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Authors' contribution. J. Moreaux performed the experiments and wrote the paper. B. Klein supervised the project and wrote the paper. D. Hose, H. Goldschmidt, M. Moos and M. Jourdan provided with bone marrow plasma cells and/or revised the paper. J. De Vos and T. Reme developed the bioinformatics tools and revised the paper. N. Robert and P. Moine provided with technical assistance.

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Key words: myeloma, TACI, gene expression profiles, c-maf.

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Abstract.

BAFF and APRIL stimulate the growth of multiple myeloma cells (MMC). BAFF and APRIL share two receptors – TACI and BCMA – and BAFF binds to a third receptor, BAFF-R. We previously reported that *TACI* gene expression is bimodal in 18 human MM cell lines (HMCL), being either present or absent, unlike BCMA that is expressed on all HMCL. BAFF-R is lacking. *TACI* expression is a good indicator of a BAFF-binding receptor in HMCL. In primary MMC, the level of TACI expression correlates with a characteristic phenotypic pattern: $TACI^{high}$ MMC resemble bone marrow plasma cells and $TACI^{low}$ resemble plasmablasts.

Using gene expression profiling, we show here that these patterns are kept in $TACI^{+}$ or $TACI^{-}$ HMCL. 80 genes/EST interrogated by Affymetrix microarrays were differentially expressed between $TACI^{+}$ and $TACI^{-}$ HMCL, particularly *c-maf*, *cyclin D2*, and *integrin beta7.*

Triggered by the finding that *TACI* and *c-maf* expressions correlate in $TACI^{+}$ HMCL, we demonstrate in this study that TACI activation influences *c-maf* expression: (1) activation of TACI by BAFF or APRIL increases *c-maf, cyclin D2*, and *integrin beta7* gene expressions in $TACI^{+}$ HMCL, (2) blocking of autocrine BAFF/APRIL stimulation in some $TACI^{+}$ HMCL by the TACI-Fc fusion protein reduces *c-maf*, *cyclin D2*, and *integrin beta7* gene expression, (3) nucleofection of siRNA to *c-maf* decreases *c-maf* mRNA levels and reduces the expression of *cyclin D2* and *integrin beta7* gene expressions, without affecting *TACI* expression. Thus we conclude that TACI activation can upregulate *c-maf* expression, that in turn controls *cyclin D2*, and *integrin beta7* gene expression.

Introduction

Multiple myeloma (MM) is an incurable plasma cell (PC) neoplasm characterized by the displacement of physiological hematopoiesis, the presence of osteolytic bone lesions and impairment of renal function due to the accumulation of malignant PC in the bone marrow and the production of monoclonal protein.

Almost all MM cells (MMC) show an aberrant or overexpression of a D-type cyclin, *i.e*. cyclin D1 (CCND1) in the case of a t(11;14) translocation or gain of 11q13, cyclin D3 (CCND3) overexpression in case of the rare t(6;14) translocation, or an overexpression of cyclin D2 (CCND2) on the background of a translocation involving *c-maf* (t(14;16)) or *FGFR3* (t(4;14)) (1-3). During the course of the disease, further cytogenetic aberrations accumulate(4). Still, survival of MMC depend on the autocrine and paracrine stimulation by growth factors, like interleukin-6 (IL-6) (5), interferon alpha (6), insulin-like growth factor (7), hepatocyte growth factor (8, 9), members of the EGF family (10-12) and members of the TNF-family (13, 14) From the latter, we and others have recently shown that BAFF (B-cell activating factor, also called BLys) and APRIL (A Proliferation-Inducing Ligand) are potent MMC growth factors (15, 16). BAFF binds to 3 receptors - BAFF-R, BCMA and TACI -, and APRIL binds to BCMA and TACI (17).

