TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Cell Growth in Aggregates Determines Gene Expression, Proliferation, Survival, Chemoresistance, and Sensitivity to Immune Effectors in Follicular Lymphoma

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Lymphomas grow as dense aggregates in patients, but whether this spatial organization affects lymphoma cell biology is unknown. We grew follicular lymphoma (FL) cells *in vitro* as multicellular aggregates of lymphoma cells to investigate this question. Gene expression analysis revealed that 612 genes were differentially expressed when cells grew in multicellular aggregates of lymphoma cells rather than in suspension. These genes correspond to several G0 biological processes, such as hypoxia, activation of NF- κ B pathway, and negative regulation of cell cycle, a gene signature also found in the transcriptomes from FL biopsies. Pimonidazole staining, HIF-1A accumulation, and VEGFA release confirmed that cells in multicellular aggregates of lymphoma cells actually respond to hypoxia. In adaptation to such conditions, they also displayed an activated NF- κ B pathway and a quiescent status far more frequently than in suspension. When cultured in three dimensions, FL cells display resistance to doxorubicin and bendamustine, two drugs largely used in FL therapy, compared to FL cultured in suspension. Finally, multicellular aggregates of lymphoma cells were also found to be less sensitive to purified natural killer cells. To conclude, our study shows that in FL, spatial organization results in dramatic changes in FL biology, including gene expression, proliferation, drug resistance, and immune escape. (*Am J Pathol* 2014, 184: 282–295; <http://dx.doi.org/10.1016/j.ajpath.2013.09.018>)

Non-Hodgkin lymphomas represent a large group of cancers, with 75,190 new cases in the United States in 2011, with diffuse large B-cell lymphomas and follicular lymphomas (FL) the most frequent subtypes. In the last decade, patients with FL have benefited from the introduction of immunochemotherapy consisting of the association of genotoxic agents such as doxorubicin with rituximab, a chimeric monoclonal antibody directed against the membrane-associated CD20 antigen. FL is still generally considered an incurable disease, however, justifying further investigations of the molecular and cellular basis of its natural history and chemosensitivity.

This disease is characterized by a low proliferation rate and subsequent slow tumor cell accumulation, natural chemoresistance reflected by relatively low incidence of

molecular response to immunochemotherapy (about 50%), and immune escape illustrated by inevitable relapse even after complete remission, suggesting that immune effectors, including natural killer (NK) cells, are inefficient in eradicating minimal residual disease. However, the molecular basis of all these features remains to be elucidated, although

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the t(14,18) and subsequent Bcl-2 overexpression has been shown to play a role in drug resistance and perhaps in negative regulation of cell proliferation.^{1,2}

To address such questions, however, most *in vitro* studies so far have involved cell suspensions that are not representative of the carcinoma-like architecture of FL spherical tumors *in vivo*. The three-dimensional (3D) organization of spheroid carcinomas drives proliferation and survival adaptations by harnessing gene expression and signaling pathways to high cell density, stress, hypoxia, and low access to nutrients.^{3,4} Further studies have shown that spatial organization confers intrinsic tumor cell resistance to genotoxic compounds,⁵ as well as to ionizing radiation, because of hypoxia.⁶ Finally, growth in aggregates results in decreased sensitivity to immune effectors such as cytotoxic T lymphocytes.^{7–9}

We designed a novel method of 3D *in vitro* culture of non-Hodgkin lymphoma cells to generate multicellular aggregates of lymphoma cells (MALC), and used these as a model to address such questions. Investigating the biological characteristics of FL cells grown in MALC revealed for the first time that this spatial organization deeply affects gene expression, cell proliferation, and survival as an adaptation to hypoxic conditions and beyond, to chemotherapeutic drugs and NK cells.

Materials and Methods

Cell Lines and Reagents

The t(14,18)⁺ FL ATCC RL cell line (ATCC, Manassas, VA) was cultured at 37°C in humidified 5% CO₂ in complete medium. Hypoxia experiments were performed in a hypoxic chamber (0.1% O₂) or with 10 µmol/L cobalt chloride (CoCl₂). PKH67 and plasmids were obtained from Sigma-Aldrich (St. Louis, MO). Vascular endothelial growth factor A (VEGFA) concentrations were determined by using the QIA51 ELISA (enzyme-linked immunosorbent assay) kit (Merck Millipore, Molsheim, France).

Peripheral blood mononuclear cells were collected from healthy donors (Etablissement Français du Sang, CHU Purpan, Toulouse) and separated by Ficoll-Hypaque density gradient (GE Healthcare, Little Chalfont, UK). Negative selection of NK cells was performed with the EasySep Human NK Cell Enrichment Kit, according to the manufacturer's instructions (STEMCELL Technologies, Grenoble, France). The purity of the enriched NK cells (around 95%) was evaluated by flow cytometry using a CD56-PE-Cy5 antibody (BD Biosciences, le Pont de Claix, France) and a CD3-PE-Cy7 antibody (Beckman Coulter, Roissy, France).

Multicellular Aggregates of Lymphoma Cells

MALC were produced by an adaptation of the hanging drop method.¹⁰ Cell suspensions (500,000 cells/mL) were prepared in complete medium containing 1% Methocult (STEMCELL Technologies), and 20 µL of this suspension

were dropped onto coverslips of a 24-well culture plate. After 24 hours incubation, all drops were transferred by returning the coverslip to a dish previously coated with 1% agarose (Invitrogen, Saint Aubin, France). All of the MALC culture medium was then renewed every 5 days. The number of cells in MALC was estimated by trypan blue counting; their volume (V) was calculated by measuring two diameters (L = longest diameter and l = shortest diameter) and using the formula:

$$V = 4/3 \times \pi \times L \times l^2/2^3. \quad (1)$$

Cell Imaging

RL cells were stained by PKH67 (Sigma-Aldrich) before MALC preparation. Samples were kept in culture medium at room temperature during acquisitions. MALC were visualized on the fluorescein channel of a LSM 7 MP Laser Scanning biphoton microscope (Zeiss, Le Pecq, France), with 20× plan apochromat objective, numerical aperture = 1, dry. Series of 90 to 150 z-sections were taken at 1-µm distance. Images were produced with ZEN-2008-LE software (Zeiss) and analyzed with Imaris version 3.1 3D software (Bitplane, Zurich, Switzerland).

Western Blot Analysis

MALC were dissociated, washed with PBS, lysed in Cyto-buster (Merck Millipore), and Western blot analysis was performed as described.¹¹ Antibodies used were against α-tubulin (Sigma-Aldrich), β-actin (Millipore, Saint-Quentin-en-Yvelines, France), hypoxia-inducible factor 1-α (HIF1A) (Cayman Biochem, Montluçon, France), phospho-Ser536 NF-κB-P65, NF-κB-P65 (total), P21 (Cell Signaling Technology, Saint-Quentin-en-Yvelines, France), P27 (Santa Cruz Biotechnology, Le-Perray-en-Yvelines, France), origin recognition complex (ORC2) (MBL International, Woburn, MA), and peroxidase-conjugated secondary anti-rabbit or anti-mouse Ig (Jackson ImmunoResearch Laboratories, Montluçon, France). Cell fractionation has been described elsewhere.¹²

Flow Cytometry

All stained cells were analyzed with a LSRII flow cytometer (BD Biosciences), and data were processed using DIVA version 6.1.2 software (BD Biosciences).

