human tumors, malignant cells constitutively express functional IDO, as a common mechanism to escape T cell–related antitumor immunity (36, 37). In agreement with their sensitivity to tryptophan deprivation, we never detected IDO expression or activity in lymphoma B cells, even after treatment with IFN-γ. IDO was recently detected in tumor endothelial cells of renal cell carcinoma in association with a reduced proliferation of malignant cells and a longer survival (38). These results indicate that the role of IDO in tumor-host interface is probably more complex than anticipated. Interestingly, IFN-γ–dependent IDO-mediated B-cell inhibition was shared by tonsil-derived stromal cells (data not shown), in line with the recent demonstration that mature stromal cells from several tissues suppress T-cell proliferation through IDO activity (39, 40). Finally, a siRNA designed to target specifically IDO-1 was sufficient to abrogate tryptophan catabolism in MSCs that expressed only very low level of IDO-2 mRNA, even after stimulation by IFN-γ (Supplementary Fig. S2). These results suggest that, as recently described for human dendritic cells (41), IDO activity is provided by IDO-1 in human MSCs. Additional experiments using tryptophan excess and tryptophan metabolites would be helpful to address the respective role of tryptophan withdrawal versus immunosuppressive catabolites.

Besides IFN-γ, IFN-α could also regulate IDO activity in human monocytes (42). In addition, a series of randomized clinical studies suggest that IFN-α2 prolongs survival and remission duration in patients with FL (29). However, IFN-α2–conditioned MSCs did not alter malignant B-cell proliferation or survival and the lack of detectable IDO activity in stromal cells treated by IFN-α2 lends further credence to the implication of IDO in MSCs–mediated B-cell inhibition. Therapeutic approaches using IFN-γ were recently suggested to be clinically effective in advanced primary cutaneous lymphomas (43). Whether these strategies could be proposed to a broader panel of patients remains to be explored. Of note, IFN-γ was already used in several solid tumors for its capacity to activate a wide range of immunocompetent cell subsets as well as for its direct cytotoxic effect on malignant cell growth. In lymphoma, the role of the dense tumor-infiltrating stromal cell meshwork should be taken into account when considering the pleiotrophic effects of IFN-γ as a potential therapeutic option.

Previously, we showed that TNF and LT collaborate in vitro to strengthen the capacity of MSCs to support malignant B-cell growth (20). TNF was proposed to work in synergy with small amounts of IFN-γ to make fibroblast-like synoviocytes able to efficiently sustain normal B-cell survival (44). A murine study suggested conversely that TNF could reverse the suppression of T-cell proliferation induced by MSCs (45), acting in this context as an IFN-γ antagonist. Low concentrations of IFN-γ did not further increase, in our current study, the capacity of MSCs treated with TNF and LT to protect lymphoma B-cell lines from serum deprivation–induced cell death. Increasing IFN-γ doses even completely abolished stromal cell feeder effect. Likewise, the TNF/LT combination synergized with IFN-γ to arrest B-cell growth.
in optimal culture conditions in agreement with the synergy between IFN-γ and TNF for the induction of IDO activity in human MSCs, as previously described in epithelial cells (46).

Using microarray and quantitative-PCR experiments, we showed that nonmalignant cells in FL biopsies overexpressed TNF, LTA, and IFNG. The influence of the relative concentrations of IFN-γ versus TNF/LT on stromal cell behavior in vivo remains to be elucidated, as well as the reciprocal localization of cytokine-producing cells, responding stromal cells, and malignant B cells. IFNG expression was significantly correlated to that of GRZA, GRZB, and CD8A, suggesting that cytotoxic cells could play a key role in FL not only through a direct effect on malignant cells but also through an indirect effect on stromal cells. In agreement, after in vitro stimulation, IFN-γ is mainly produced by CD3posCD56neg T cells and CD3negCD56pos NK cells within normal and malignant lymphoid organs (data not shown). Interestingly, gene expression and immunohistochemical studies have recently generated a huge amount of data that converge to support a model of FL as a disease in which the composition and organization of the immune microenvironment determine clinical outcome (47–50). Within neoplastic follicles, malignant B cells are admixed with CD4posCD40pos T cells, and using soluble CD40 ligand in combination with IL-2 and IL-4, we were able to trigger proliferation of purified FL B-cells. Interestingly, cell proliferation was enhanced by MSCs, but this feeder effect was abolished by treatment with IFN-γ. These results are in agreement with the demonstration that FL B-cells retain typical features of normal germinal center–derived B cells.

Collectively, our results depict new facets of the complex interactions between stromal cells and mature B cells, both in normal and malignant settings, and emphasize the role of the cytokine context as a master regulator of this crosstalk (Supplementary Fig. S3). Drawing a more complete picture of lymphoma cell niches would potentially provide new therapeutic modalities to alter FL-cell growth by combining direct antitumor activity and indirect action through specialized microenvironment cell subsets.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

Received 8/4/08; revised 12/31/08; accepted 1/2/09; published OnlineFirst 3/10/09.

**Grant support:** Région Bretagne, ADHO association, Roche SA, Institut National du Cancer (INCA libre 2005-P1.070), and Agence Nationale de la Recherche (ANR-06-PHYSIO-019-023). H-M El Hajjami was partially supported by the SFH.

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We thank Mrs. Buaux, Leguerrier and Thauran for providing human tonsil and bone marrow samples, Nathalie Le Floch-Barban for technical assistance in evaluation of IDO activity, and Delphine Rossille for microarray data arrangement.

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