The activation of nuclear factor (NF)-κB by TACI, BCMA and BAFF-R(18) is consistent with the antiapoptotic role of BAFF since NFκB enhances the expression of several cell survival genes (19, 20). Depending on the B-cell maturation stage, BAFF was reported to induce the anti-apoptotic proteins Bcl-2, A1, and Bcl-XL and to reduce the pro-apoptotic protein Bak (18, 21, 22). BAFF also activates JNK, Elk-1, p38 kinase, AP-1 and NF-AT in various models (23). We recently identified that BAFF and APRIL activate MAPK, PI3K/AKT and NFκB pathways in MMC leading to an

upregulation of Mcl-1 and Bcl-2 antiapoptotic proteins (16). Recently Tai *et al.* shown that MMC express BCMA and TACI but very low level of BAFF-R (24). They demonstrated that BAFF induces activation of NFκB and PI3K/AKT pathways confirming our previous results. Furthermore, they showed BAFF could activate the canonical and the non canonical NFκB pathways in MMC.

Using gene expression profiling (GEP) with Affymetrix microarrays, we found that all primary MMC as well as HMCL express *BCMA* (25). *TACI* is also expressed on almost all MMC as well as normal bone marrow plasma cells (BMPC), plasmablasts and CD27-positive B-cells, but only on about one third (8/18) of HMCL. We have shown *TACI* expression to be necessary for BAFF binding on HMCL and that primary MMC with high expression of TACI ($TACI^{high}$) have a gene expression signature resembling bone marrow plasma cells (BMPC) dependant on the interaction with the bone marrow environment (25). In contrast, primary MMC with low TACI expression ($TACI^{low}$) have a signature resembling proliferating polyclonal plasmablasts (25). The TACI ligands are produced by the bone marrow microenvironment, and in particular, APRIL by osteoclasts (25). Some HMCL, e.g. RMPI8226, L363 and LP1, are rendered independent of this paracrine stimulation and have acquired the property of autocrine BAFF and/or APRIL production (16).

Here, we show that HMCL have kept the signature seen in primary MMC, namely a signature resembling BMPC in TACI-expressing HMCL ($TACI^{+}$), and a signature resembling plasmablasts in $TACI^{-}$ HMCL. We found 80 genes/EST to be differentially expressed between $TACI^{+}$ and $TACI^{-}$ HMCL, in particular *c-maf*, *cyclin D2* and *integrin beta7*. As *TACI* and *c-maf* expression are tightly correlated, we have provided evidence that *c-maf* is a target of the TACI/BAFF/APRIL signaling pathway.

Materials and methods

Cell samples

XG-1, XG-2, XG-3, XG-4, XG-5, XG-6, XG-7, XG-10, XG-11, XG-12, XG-13, XG-16, XG-19, and XG-20 human myeloma cell lines (HMCL) were obtained and characterized in our laboratory (26-29). SKMM, OPM2, LP1 and RPMI8226 were purchased from ATTC (Rockville, MD). Normal bone marrows were obtained from healthy donors after informed consent was given and BMPC were purified using anti-CD138 MACS microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) as described (25). Polyclonal plasmablasts were generated by differentiating peripheral blood $CD19^+$ B cells *in vitro* (30).

Flow cytometry analysis

The expression of TACI on HMCLs was evaluated by incubating 5 x 10^5 cells with an anti-TACI monoclonal antibody (MoAb) biotinylated in PBS containing 30% human AB serum at 4°C for 30 min followed by incubation with PE-conjugated streptavidin (Beckman-Coulter, Marseille, France). Flow cytometry analysis was done on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

Modulation of the gene expression profile by addition or deprivation of BAFF/APRIL in MMC

The modulation of gene expression by addition of BAFF and APRIL was investigated with the XG-7, XG-13 and XG-20 HMCL. XG-7, XG-13 and XG-20 cells were starved of IL-6 for 3 hours and washed. Then BAFF (Peprotech, Rocky Hill, NJ) and APRIL (R&D Systems, Abington, UK) (200 ng/mL each) were added in one culture group for 12 hours in RPMI1640/10% FCS. The modulation of gene expression by deprivation of BAFF/APRIL in RPMI8226 and LP1 HMCL was also investigated. RPMI8226 and LP1 HMCL were starved for 3 hours and washed. Then TACI-Fc (R&D Systems) (10

μg/mL) was added in one culture group for 12 hours in RPMI1640/10% FCS. RNA were extracted for gene expression profiling or real-time PCR analysis.