Phenotype

MALC were dissociated in PBS by mechanical force; cells were then washed with cold PBS and stained with the following fluorochrome-conjugated antibodies; briefly, phycoerythrin (PE)-conjugated intercellular adhesion molecule (ICAM; CD54) (Beckman Coulter), fluorescein isothiocyanate (FITC)-conjugated human leukocyte antigen (HLA)-ABC, PE-conjugated HLA-E (eBiosciences, Paris,

France), and FITC-conjugated FAS antibody (Beckman Coulter) were incubated on cells for 20 minutes at 4°C, and then washed with cold PBS prior to analysis by flow cytometry.

Degranulation Assay and CD107a Expression

For the degranulation assay, MALC cells or RL cells in suspension were harvested and incubated at 37°C with NK cells at a 1:1 ratio for 4 hours. Reactions were stopped on ice, and cells were stained with fluorochrome-conjugated anti-human CD107a (BD Pharmingen; BD Biosciences) or isotype-matched control antibody in staining buffer containing 1% fetal calf serum. Fluorescence was analyzed by flow cytometry.

Pimonidazole Assay

Oxygen deprivation was measured with a Hypoxyprobe-1 Plus Kit (NPI Inc, Burlington, MA). MALC were incubated in medium containing 100 µmol/L pimonidazole for 2 hours at 37°C, and then dissociated, washed, fixed with 2% paraformaldehyde for 15 minutes at room temperature, washed again, and finally incubated for 20 minutes with 1% FITC-conjugated anti-pimonidazole antibody.

5-Bromo-2-Deoxyuridine Incorporation Assay and Cell-Cycle Analysis

MALC medium was replaced by medium containing 10 µmol/L 5-bromo-2-deoxyuridine (BrdU) and incubated 1 hour at 37°C. MALC were then dissociated and washed (PBS 0.5% bovine serum albumin). Ice-cold ethanol (100 µL) was added to cells and incubated (20 minutes at room temperature). Cells were washed and incubated in 500 µL of 2 mol/L HCl buffer (20 minutes at room temperature) before incubation (5 minutes) with 0.1 mol/L Na₂B₄O₇ (pH 8.5). Anti-BrdU-FITC (BD Biosciences) was added to cells (20 minutes at room temperature). Cells were then washed twice and incubated (30 minutes at room temperature in the dark) with 10 µg/mL propidium iodide (Invitrogen) and 500 µg/mL RNase A (Invitrogen).

High-Resolution Cell-Cycle Analysis

Quiescent cells were detected by a high-resolution cell-cycle analysis technique.¹³ After fixation (20 minutes at room temperature in ice-cold ethanol), cells were stained for Ki-67 (clone Ki-67; BioLegend, Saint Quentin en Yvelines, France), washed, and then labeled with 7-AAD (BD Biosciences) for 30 minutes at room temperature to visualize their DNA content. The raw percentages from cytometry are shown as ratios: induction of quiescence (fold change) = (% quiescent among treated cells)/(% quiescent among untreated cells).

Cell-Cycle Fractionation

We used a cell-cycle fractionation technique¹⁴ to sort the quiescent and cycling viable cells from MALC. The DNA content were stained with 10 µg/mL Hoechst 33342 (Invitrogen) for 45 minutes at 37°C, washed, and then stained with 2 µmol/L pyronin Y (Sigma-Aldrich) for 45 minutes at 37°C. Quiescent cells are pyronin Y^{dull} and Hoechst²ⁿ, whereas proliferating cells are pyronin Y^{bright}. To sort cells for culture, this technique was replaced by a single pyronin Y staining. Drug sensitivity of both types of cells was evaluated by trypan blue counting.

Cytotoxicity Assay

The sensitivity of cells to NK cytotoxicity was measured by a conventional 4-hour ⁵¹Cr release assay by using triplicate cultures in round-bottom 96-well plates. Target cells (1 × 10⁶ cells) (suspension RL cells or cells dissociated from MALC) were labeled with 100 µCi of ⁵¹Cr (sodium chromate, 1 mCi/mL; PerkinElmer, Courtaboeuf, France). After 1-hour incubation at 37°C, cells were washed three times in RPMI. NK effector cells were added to ⁵¹Cr-labeled target cells in replicate at various effector-to-target ratios (10:1, 5:1, 2.5:1, and 1.25:1) in a total volume of 200 µL of RPMI containing 5% fetal calf serum per well of 96-well round-bottomed microtiter plates. Microtiter plates were centrifuged at 300 × g for 5 minutes and incubated at 37°C. After 4 hours of culture, 50 µL of cell-free supernatants were transferred to a LumaPlate microplate (PerkinElmer), and the radioactivity was measured on a TopCount microplate scintillation and luminescence counter (PerkinElmer). The specific cytotoxicity was calculated. Spontaneous release was determined from wells containing target cells alone. Maximum release was determined from wells containing target cells lysed in 1% Triton X-100. Percent-specific cytotoxicity was calculated conventionally as described earlier.

Immunohistochemistry of MALC

Aggregates were transferred in agarose-precoated transwells (R&D Systems, Lille, France). Medium was replaced by 10% buffered formalin for fixation; MALC were embedded in agarose and then in paraffin. Immunostaining was performed on sections obtained with an automated stainer (Ventana Medical Systems, Tucson, AZ). Antibodies used were anti-Ki-67 (clone MIB-1; BioLegend) and anti-pimonidazole (NPI). After incubation with antibodies, sections were incubated with the avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA) followed by staining with diaminobenzidine (DAB) chromogen solution.

Lentivector Production and RL Cell Transduction

The pLKO.1-puro-CMVtagFP635 vector was used for all transduction experiments. *HIF1A* gene inhibition used the

Table 1 Sequences Used for *HIF1A* Gene Inhibition (sh3810 and sh10819) and Control (shc013)

shRNA	Sequence
sh3810	5'-CCGGGTGATGAAAGAATTACCGAATCTCGAGATTCGGTAATTCTTTCATCACTTTT-3'
sh10819	5'-CCGGTGCTCTTTGTGGTTGGATCTACTCGAGTAGATCCAACCACAAAGAGCATTTT-3'
shc013	Empty vector

sequences sh3810 and sh10819 (Table 1) and, as control, shc013 (empty vector) (plasmids maps are provided in Supplemental Figure S1). RL cells were transduced with lentivector particles carrying these constructs produced by the INSERM UMR1037 vector facility (Cancer Research Center of Toulouse, Toulouse, France). After subcloning of transduced cells and puromycin selection, the subclones specified in the text were used.