Modulation of the gene expression profile after NF-kappaB pathway inhibition

To investigate the modulation of gene expression by NF-kappaB pathway inhibition, RPMI8226 and LP1 cells were cultured for 12 hours with an inhibitory peptide of NF-kappaB pathway (100 μg/ml, SN50) or the corresponding inactive peptide (BIOMOL, Plymouth meeting, PA), or TACI-Fc (R&D Systems, 10 μg/mL) in RPMI1640/10% FCS. RNA was extracted and gene expression assayed by real-time PCR.

Preparation of complementary RNA (cRNA) and microarray hybridization

RNA was extracted using the RNeasy Kit (Quiagen, Hilden, Germany). Biotinylated cRNA was amplified with a double *in vitro* transcription and hybridized to the Affymetrix HG U133 set microarrays, according to the manufacturer's instructions (Affymetrix, Santa Clara, CA). Fluorescence intensities were quantified and analyzed using the GCOS 1.2 software (Affymetrix). Gene expression data were normalized with the MAS5 algorithm by scaling each array to a target value of 100 using the "global scaling" method.

Western blot analysis

Cells were lysed in 10 mM tris-HCl (pH 7.05), 50 mM NaCl, 50 mM NaF, 30 mM sodium pyrophosphate (NaPPi), 1% Triton X-100, 5 μM ZnCl2, 100 μM Na3VO4, 1 mM Dithiothreitol (DTT), 20 mM β-glycerophosphate, 20 mM *p*-nitrophenolphosphate (PNPP), 2.5 μg/ml aprotinin, 2.5 μg/ml leupeptin, 0.5 mM phenylmethylsulphonyl fluoride (PMSF), 0.5 mM benzamidine, 5 μg/ml pepstatin and 50 nM okadaic acid. Lysates were cleared by centrifugation at 10,000 *g* for 10 min and resolved by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) before transfer to a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany).

Membranes were blocked for 1 hour at room temperature in 140 mM NaCl, 3 mM KCl, 25 mM tris-HCl (pH 7.4), 0.1% Tween 20 (TBS-T), 5% BSA, then incubated for 3 hours at room temperature with anti-c-maf MoAb (Abnova, Taiwan) at a 1:1000 dilution in 1% BSA TBS-T. The primary antibodies were visualized with goat anti-rabbit (Sigma) or goat anti-mouse (Bio-Rad, Hercules, CA) peroxidase-conjugated antibodies using an enhanced chemiluminescence detection system. As a control for protein loading, we used anti-Beta actin (1:2000) (Santa Cruz Biotechnology, Santa Cruz, CA) antibody.

siRNA transduction

The *c-maf* siRNA duplex construct ACGGCUCGAGCAGCGACAA (Dharmacon Inc, IL, USA) was transduced by electroporation (Amaxa, Köln, Germany) using nucleofaction. We also used Dharmacon's negative control siRNA (ON-TARGETplus siCONTROL Non-Targeting siRNA) as control. RPMI8226 and LP1 HMCL were electroporated using respectively the programs T-001 or A-023 and the T solution according to the manufacturer's instructions.

Real-time RT-PCR

Total RNA was converted to cDNA using the Superscript II reverse transcriptase (Invitrogen, Cergy Pontoise, France). The assays-on-demand primers and probes and the *Taq*Man Universal Master Mix were used according to the manufacturer's instructions (Applied Biosystems, Courtaboeuf, France). The measurement of gene expression was performed using the ABI Prism 7000 Sequence Detection System and analyzed using the ABI PRISM 7000 SDS Software. For each primer, serial dilutions of a standard cDNA were amplified to create a standard curve, and values of unknown samples were estimated relative to this standard curve in order to assess the PCR efficiency. Ct values were obtained for GAPDH and the respective genes of

interest during log phase of the cycle. Gene of interest levels were normalized to GAPDH for each sample (δCt = Ct gene of interest – Ct GAPDH) and compared with the values obtained for a known positive control using the following formula $100/2^{\delta\delta Ct}$ where $\delta\delta$Ct = δCt unknown – δCt positive control.

Statistical analysis

Gene expression data were normalized with the MAS5 algorithm and analyzed with our bioinformatics platform (RAGE, http://rage.montp.inserm.fr/) or the SAM (Significance Analysis of Microarrays) software (31). Statistical comparisons were done with Mann-Whitney, Chi-square, or Student t-tests. Probesets differentially expressed between $TACI^+$ and $TACI^-$ HMCL were picked by 2 methods. First, we selected 109 probesets that were differentially expressed between $TACI^+$ and $TACI^-$ HMCL with a Mann-Whitney test ($P \leq .01$) and with a ratio of the mean expression in $TACI^+$ and $TACI^-$ HMCL that was ≥ 2 or ≤ 0.5. Secondly, we used the SAM software based on a Wilcoxon test, filtering the probesets with the 3-presence and 2-ratio filters. This SAM selection yielded 330 probesets with a false discovery rate of 25.5% using 100 permutations. Crossing the 2 gene lists yielded 86 probesets, corresponding to 80 genes/EST, that were differentially expressed between $TACI^+$ and $TACI^-$ HMCL.

Results

Gene expression profile associated with TACI expression in HMCL

As *TACI* expression yields a functional BAFF-binding receptor in our 18 HMCL (25), we compared the gene expression profiles of 7 $TACI^+$ HMCL and of 11 $TACI^-$ HMCL. 109 probesets out of the 49000 interrogated with U133 set Affymetrix microarrays were differentially expressed between $TACI^+$ and $TACI^-$ HMCL ($P \leq .01$ with a Mann Whitney test and ratio of the mean expressions ≥ 2 or ≤ 0.5). The analysis performed on the same samples using the SAM software with a 3-presence and a 2-ratio filters on probesets and 1000 permutations leaded to a larger 330 probeset list with a higher false discovery rate (FDR) of 25.5 %. This high FDR is due to the number of samples. For the further analysis, we considered the probesets picked up by the two methods, *i.e.* 86 probesets corresponding to 80 genes/EST. This gene/EST list is available in supplementary Tables A ($TACI^+$ probesets) and B ($TACI^-$ probesets).

Some genes are noteworthy, particularly *c-maf, cyclin D2, integrin beta7, MAGE-A3, kappa and lambda immunoglobulin (Ig)-light chains*. The differential expression of these genes was validated with real-time RT-PCR for *TACI*, *c-maf*, *cyclin D2* and *integrin beta7* (Figure 1) and with flow-cytometry for kappa/lambda Ig (data not shown). Interestingly, 7/7 $TACI^+$ HMCL expressed lambda Ig light chains, whereas among the 11 $TACI^-$ HMCL, 6 expressed kappa chains and 5 expressed lambda chains. 46 of the 80 genes /EST (58%) mentioned above (28 genes overexpressed in $TACI^+$ HMCL and 18 genes overexpressed in $TACI^-$ HMCL) could be assigned to 8 functional categories using Gene Ontology terms (Table 1). $TACI^+$ HMCL express a higher percentage of genes coding for cell communication signals or signal transduction ($P < .05$, Table 1). Conversely, $TACI^-$ HMCL overexpressed genes coding for proteins involved in nuclear functions (Table 1).