Gene Expression Analysis

RNA was extracted from cells grown either in suspension or in MALC. cDNA were prepared from 200 ng of RNA per sample, and hybridized to GeneChip Human Genome U133 Plus 2.0 Affymetrix (Santa Clara, CA) microarrays (ProfileXpert, Lyon, France). Raw data corresponding to both experiments (MALC#2D and quiescent#cyclin) are available on the NCBI Gene Expression Omnibus website (<http://www.ncbi.nlm.nih.gov/geo>; accession number GSE41856). Additional raw data files from purified germinal center cells (GC) ($n = 10$), FL cells purified from biopsies ($n = 38$), and five B-EBV lymphoblastoid cell lines were downloaded from the NCBI GEO

website (GEO dataset GSE12195¹⁵). Raw data were analyzed using robust multiarray average algorithms, then normalized by dChip (<http://www.hsph.harvard.edu/cli/complab/dchip>, last accessed September 15, 2012), log2 converted and collapsed to 20,606 unique genes with Gene Set Enrichment Analysis (GSEA, <http://www.broadinstitute.org/gsea>, last accessed September 15, 2012). The 612 experimental genes that were differentially expressed by cells from MALC (fold change ≥ 2 , P value < 0.05 versus cells in suspension) were analyzed with AutoCompare¹⁶ (Fred's Softwares, AutoCompare_win-400.zip, <https://sites.google.com/site/fredsoftwares/products/data-mining>, last accessed September 20, 2012). Expression levels of genes shared by reference pathways and experimental gene set were compared between sample groups as specified in the text. For these comparisons, expression levels were normalized and heat maps were generated by Genesis software version 1.7.6 (Genomics and Bioinformatics Graz, http://genome.tugraz.at/genesis/client/genesisclient_download.shtml, last accessed October 1, 2012). Significantly differently expressed genes were defined according to the false discovery rate—corrected Q-value.

Figure 1 MALC imaging, modeling, and gene expression. **A:** Fluorescence image of PKH67 MALC was captured by two-photon microscopy 2 days after transfer. **B:** Analysis and 3D reconstitution were performed with Imaris version 3.1 3D software. **C:** Heat map represents the clustering of genes deregulated in RL cells cultured in suspension (2D) or as aggregates (MALC).

Table 2 List of Specific Pathways with the Corresponding Genes Represented by the Analyzed 612 Genes

Response to hypoxia	I-κB kinase-NF-κB cascade	Negative regulation of apoptosis	Regulation of cell cycle
<i>BNIP3</i>	<i>CFLAR</i>	<i>BCL2A1</i>	<i>ATM</i>
<i>CD24</i>	<i>HTR2B</i>	<i>BNIP3</i>	<i>CDC25A</i>
<i>CXCR4</i>	<i>IRAK2</i>	<i>BNIP3L</i>	<i>CDC45L</i>
<i>EGLN1</i>	<i>LGALS1</i>	<i>CFLAR</i>	<i>CDC6</i>
<i>VEGFA</i>	<i>LITAF</i>	<i>FAIM3</i>	<i>CDKN1B</i>
	<i>MAP3K3</i>	<i>HRK</i>	<i>FOXN3</i>
	<i>SQSTM1</i>	<i>NOL3</i>	<i>GADD45A</i>
	<i>TNFAIP3</i>	<i>NOTCH2</i>	<i>HEXIM2</i>
	<i>TRAF5</i>	<i>TNFAIP8</i>	<i>NOTCH2</i>
		<i>TNFRSF10D</i>	<i>SESN1</i>
		<i>VEGFA</i>	<i>TIPIN</i>

Statistics

Comparisons of datasets or gene expression levels between the specified groups of samples were based on two-sided Student’s *t*-test of paired samples or of groups of unequal variance, according to the datasets. As specified in the text, *P* values of <1% or <5% were considered significant. Gene enrichment analysis was based on hypergeometric distributions and Bonferroni corrections for multiple comparisons.¹⁷

Results

Growth in MALC Impacts Gene Expression

We produced MALC from the RL cell line and maintained these for 3 weeks by *in vitro* cell culture. In general, MALC were visible to the eye and routinely reached volumes of about 30 mm³ within 3 weeks of culture. Visualization of 3-day-old MALC from PKH67-stained RL cells by

biphoton microscopy showed oblate spheroids composed of a dense and homogeneous cell population (Figure 1, A and B). Immunohistochemistry of 10-day-old MALC revealed a CD20⁺, CD10⁺, CD5[−], and CD34[−] phenotype that was similar to that seen in two-dimensional (2D) cultures and typical of FL cells (data not shown).

We then investigated the gene expression pattern of cells from this 3D structure. mRNA from RL cells grown either in conventional 2D (exponential phase) or in MALC (10 days culture) were isolated and hybridized on Affymetrix microarrays. Comparison of gene expression patterns in both conditions revealed that >7000 genes were differentially expressed in MALC (*P* < 0.05), 612 of which displayed significant changes of expression level (>2-fold, both up and down) (Figure 1C). The functional significance of the 612 genes most differentially expressed by MALC cells was analyzed by using AutoCompare for matches with the Gene Ontology’s (GO) biological process compendium. As a whole, these genes were significantly involved in *negative regulation of cellular process* (38 genes, *P* = 2.27 × 10^{−5}), *negative regulation of biological process* (39 genes, *P* = 2.82 × 10^{−5}), *negative regulation of cellular metabolic process* (18 genes, *P* = 0.0004), and *negative regulation of apoptotic process* (10 genes, *P* = 0.002). More specifically, these 612 genes were involved in *response to hypoxia* (5 genes, *P* = 0.00106), *I-κB kinase/NFκ-B cascade* (9 genes, *P* = 0.00538), *negative regulation of apoptosis* (11 genes, *P* = 0.00389), and *regulation of cell cycle* (11 genes, *P* = 0.0156) (Figure 1C). These genes are listed in Table 2.

Thus, FL cells could stably grow in tridimensional aggregates. In such conditions, they displayed a distinct gene expression pattern that reflected adaptation to hypoxia, induction of prosurvival pathway, and an antiproliferative status, the latter being expected from other 3D culture models.

Figure 2 Response to hypoxia. **A:** Venn diagram represents genes included in the *response to hypoxia* GO biological process acquired by MALC, compared to 2D RL, and by FL nodules, compared to normal GC. **B:** Pimonidazole staining in RL cells grown in 2D normoxia, 2D hypoxia, or in MALC. Analysis was performed with flow cytometry. Additional pimonidazole staining was realized by immunohistochemistry. **C:** Western blot analysis of HIF1A was performed with protein extracts from RL cells grown in 2D normoxia, 2D hypoxia, or in MALC. **D:** ELISA dosage of VEGFA in 2D normoxia or in MALC. ****P* = 0.001.