TACI$^+$ HMCL have a gene signature of mature bone marrow plasma cells and TACI$^-$ HMCL of plasmablasts

Based on our recent finding that TACI^{high} primary MMC have a gene signature resembling normal mature BM plasma cells, whereas TACI^{low} MMC have a plasmablastic gene signature, we investigated whether TACI$^+$/TACI$^-$ HMCL keep these properties. The GEP of 7 normal BMPC and 7 normal plasmablasts were determined with U133 Affymetrix microarrays. Using an unsupervised clustering with the above-mentioned 80 genes/EST, the TACI$^+$ HMCL clustered together with BMPC whereas 6 out of 7 TACI$^-$ HMCL clustered with plasmablasts (Figure 2A). In addition, out of 80 genes/EST differentially expressed between TACI$^+$ and TACI$^-$ HMCLs, 19 are upregulated in BMPC compared to plasmablasts, and 15 in plasmablasts versus BMPC. 19 out of the 19 BMPC genes were upregulated in TACI$^+$ HMCL compared to TACI$^-$ HMCL and conversely, 11 of the 15 plasmablast-genes were overexpressed in TACI$^-$ HMCL, confirming that TACI$^+$ HMCL have a BMPC gene signature and TACI$^-$ one of plasmablast. These “BMPC” and “plasmablast-genes” are indicated in supplementary Tables C and D. In Figure 2B, we show the expression of some remarkable TACI$^+$ HMCL or TACI$^-$ HMCL genes in BMPC and PPC. TACI$^+$ HMCL overexpressed integrin beta8 that is expressed by BMPC only in normal B cell differentiation (32) (Figure 2B). In the TACI$^+$ gene signature, CX3CR1 and CD74 are also overexpressed in BMPC compared to plasmablasts (Figure 2B).

***TACI* expression is correlated with *c-maf* expression in HMCL**

TACI$^+$ HMCL showed a significantly higher mean expression of *c-maf* compared to TACI$^-$ HMCL (mean expression in TACI$^+$ of 209.7 versus 25.6 TACI$^-$ HMCL, ratio = 8.4, $P < .01$). In the TACI$^+$ HMCL, *TACI* and *c-maf* expressions correlated well (r = .94, $P < .01$) (Figure 3A). This correlation was found with expression signals

determined by Affymetrix microarrays and by real-time RT-PCR as well. We looked further for c-maf protein in 3 TACI$^+$ HMCLs and 3 TACI$^-$ HMCLs (Figure 3b). *C-maf* Affymetrix expression was significantly correlated with c-maf protein (r = .8, *P* < .05). TACI$^+$ HMCLs also showed higher expressions of *cyclin D2* (mean expression in TACI$^+$ of 2059.4 versus 588.2 TACI$^-$ HMCL, ratio = 3.5, *P* < .01) and *integrin beta7* (mean expression in TACI$^+$ of 1842.4 versus 458.5 TACI$^-$ HMCL ratio = 4, *P* < .01) (Figure 3B).

TACI influences c-*maf* expression

In order to determine whether signaling via TACI could induce *c-maf* expression, we exposed the XG-13 and XG-20 TACI$^+$ HMCL, whose growth can be stimulated by BAFF and APRIL (16), for 12-hours with BAFF and APRIL. For XG-13 and XG-20 HMCL, BAFF/APRIL stimulation induced a significant upregulation of *c-maf*, *cyclin D2* and *integrin beta7* expressions in 5 separate experiments (Figure 4A, *P* = .01, *P* = .03 and *P* = .02, respectively). In the TACI$^-$ XG-7 HMCL, in which growth or proliferation cannot be stimulated by BAFF/APRIL, no increased expression of these genes by BAFF/APRIL stimulation was found (Figure 4A). The effect of IL-6 was also investigated. For XG-7, XG-13 and XG-20, IL-6 stimulation did not modify *c-maf* and *integrin beta7* gene expressions in 5 separate experiments (Figure 4B). C*yclin D2* expression was upregulated by IL-6 stimulation in XG-13 and XG-20 cells, unlike XG-7 cells (Figure 4B). Next we investigated the RPMI8226 and LP1 cells, which produces BAFF/APRIL as autocrine growth factors (16). A blocking of the BAFF/APRIL autocrine loop by a TACI-Fc fusion protein that acts as decoy-receptor for BAFF and APRIL resulted in a reduction of *c-maf* expression by 32% in RPMI8226 cells (*P* = .01) and by 40% in LP1 cells (*P* = .005). The expression of c*yclin D2* was also reduced by 35% (*P* = .005) and 28% (*P* = .01) in these 2 HMCL

as well as that of *integrin beta7* (39% of inhibition in RPMI8226, *P* = .006 and 27% of inhibition in LP1, *P* = .01) (Figure 4C).

As RPMI8226 and LP1 could be nucleotransfected with siRNA, unlike XG HMCL, we used these tow cell lines to investigate further the link between TACI activation and c-maf expression. The nucleofection with c-maf siRNA significantly (*P* = .001) decreases the expression of *c-maf* (55 and 45% in RPMI8226 and LP1 HMCL, respectively) as well as the expression of *cyclin D2* (41 and 47% in RPMI8226 and LP1, respectively) and *integrin beta7* (40 and 35% in RPMI8226 and LP1, respectively) (*P* ≤ .05) (Figure 4D). The c-maf siRNA nucleofection did not affect *TACI* expression in these HMCL. Addition of BAFF/APRIL could not reverse the downregulation of *cyclin D2* and *integrin beta 7* expression induced by the c-maf siRNA (Figure 4E).

NF-kappaB pathway is activated by BAFF/APRIL stimulation in MMC (16). We found here that the expression of *c-maf* was not affected by a peptide inhibitor of the NF-kappaB pathway (SN50), unlike TACI-Fc (Figure 5). This SN50 peptide inhibitor efficiently inhibited NF-kappaB activation in MMC (Figure S1 in supplementary data).

Discussion

The aim of this work was to further characterize the role of TACI-expression in MM. We have previously shown that BAFF and APRIL are important growth factors for MMC, and that the respective receptors, namely TACI, BCMA and BAFF-R, show a characteristic expression pattern in MMC. BAFF-R is not expressed (33) and BCMA is expressed by all primary MMC and HMCL(25). MMC expressing only BCMA seems not to be able to bind BAFF/APRIL. Indeed, the ability of HMCL to bind BAFF is strictly restricted to $TACI^+$ HMCL (16). Interestingly, the level of TACI expression in primary MMC correlated with a characteristic phenotypic pattern, namely, $TACI^{high}$ MMC with an expression pattern resembling BMPC, and $TACI^{low}$ MMC with a plasmablastic expression pattern (25).

First we showed that these expression patterns are kept in HMCL. Using GEP determined with Affymetrix microarrays, $TACI^+$ HMCL have a gene signature of BMPC, indicative of a dependence on the microenvironment whereas $TACI^-$ HMCL have a plasmablastic gene signature. Indeed, an unsupervised clustering shows that $TACI^+$ HMCL clustered together with BMPC whereas 6 out of 7 $TACI^-$ HMCL clustered with plasmablasts. Secondly, $TACI^+$ HMCL overexpressed genes coding for cell communication, noteworthy the adhesion molecules (integrin alpha8, integrin beta2 and integrin beta7), the CX3CR1 chemokine receptor and CD74. Integrin alpha8 is an adhesion protein characteristic of terminally differentiated BMPC (32). $TACI^-$ HMCL overexpressed cancer testis antigens *MAGE-A1, MAGE-A3* and *MAGE-A6*. The thyrosine phosphatase CD45 is a marker of normal plasmablasts (34)and of proliferating plasmablastic myeloma cells (35). CD45 gene was not picked up in this study because there is only a trend (P = .01) of higher CD45 expression in $TACI^-$

HMCL (7 of 11, 64%) compared to $TACI^+$ HMCL (2 of 7, 28%) using Affymetrix data or FACS analysis.