Table 3 Comparison of Genes Up-Regulated Using Gene Signature Response to Hypoxia According to GO-Biological Process Database

All	FL vs GC	MALC vs 2D	Intersection
ALAS2	ARNT2	BNIP3	CD24
ANG	BNIP3	CD24	EGLN1
ARNT2	CD24	CXCR4	VEGF
BNIP3	EGLN1	EGLN1	
CD24	EPAS1	PLOD1	
CHRNA7	HIF1A	SMAD3	
CHRN2	HSP90B1	VEGF	
CLDN3	NARFL		
CREBBP	PLOD2		
CXCR4	SMAD4		
EGLN1	VEGF		
EGLN2			
EP300			
EPAS1			
HIF1A			
HSP90B1			
MT3			
NARFL			
NF1			
PDIA2			
PLOD1			
PLOD2			
PML			
SMAD3			
SMAD4			
TGFB2			
VEGF			

MALC Models Hypoxia Occurring in FL

We first asked whether such conditions might be representative of authentic FL nodules, in terms of molecular profile. To assess this, we looked for the gene signature *response to hypoxia* in MALC, and compared it with the one of FL cells from biopsies relative to normal GC. In this aim, we downloaded transcriptomes from a single study comprising such samples¹⁵ and compared the expression level of the 28 genes defining *response to hypoxia* according to the GO biological process database. Seven of these 28 genes were differentially up-regulated in MALC versus 2D cultures ($P < 0.01$), including the above-mentioned 5 genes (Figure 1C) with fold change >2 and P value >0.05 (Table 2). In addition, 11 of the 28 genes were also significantly up-regulated ($P < 0.01$) in FL cells relative to normal GC (Supplemental Figure S2), three of which were up-regulated and shared with MALC (Figure 2A and Table 3).

This suggested that by culturing FL cell lines in 3D, they acquire a gene expression profile closer to the one observed in FL biopsies.

The next point was to investigate whether this gene signature of *response to hypoxia* actually reflected the presence of hypoxic conditions in MALC. RL cells were

grown in 2D normoxic or hypoxic conditions, and in MALC. Hypoxia was determined in these samples through flow cytometry—based analysis of hypoxic cells following the pimonidazole assay (Figure 2B). This experiment showed that the classic 2D cultures do not comprise hypoxic cells unless they are maintained for 48 h in hypoxic chambers. By contrast, nearly half of the cells were in hypoxia when grown in MALC. Further immunohistochemistry of pimonidazole-stained MALC showed that hypoxic cells were mostly located in the core of MALC (Figure 2B), hence the gene signature of MALC reflected the sensing of hypoxic conditions in the center of MALC. Because adaptation to hypoxic conditions usually involves stabilization of the HIF1A protein,¹⁸ we asked whether HIF1A was present in the cell lysates from MALC. The Western blots showed that HIF1A protein in MALC was expressed to the same extent as in 2D culture submitted to severe hypoxia conditions (0.1% O₂) (Figure 2C). Interestingly, confluent cell cultures did not display HIF1A expression (data not shown). Furthermore, because HIF1A protein induces the production of proangiogenic factors such as VEGFA,¹⁹ we asked whether this also occurs in MALC. VEGFA production was found to be increased in MALC relative to 2D cultures (Figure 2D). Thus, a functional pathway induced by hypoxia was identified in MALC.

NF- κ B Is Found Activated in MALC

The NF- κ B pathway is reportedly found activated in non-Hodgkin lymphoma cells,²⁰ so we wondered whether this feature was also recovered in the MALC model. The above-depicted gene expression signature of MALC (Figure 1C) encompasses up-regulation of a set of nine genes corresponding to *I- κ B kinase/NF κ B cascade*. At this point, we wondered whether this activation pattern might be found in FL nodules. To address this possibility, we compared the gene signature of MALC versus 2D with the one of FL versus normal GC cells (see above) in terms of the 102 genes involved in the *NF- κ B cascade*, because they are listed in the GO biological process compendium. Sixteen of these 102 genes were differentially regulated in MALC versus 2D ($P < 0.01$). In addition, we found that 66 of the 102 *NF- κ B cascade* genes were significantly ($P < 0.01$) up-/down-regulated in FL nodules relative to normal GC (Supplemental Figure S3). Seven of these genes were similarly up-/down-regulated in both MALC and FL samples, six of them being up-regulated; *PTPLAD1* is the only down-regulated gene in FL as in MALC (Figure 3A, Table 4). Of note, these 65 *NF- κ B cascade* genes were also differentially expressed by the constitutively activated B-EBV cell lines.^{21,22} Furthermore, among the genes deregulated by both MALC and FL cells, *PTPLAD1*, *CASP8*, *TNFAIP3*, and *CFLAR* are wired through a functional network of protein interaction defined as *I- κ B kinase/NF- κ B cascade* by the STRING database (<http://string-db.org>, last

Figure 3 NF- κ B pathway activation in MALC. **A:** Venn diagram represents genes included in *I- κ B kinase/NF- κ B cascade* GO biological process, regulated in a similar manner in MALC (compared to RL grown in 2D), and in FL nodules (compared to normal GC). **B:** Functional interaction network part of NF- κ B pathway. **C:** Western blot analysis of total and phosphorylated (Ser536) forms of RELA-P65 in RL grown in suspension and in MALC (three samples). **D:** Western blot analysis of RELA-P65 in nuclear extracts of RL grown in suspension or in MALC (two samples) is on the **left**. Nuclear protein control of this fraction is shown on the **right** with ORC2 expression, analyzed by Western blot. **E:** ICAM1 expression in MALC cells relative to 2D was analyzed by flow cytometry. *** $P = 0.001$.

accessed October 10, 2012) (Figure 3B). These genes are listed in Table 4.

We were thus prompted to check whether the NF- κ B pathway was really activated at the cellular level. To this aim, we analyzed the phosphorylation status and nuclear translocation of RELA-P65 protein in RL cells grown either in suspension or in MALC. We found that both total RELA-P65 and its Ser536-phosphorylated form were accumulated in MALC, compared to 2D culture (Figure 3C). In addition, the RELA-P65 protein was more abundant in the nuclear fraction of cells grown in MALC than in the same nuclear fraction of cells grown in 2D (Figure 3D) (the quality control for cell fractionation is shown in Supplemental Figure S4). Finally, we investigated the expression of ICAM1, a protein regulated by the NF- κ B pathway.²³ We found that, indeed, ICAM1 was overexpressed in MALC relative to 2D cultures (Figure 3E).

Thus, a functional NF- κ B pathway was found in MALC. This suggests that by culturing lymphoma cells in aggregates, we induce a proinflammatory phenotype.

MALC Growth Induces a Cell-Cycle Exit

We next investigated cell-cycle regulators in MALC versus 2D as well as in FL versus normal GC. Thus, *CDKN1B*, *CDT1*, *DDB1*, *HCFC1*, *PKMYT1*, *PPM1G*, and *UBE2C*, all

were similarly deregulated in both MALC (relative to 2D) and in the FL biopsy samples (relative to normal GC) (Figure 4A, Supplemental Figure S5, and Table 5): *CDKN1B* was the only one up-regulated in MALC as in FL; the others genes were all underexpressed in both groups. This transcriptome profile was highly suggestive of a low proliferation rate.