Of note, comparing the gene lists making it possible to distinguish $TACI^+$ and $TACI^-$ HMCLs and $TACI^{high}$ and $TACI^{low}$ primary MMC - see our previous report (25)) - only 4 genes/EST were common: TACI, lambda Ig light chain, a gene coding for a cell cycle protein and one EST. In particular, *c-maf* gene was not significantly overexpressed in $TACI^{high}$ MMC and no correlation between *c-maf* and *TACI* expression in 65 primary MMC could be found (data not shown). Thus the patterns of cell communication and signaling of $TACI^{high}$ MMC and of plasmablast of $TACI^{low}$ MMC are conserved in $TACI^+$ and $TACI^-$ HMCL but not the individual genes making it possible to define these patterns. This might be explained by the fact that the clear cut expression of *TACI* found in HMCL (absent or present using real time RT-PCR or Affymetrix microarrays) is not found in primary MMC, in which *TACI* expression is always present. Using labeling of primary MMC with an anti-TACI antibody, we looked for TACI expression by primary MMC of five consecutive newly-diagnosed patients (Table S1 in supplementary data). TACI expression was heterogeneous in primary MMC patients ranging from 1.1% to 87.1% of MMC. These data suggest that there are likely MMC at different stages of dependency on the microenvironment in a given patient. This may be due to a differentiation of the MM tumor *in vivo*, eventually as the counterpart of the normal plasma cell differentiation that is poorly known. This might be also due to a proceeding oncogenic process, rendering MMC less dependent on their dependency on the microenvironment for their survival, proliferation and differentiation. When obtaining an HMCL, which is almost only possible in patients with extramedullary proliferation, only one clone of MMC, frozen at a specific stage of dependency on the BM environment, might be selected.

Driven by the observation that *TACI* and *c-maf* expressions correlated in TACI$^+$ HMCL, we have shown that TACI can signal via c-maf. Indeed, we have shown that addition or capturing of BAFF/APRIL yields an up- or a downregulation of *c-maf* expression whereas IL-6 did not affect the expression of *c-maf*. It also yields a concomitant increase or decrease of *cyclin D2* and *integrin beta7* expressions. A recent study has shown that these two genes are upregulated in response to c-maf (36). It suggested that c-maf could promote malignant transformation of plasma cells by enhanced proliferation and adhesion with BM stromal cells known to provide survival signals to plasma cells (36, 37). A regulation of *cyclin D2* and *integrin beta7* genes by c-maf was also shown in a model of murine lymphoma (38). Blocking *c-maf* RNA we confirmed that a decrease of *c-maf* mRNA levels reduce the expression of *cyclin D2* and *integrin beta7*. Blocking *c-maf* RNA did not affect *TACI* expression and addition of BAFF/APRIL could not reverse the downregulation of *cyclin D2* and *integrin beta 7* expression induced by the c-maf siRNA. These results indicate that TACI activation can upregulate *c-maf* expression, that in turn controls *cyclin D2*, and *integrin beta7* gene expressions as reported (36).

The mechanisms of regulation of *c-maf* expression are poorly known. TACI activates several transduction pathways in human myeloma cells, the ERK, PI-3-Kinase and NF-kappa B pathways (16). We show here that an inhibitor of the canonical NFκB pathway did not influence *c-maf* expression. BAFF/APRIL could also activate the non canonical NFκB pathway that could participate to the regulation of *c-maf* expression driven by TACI, in MMC. Furthermore, it was recently identified that MMC with a dysregulated expression of TACI showed increased NFκB2 p52/p100 ratios, consistent with activation of the non-canonical NFκB pathway (39). This regulation of *c-maf* expression by TACI could be explained in part by its activation of ERK that

triggers *c-maf* expression (40). But this is not the only mechanism since *c-maf* expression is not activated in some TACI$^-$ HMCL that are stimulated by IL-6, which also triggers ERK pathway (41).

Given the importance of the TACI/BAFF/APRIL pathway, we recently initiated a clinical trial with the TACI receptor fused with Ig-Fc fragment (Ares Serono, TACI-Fc5), a BAFF and APRIL inhibitor. Preliminary results indicate that TACI-Fc5 treatment decreases the level of polyclonal Ig in patients with MM (42), fostering a role of TACI/BAFF/APRIL signaling in BMPC survival. It is of interest to investigate whether the different level of TACI-expression together with the associated patterns of gene expression that we have shown to be present in MMC (25) and HMCL will transmit into differences in responsiveness to TACI-Fc5 treatment. In particular, it will be important to investigate whether in some patients the TACI-Fc treatment may select for TACI$^-$ MMC subclones with a plasmablastic gene signature.