These results encouraged us to investigate possible differences in terms of cell proliferation between MALC and 2D culture. The volume of MALC increased with time (Figure 4B). During the first week of *in vitro* culture, cells from MALC grew quickly but slower than in 2D (mean doubling time of 42 hours, versus 31 hours, respectively), and increased from 10,000 cells at day 0 to roughly 600,000 at day 7. After 1 week, MALC proliferation was sustained with a mean doubling time of 7 days. Along with the culture, however, the cell death rate remained consistently below 10%. Cell cycle, when analyzed by BrdU/propidium iodide double staining, revealed that cells from MALC accumulated in the G0/G1 phase after 7 days of culture, whereas their S-phase decreased (Figure 4C). We hypothesized that this might result from cell-cycle exit rather than G1/S blockade, and thus tested this by high-resolution cell-cycle analysis of Ki-67/7-AAD double staining. We found a progressive increase of cells with the 7-AAD^{diploid}, Ki-67^{negative} phenotype revealing accumulation of quiescent cells in MALC. Quiescent cells represented 49% of all cells

Table 4 Comparison of Genes Up-Regulated Using Gene Signature *NF-κB Cascade* According to the GO Biological Process Database

All		B-EBV and FL vs GC		MALC vs 2D	Intersection
<i>APOL3</i>	<i>NEK6</i>	<i>APOL3</i>	<i>TMEM9B</i>	<i>BCL10</i>	<i>BCL10</i>
<i>ATP2C1</i>	<i>NUP62</i>	<i>ATP2C1</i>	<i>TNFAIP3</i>	<i>BCL3</i>	<i>CASP8</i>
<i>BCL10</i>	<i>OTUD7B</i>	<i>BCL10</i>	<i>TNFRSF10B</i>	<i>CASP8</i>	<i>CFLAR</i>
<i>BCL3</i>	<i>PLEKHG5</i>	<i>CARD10</i>	<i>TNFRSF1A</i>	<i>CFLAR</i>	<i>LITAF</i>
<i>BIRC2</i>	<i>PLK2</i>	<i>CASP1</i>	<i>TNFSF10</i>	<i>IKBKG</i>	<i>MAP3K7IP2</i>
<i>BST2</i>	<i>PPM1A</i>	<i>CASP8</i>	<i>TNFSF14</i>	<i>IRAK2</i>	<i>PTPLAD1</i>
<i>CANT1</i>	<i>PPP5C</i>	<i>CD40</i>	<i>TRADD</i>	<i>LITAF</i>	<i>TNFAIP3</i>
<i>CARD10</i>	<i>PRDX4</i>	<i>CFLAR</i>	<i>TRAF3IP2</i>	<i>MAP3K7IP2</i>	
<i>CARD8</i>	<i>PTPLAD1</i>	<i>CXXC5</i>	<i>TRIM13</i>	<i>MIB2</i>	
<i>CARD9</i>	<i>REL</i>	<i>ECT2</i>	<i>TRIM38</i>	<i>PTPLAD1</i>	
<i>CASP1</i>	<i>RELA</i>	<i>F2R</i>	<i>TSPAN6</i>	<i>RIPK2</i>	
<i>CASP8</i>	<i>RHOA</i>	<i>FAF1</i>	<i>VAPA</i>	<i>SLC20A1</i>	
<i>CC2D1A</i>	<i>RHOC</i>	<i>FASLG</i>	<i>ZDHHC13</i>	<i>SLC35B2</i>	
<i>CD40</i>	<i>RHOH</i>	<i>FKBP1A</i>	<i>ZDHHC17</i>	<i>TMEM101</i>	
<i>CFLAR</i>	<i>RIPK1</i>	<i>FLNA</i>		<i>TNFAIP3</i>	
<i>CHUK</i>	<i>RIPK2</i>	<i>GJA1</i>		<i>UBE2N</i>	
<i>CXXC5</i>	<i>RPL17</i>	<i>GOLT1B</i>			
<i>ECM1</i>	<i>SECTM1</i>	<i>GPR177</i>			
<i>ECOP</i>	<i>SLC20A1</i>	<i>HMOX1</i>			
<i>ECT2</i>	<i>SLC35B2</i>	<i>HTR2B</i>			
<i>EDARADD</i>	<i>SLC44A2</i>	<i>IRAK1</i>			
<i>EEF1D</i>	<i>SQSTM1</i>	<i>IRAK1BP1</i>			
<i>ERC1</i>	<i>STAT1</i>	<i>LGALS1</i>			
<i>F2R</i>	<i>TFG</i>	<i>LITAF</i>			
<i>FADD</i>	<i>TIAF1</i>	<i>LTBR</i>			
<i>FAF1</i>	<i>TICAM1</i>	<i>MALT1</i>			
<i>FASLG</i>	<i>TLR6</i>	<i>MAP3K3</i>			
<i>FKBP1A</i>	<i>TMED4</i>	<i>MAP3K7</i>			
<i>FLNA</i>	<i>TMEM101</i>	<i>MAP3K7IP2</i>			
<i>GJA1</i>	<i>TMEM9B</i>	<i>MIER1</i>			
<i>GOLT1B</i>	<i>TNFAIP3</i>	<i>NDFIP1</i>			
<i>GPR177</i>	<i>TNFRSF10B</i>	<i>OTUD7B</i>			
<i>HMOX1</i>	<i>TNFRSF1A</i>	<i>PLK2</i>			
<i>HTR2B</i>	<i>TNFSF10</i>	<i>PPM1A</i>			
<i>IKBKE</i>	<i>TNFSF14</i>	<i>PRDX4</i>			
<i>IKBKG</i>	<i>TNFSF15</i>	<i>PTPLAD1</i>			
<i>IRAK1</i>	<i>TRADD</i>	<i>REL</i>			
<i>IRAK1BP1</i>	<i>TRAF2</i>	<i>RELA</i>			
<i>IRAK2</i>	<i>TRAF3IP2</i>	<i>RHOA</i>			
<i>LGALS1</i>	<i>TRAF5</i>	<i>RHOC</i>			
<i>LGALS9</i>	<i>TRAF6</i>	<i>RHOH</i>			
<i>LITAF</i>	<i>TRIM13</i>	<i>RIPK1</i>			
<i>LTBR</i>	<i>TRIM38</i>	<i>RPL17</i>			
<i>MALT1</i>	<i>TRIP6</i>	<i>SECTM1</i>			
<i>MAP3K3</i>	<i>TSPAN6</i>	<i>SLC20A1</i>			
<i>MAP3K7</i>	<i>UBE2N</i>	<i>SLC44A2</i>			
<i>MAP3K7IP2</i>	<i>VAPA</i>	<i>SQSTM1</i>			
<i>MIB2</i>	<i>VISA</i>	<i>STAT1</i>			
<i>MIER1</i>	<i>ZDHHC13</i>	<i>TFG</i>			
<i>NDFIP1</i>	<i>ZDHHC17</i>	<i>TIAF1</i>			
<i>NDFIP2</i>	<i>ZNF675</i>	<i>TLR6</i>			

in 3-week-old MALC (Figure 4D), in contrast to confluent cell cultures, which did not contain quiescent cells (data not shown). Loss of Ki-67 expression without associated necrosis was confirmed by immunohistochemistry in MALC (Figure 4D). In addition, both P27 (*CDKN1B*) and P21

(*CDKN1A*) regulators of quiescence entry in fibroblasts^{24,25} were more abundant in cells from MALC than in 2D (Figure 4E). Thus, growth in aggregates resulted in a dramatic decrease in cell proliferation and entry into quiescence.

Table 5 Comparison of Cell-Cycle Regulators in MALC

All			FL vs GC		MALC vs 2D	Intersection
<i>AIF1</i>	<i>CHEK2</i>	<i>NEK11</i>	<i>AIF1</i>	<i>IL8</i>	<i>ATM</i>	<i>CDKN1B</i>
<i>ALOX15B</i>	<i>CHFR</i>	<i>NEK2</i>	<i>ANLN</i>	<i>JMY</i>	<i>BCCIP</i>	<i>CDT1</i>
<i>ANAPC2</i>	<i>CITED2</i>	<i>NEK6</i>	<i>ATR</i>	<i>KHDRBS1</i>	<i>CCNG1</i>	<i>DDB1</i>
<i>ANLN</i>	<i>CKS1B</i>	<i>NOTCH2</i>	<i>BIRC5</i>	<i>KNTC1</i>	<i>CD28</i>	<i>HCFC1</i>
<i>APBB1</i>	<i>CKS2</i>	<i>NPM2</i>	<i>BMP2</i>	<i>LATS2</i>	<i>CDK5RAP1</i>	<i>PKMYT1</i>
<i>APBB2</i>	<i>CUL1</i>	<i>NUSAP1</i>	<i>BRSK1</i>	<i>MAD2L1</i>	<i>CDKN1B</i>	<i>PPM1G</i>
<i>APC</i>	<i>CUL2</i>	<i>PA2G4</i>	<i>BTG3</i>	<i>MAD2L2</i>	<i>CDT1</i>	<i>UBE2C</i>
<i>ASNS</i>	<i>CUL3</i>	<i>PCBP4</i>	<i>BUB1</i>	<i>MAP2K6</i>	<i>CUL2</i>	
<i>ATM</i>	<i>CUL4A</i>	<i>PIN1</i>	<i>BUB1B</i>	<i>MFN2</i>	<i>DDB1</i>	
<i>ATR</i>	<i>CUL5</i>	<i>PKMYT1</i>	<i>CCNA2</i>	<i>MLF1</i>	<i>EIF4G2</i>	
<i>BCCIP</i>	<i>DDB1</i>	<i>PLAGL1</i>	<i>CCND1</i>	<i>NEK2</i>	<i>ERCC2</i>	
<i>BIRC5</i>	<i>DHRS2</i>	<i>PML</i>	<i>CCND2</i>	<i>NOTCH2</i>	<i>HCFC1</i>	
<i>BMP2</i>	<i>DIRAS3</i>	<i>PPM1G</i>	<i>CCND3</i>	<i>NUSAP1</i>	<i>ING4</i>	
<i>BMP7</i>	<i>DLG1</i>	<i>PPP1R13B</i>	<i>CCNE2</i>	<i>PCBP4</i>	<i>MFN2</i>	
<i>BRSK1</i>	<i>EIF4G2</i>	<i>PPP1R15A</i>	<i>CCNT1</i>	<i>PKMYT1</i>	<i>PKMYT1</i>	
<i>BTG3</i>	<i>ERCC2</i>	<i>PPP1R9B</i>	<i>CD28</i>	<i>PPM1G</i>	<i>PPM1G</i>	
<i>BUB1</i>	<i>EREG</i>	<i>PRMT5</i>	<i>CDC16</i>	<i>PPP1R13B</i>	<i>RAD9A</i>	
<i>BUB1B</i>	<i>ERN1</i>	<i>PTEN</i>	<i>CDC25A</i>	<i>PPP1R15A</i>	<i>SMAD3</i>	
<i>C9ORF127</i>	<i>FANCG</i>	<i>PTPRC</i>	<i>CDC25C</i>	<i>PTEN</i>	<i>TBRG4</i>	
<i>CCNA2</i>	<i>FBX05</i>	<i>RAD1</i>	<i>CDC2L5</i>	<i>RAD1</i>	<i>TRIAP1</i>	
<i>CCND1</i>	<i>FOXC1</i>	<i>RAD17</i>	<i>CDC45L</i>	<i>RAD17</i>	<i>UBE2C</i>	
<i>CCND2</i>	<i>GADD45A</i>	<i>RAD9A</i>	<i>CDC6</i>	<i>RASSF1</i>		
<i>CCND3</i>	<i>GAS1</i>	<i>RASSF1</i>	<i>CDC7</i>	<i>RBBP8</i>		
<i>CCNE2</i>	<i>GAS7</i>	<i>RB1</i>	<i>CDK5RAP3</i>	<i>RHOB</i>		
<i>CCNG1</i>	<i>GML</i>	<i>RBBP8</i>	<i>CDKN1B</i>	<i>RINT1</i>		
<i>CCNG2</i>	<i>GMNN</i>	<i>RHOB</i>	<i>CDKN1C</i>	<i>RUNX3</i>		
<i>CCNK</i>	<i>GTF2H1</i>	<i>RINT1</i>	<i>CDKN2B</i>	<i>SESN1</i>		
<i>CCNT1</i>	<i>GTPBP4</i>	<i>RPRM</i>	<i>CDKN2C</i>	<i>SPHK1</i>		
<i>CD28</i>	<i>HCFC1</i>	<i>RUNX3</i>	<i>CDKN2D</i>	<i>TBRG4</i>		
<i>CDC16</i>	<i>HERC5</i>	<i>SERTAD1</i>	<i>CDKN3</i>	<i>TBX3</i>		
<i>CDC23</i>	<i>HEXIM1</i>	<i>SESN1</i>	<i>CDT1</i>	<i>TGFA</i>		
<i>CDC25A</i>	<i>HEXIM2</i>	<i>SMAD3</i>	<i>CHEK1</i>	<i>TIMELESS</i>		
<i>CDC25C</i>	<i>HPGD</i>	<i>SMC1A</i>	<i>CHEK2</i>	<i>TIPIN</i>		
<i>CDC2L5</i>	<i>HUS1</i>	<i>SPHK1</i>	<i>CKS1B</i>	<i>TP53</i>		
<i>CDC37</i>	<i>IL8</i>	<i>STK11</i>	<i>CKS2</i>	<i>TP53BP2</i>		
<i>CDC45L</i>	<i>ING4</i>	<i>TBRG1</i>	<i>CUL3</i>	<i>TRIAP1</i>		
<i>CDC6</i>	<i>INHA</i>	<i>TBRG4</i>	<i>CUL4A</i>	<i>TTK</i>		
<i>CDC7</i>	<i>INHBA</i>	<i>TBX3</i>	<i>CUL5</i>	<i>UBE2C</i>		
<i>CDK10</i>	<i>JMY</i>	<i>TGFA</i>	<i>DDB1</i>	<i>UHMK1</i>		
<i>CDK2</i>	<i>KHDRBS1</i>	<i>TGFB1</i>	<i>EIF4G2</i>	<i>UHRF2</i>		
<i>CDK5R1</i>	<i>KNTC1</i>	<i>TGFB2</i>	<i>ERN1</i>	<i>ZAK</i>		
<i>CDK5RAP1</i>	<i>LATS1</i>	<i>TIMELESS</i>	<i>FANCG</i>	<i>ZWINT</i>		
<i>CDK5RAP3</i>	<i>LATS2</i>	<i>TIPIN</i>	<i>FBX05</i>			
<i>CDK7</i>	<i>MAD2L1</i>	<i>TP53</i>	<i>FOXC1</i>			
<i>CDKN1B</i>	<i>MAD2L2</i>	<i>TP53BP2</i>	<i>GAS1</i>			
<i>CDKN1C</i>	<i>MADD</i>	<i>TRIAP1</i>	<i>GAS7</i>			
<i>CDKN2A</i>	<i>MAP2K6</i>	<i>TTK</i>	<i>GMNN</i>			
<i>CDKN2B</i>	<i>MAPK12</i>	<i>UBE2C</i>	<i>GTF2H1</i>			
<i>CDKN2C</i>	<i>MFN2</i>	<i>UHMK1</i>	<i>HCFC1</i>			
<i>CDKN2D</i>	<i>MLF1</i>	<i>UHRF2</i>	<i>HEXIM1</i>			
<i>CDKN3</i>	<i>MNAT1</i>	<i>ZAK</i>	<i>HEXIM2</i>			
<i>CDT1</i>	<i>MYC</i>	<i>ZBTB17</i>	<i>HPGD</i>			
<i>CHEK1</i>	<i>NBN</i>	<i>ZWINT</i>	<i>HUS1</i>			

Because hypoxia induces quiescence in hematopoietic stem cells,²⁶ we questioned whether the quiescent MALC cells arose by adaptation to hypoxic conditions. RL cells were cultured for 2 days in either hypoxic or normoxic

conditions and were analyzed for cell-cycle status as above. The hypoxic cultures comprised three times more quiescent cells than the normoxic cultures did (Figure 4F). Along with the response to hypoxia, stabilization of the

Figure 4 Negative regulation of cell cycle. **A:** Venn diagram represented genes included in regulation of cell cycle GO biological process, regulated in a similar manner in MALC (compared to RL grown in 2D), and in FL nodules (compared to normal GC). **B:** Number of cells per MALC was evaluated by trypan blue counting, and the volume was calculated using the formula described in *Materials and Methods*, at different culture times. **C:** Cell-cycle regulation in 2D RL and MALC was studied by BrdU/propidium iodide co-staining. **D:** Quiescence entry of MALC cells was quantified by high-resolution cell-cycle analysis. Quiescence was also measured by immunohistochemical staining for Ki-67 at day (d) 7 and day 21. **E:** Western blot analysis of P27 and P21 was performed with protein extracts from 2D RL in suspension and from MALC at days 7, 14, and 21. **F:** Quantification of quiescence entry in RL cells grown in 2D normoxia and 2D hypoxia. **G:** Effects of CoCl₂ in 2D RL cells: wild type (ATCC), transduced with empty vector, and transduced with *HIF1A* shRNA (sh3810-1, 3810-2, 3810-3, and sh10819-2). * $P < 0.05$, *** $P < 0.01$, and *** $P < 0.001$. F.C., fold change; PI-PE, propidium iodide - phycoerythrin.

HIF1A protein mediates cell-cycle arrest in mouse embryonic fibroblasts and splenic B lymphocytes.²⁷ Thus, we investigated the role of HIF1A in the quiescence of MALC cells. RL cells grown in suspension were treated with the HIF1A protein stabilizer CoCl₂, and the induced quiescent cells were measured. First, CoCl₂-treated RL cells comprised twice as many quiescent cells as in the untreated counterpart. By contrast, CoCl₂-treated RL cell clones previously transduced with various shRNA targeting the *HIF1A* gene did not enter into quiescence (Figure 4G). Altogether, these results suggest that, in MALC, hypoxia, through a HIF1A-dependent mechanism, regulated cell-cycle exit and subsequent MALC expansion.

Gene Expression Changes and Their Functional Consequences in a Quiescent Cell Subpopulation

On the basis of segregation between cycling and quiescent cells, we hypothesized that the latter displayed specific

changes in gene expression profile, compared to the former, and that these changes might account for chemoresistance.

Thus, MALC were sorted by flow cytometry after double staining for pyronin Y/Hoechst 33342, yielding purified quiescent cells of pyronin Y^{dull}, Hoechst 33342^{diploid}, and Ki-67^{dull} phenotypes as well as their cycling counterparts (Figure 5A). In the quiescent cell fraction, >80% of cells were Ki-67 negative. These cells recovered full proliferative potential after 3 days (Figure 5B). The transcriptomes of quiescent and cycling cells were then produced and analyzed by Affymetrix microarrays as above: 4273 genes were differentially expressed by quiescent cells relative to the cycling ones. As illustrated in Table 6, the most significant changes involved negative regulation of proliferation, overexpression of drug transporters, tolerance to DNA damage, and inhibition of apoptosis. Moreover, the major doxorubicin target²⁸ topoisomerase II- α was strongly (10-fold reduction) down-regulated at both mRNA (Table 6) and protein levels (data not shown); *CHEK1* was also down-

Figure 5 Entry to quiescence and chemoresistance in MALC. **A:** Sorting efficacy of Hoechst 33342/pyronin staining. **B:** Growth potential of quiescent cells. **C:** Differential sensitivity of quiescent and proliferating cells to drugs. ** $P < 0.01$, *** $P < 0.001$.

regulated at the protein level (data not shown). All these changes in quiescent MALC cells suggest a coordinated process converging toward protection and survival. To check whether quiescent cells actually were more chemoresistant, we compared the sensitivity of these and cycling cells to the FL drugs doxorubicin, rituximab, bendamustine, and rapamycin. The quiescent cells were more resistant to doxorubicin and bendamustine, but not to rituximab or rapamycin (Figure 5C).

Sensitivity of MALC Cells to NK Cells

Finally, we asked whether 3D organization could modulate FL cell resistance to NK cells. Thus, we performed Cr⁵¹ release assay by coculturing cells either dissociated from MALC aggregates, or in suspension with NK cells. A dramatic decrease in sensitivity of the former to NK cells at each effector-to-target ratio was indeed observed (Figure 6A). However, by using anti-CD107A, we found no differences between NK cells cultured with 3D cells and NK cells cultured with 2D cells, suggesting that NK degranulation was similar (Figure 6B). Moreover, we found no

differences between 3D and 2D FL cells for the expression of Fas receptor as well as HLA class I molecules (HLA-A, -B, -C, and -E) (Figure 6C).

Discussion

This study depicts for the first time a method for *in vitro* 3D culture of FL cells. This method sets up appropriate conditions to produce stiff, spheroid-like aggregates growing stably for >3 weeks. The resulting MALC show interesting characteristics such as compatibility with immunohistochemistry and biphoton microscopy imaging, as well as capacity for drug testing. Our study indeed shows that MALC represents an invaluable model for screening new genotoxic drugs and therapeutic monoclonal antibodies in lymphoma, because spheroids have been used for preclinical studies of targeted therapy in solid tumors.^{29,30} MALC could be obtained with Raji (Burkitt) and DEAU (diffuse large B-cell lymphoma) cell lines, but not with primary neoplastic B cells (including chronic lymphocytic leukemia) and even some B lymphoma cell lines such as Karpas 422 and DOHH2 (data not shown).

Table 6 List of Fold Changes in the mRNA Levels of Various Genes in Quiescent MALC Cells

Family/function	Gene symbols	Folds	P value (Student's <i>t</i> -test)
Quiescence	<i>CDKN1B</i>	2.11	1.46E-03
	<i>MXI 1</i>	1.96	1.50E-05
	<i>CDKN1A</i>	1.32	4.96E-02
Detoxification	<i>TXNDC13</i>	1.87	2.47E-03
	<i>GSTA1</i>	1.65	6.13E-03
	<i>GSTM4</i>	1.62	6.71E-03
Survival	<i>BCL2</i>	2.11	1.98E-04
	<i>RELA</i>	1.68	2.98E-03
	<i>REL</i>	1.59	5.51E-03
	<i>PKC eta</i>	1.52	2.28E-02
	<i>RELB</i>	1.5	2.52E-02
	<i>CASP3</i>	0.73	4.83E-02
DNA damage tolerance	<i>CHEK1</i>	0.25	3.22E-05
	<i>BRCA1</i>	0.22	1.19E-04
Drug resistance	<i>ABCA1</i>	1.68	3.82E-02
	<i>ABCA7</i>	1.65	1.11E-02
	<i>ABCD4</i>	1.48	6.84E-03
Drug targets	<i>TOP2A</i>	0.11	3.04E-06

This study shows that the expansion of MALC comprised two steps. During the first week, FL cells actively proliferated and thus MALC grew quickly, although later, cell-cycle exit of an increasing rate of MALC cells progressively stopped its growth, as reported for prostate carcinoma cell-derived spheroids.³¹ Isolated Ki-67^{negative} cells did overexpress the *CDKN1A*^(p21), *CDKN1B*^(p27), and *MXI-1* genes that mediate quiescence in various cellular models.^{32–34} In a deterministic interpretation, we view quiescence as an adaptive response to defective nutrient, oxygen, and glucose supply, and the resulting acidosis. Moreover, both experimental studies and mathematical models support the general concept of a metabolic deterioration gradient increasing in the spheroid.^{35,36} Accordingly here, hypoxia was located in the core of MALC. The fact that hypoxia induced cell-cycle exit in 2D cultures suggests its role of quiescence promoter in MALC. We showed that HIF1A mediates this effect, and that *HIF1A* possibly acts by transcriptionally reactivating genes causing quiescence. This is supported by the regulatory role of *HIF1A* over P27.³⁷ The contribution of hypoxia to the G0 status of MALC cells does not rule out, however, the contribution of additional stresses such as mechanical compression by high cell densities or stiffness because of an extracellular matrix (data not shown).

At the protein level, our study reveals interesting similarities between MALC and FL clinical settings, including reduced KI-67 expression and increased HIF1A expression in biopsies,^{38,39} and elevated VEGFA concentration in serum.⁴⁰ At the genomic level, we have found major differences in terms of gene expression between MALC and 2D cultures, both quantitative (more than 7000 genes) and qualitative (up to 10-fold variation). Among these modifications, we have detected several set of genes involved in *response to hypoxia, cell*

proliferation inhibition, and *activation of NF-κB pathway* by using the AutoCompare software, a method that has been previously validated.⁴¹ Bioinformatic meta-analysis of transcriptome dataset of Compagno et al¹⁵ pinpointed interesting similarities in the gene expression profile between FL biopsies and MALC. Thus, it is possible that previous studies based on conventional (suspension) cultures have underestimated these pathways shown here as conditioned by aggregation. This is obviously the case for the NF-κB pathway. It is important to note that although NF-κB has received very little attention in FL compared to diffuse large B-cell lymphoma,²⁰ there is some evidence that this pathway is indeed activated in FL tissue samples.⁴² We nevertheless propose here that HIF1A- or NF-κB-targeting drugs might represent interesting candidates for FL therapy.

Figure 6 MALC display resistance to NK cell lysis independent of degranulation modification by effector or phenotypic modification on target cells. **A:** ⁵¹Cr release assay. Results are presented as percentages of the specific lysis obtained for the different effector-to-target ratios. **B:** Degranulation of NK cells cultured for 4 hours either with MALC cells or suspension cells was measured by CD107A staining with PE-Cy5 antibody and detected by flow cytometry. **C:** HLA-A, -B, -C, and -E and FAS expression in MALC cells relative to 2D was analyzed by flow cytometry.

Our study showed that in MALC, the quiescent cells were chemoresistant, not only to doxorubicin, an anthracycline used for years in the treatment of FL, but also to bendamustine, an alkylating agent with promising potential for therapeutic combinations in this disease. Interestingly, with MALC, we did not find significant differences between cycling and quiescent cells in terms of sensitivity to rituximab and mammalian target of rapamycin (mTOR) inhibitor. The high degree of chemoresistance raises several hypotheses related to doxorubicin's mechanism of action. Indeed, after transmembrane penetration, this drug operates through reactive oxygen species production⁴³ as well as DNA double-strand breaks because of topoisomerase II/DNA covalent interaction and subsequent apoptosis. Transcriptome analysis suggested that quiescent cells exhibit defense mechanisms against each intoxication step. Among these, there is overexpression of detoxifying enzymes, together with antiapoptotic signaling through enhanced NF- κ B, BCL2, and protein kinase C (PKC) pathways. It is important to note that previous studies have already documented in FL tissue samples activation of NF- κ B pathway,⁴² up-regulation of PKC expression,⁴⁴ as well as some degree of BCL2 regulation correlated with cell proliferation in parallel with t(14;18).² Furthermore, the topoisomerase II α target of doxorubicin²⁸ was decreased. Reduction in DNA damage controllers such as *CHEK1* and *BRCA1* may also increase tolerance to DNA breaks induced by drugs, ionizing radiation, or even endogenous reactive oxygen species production, although it might also favor DNA instability.

Our study shows that MALC acquire resistance to NK cells. This mechanism may contribute to immune escape. However, the mechanism by which MALC become resistant to NK remains to be identified. We were unable to detect significant changes in the expression of molecules involved in the recognition of target by NK cells or in the intoxication process, including Fas expression (Figure 6C) or the expression of PI-9, a granzyme B inhibitor (data not shown). Another hypothesis is that the mechanism could involve a defect in the formation of the immunological synapse as it has been described for FL cells derived from patients and co-incubated with autologous NK cells.⁴⁵ *In vivo*, it is possible that intrinsic resistance of FL cells to natural immune effectors associates with a defect in immune infiltration, both mechanisms converging toward immune escape. The latter hypothesis is currently under investigation in our laboratory. In the same perspective, the sensitivity of MALC to other immune effectors, such as cytotoxic T lymphocytes, should also be investigated.

To conclude, our study based on MALC, a new *in vitro* model, shows that aggregated growth of FL tumor cells profoundly affects their proliferation, signaling pathway, cellular defenses, and sensitivity to immune effectors. The MALC model should provide new targets for increasing sensitivity to genotoxic drugs and immune intervention.

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P.G. realized experiments, analyzed and interpreted data, made the figures, and wrote the paper. C.J. realized experiments and analyzed and interpreted data. J.F. performed data mining and statistical analysis. E.D. and A.B. performed experiments. C.B. and A.S. provided IHC images. C.L. provided samples and interpreted IHC. J.J.F. and G.L. interpreted data and wrote the paper.

Supplemental Data

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