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Figure legends

Figure 1. Validation of Affymetrix data.

Gene expressions of *TACI*, *c-maf, cyclin D2 and integrin beta7* in TACI$^+$ HMCL were assayed with real time RT-PCR and normalized with *GAPDH* expression. The correlation between Affymetrix and real-time RT-PCR values was determined with a spearman test and the coefficient correlations and *P* value are provided in the panels.

Figure 2. TACI$^+$ HMCL have a gene signature of BMPC and TACI$^-$ HMCL a plasmablastic gene signature.

(A) Hierarchical clustering of HMCL, BMPC, and PPC identifies a signature of BMPC for TACI$^+$ HMCL and a plasmablastic signature for TACI$^-$ HMCL.

Unsupervised hierarchical clustering analysis of the gene expression profiles of 18 HMCL, 7 PPC samples and 7 BMPC samples show that TACI$^-$ HMCL cluster (green) together with PPC (blue) whereas TACI$^+$ cluster (red) with normal BMPC (purple). The clustering was performed on the 80 genes/EST differentially expressed between TACI$^+$ and TACI$^-$ HMCL.

(B) Histograms show the expression of 3 TACI$^+$ HMCL and 3 TACI$^-$ HMCL genes in PPC and BMPC.

Figure 3. *TACI* and *c-maf* expressions are correlated in HMCL.

(A) Correlation between *TACI* and *c-maf* expressions in TACI$^+$ HMCL using Affymetrix microarrays or real time RT-PCR.

(B) Expression level of *c-maf*, *cyclin D2* and *integrin beta7* in TACI$^+$ and TACI$^-$ HMCL using Affymetrix microarrays.

(C) Expression level of TACI and c-maf in HMCL using Affymetrix microarrays, western blot and flow cytometry. For each cell line, the ratios of c-maf and beta actin

proteins were determined in order to compare c-maf protein expression between cell lines.

Figure 4. BAFF and APRIL regulate the expression of *c-maf*, *cyclin D2* and *integrin beta7* in TACI$^+$ HMCL.

(A) BAFF/APRIL upregulate *c-maf*, *cyclin D2* and *integrin beta7* expressions in the XG-20 and XG-13 TACI$^+$ HMCL unlike the XG-7 TACI$^-$ HMCL. C*yclin D2* and *integrin beta7* expressions were determined by real-time RT-PCR and normalized with *GAPDH* expression. For each experiment, the expression of the studied gene in BAFF/APRIL stimulated myeloma cells was compared to that of untreated myeloma cells that was assigned the 1 arbitrary value. Data are mean values of 5 independent experiments. * The mean value is statistically significantly different from that obtained without BAFF/APRIL stimulation (control) using a Student *t* test ($P \leq .05$).

(B) C*yclin D2* and *integrin beta7* expressions were determined by real-time RT-PCR with or without IL-6 stimulation for XG-13, XG-20 and XG-7 HMCL. Expression values were normalized using those of *GAPDH*. For each experiment, the expression of the studied gene in IL-6 stimulated myeloma cells was compared to that of untreated myeloma cells that was assigned the 1 arbitrary value. Data are mean values of 5 independent experiments. * The mean value is statistically significantly different from that obtained without IL-6 stimulation (control) using a Student *t* test ($P \leq .05$).

(C) BAFF/APRIL deprivation using TACI-Fc inhibitor induces a downregulation of *c-maf*, *cyclin D2* and *integrin beta7* expressions in RPMI 8226 and LP1 HMCL. *C-maf*, *cyclin D2* and *integrin beta7* expressions were determined by real-time RT-PCR and normalized with *GAPDH* expression. For each experiment, the expression of the studied gene in TACI-Fc treated myeloma cells was compared to that of untreated

myeloma cells that was assigned the 1 arbitrary value.. Data are mean values of 5 independent experiments. * The mean value is statistically significantly different from that obtained without TACI-Fc inhibitor (control) using a Student *t* test ($P \leq .05$).

(D) Real time RT-PCR assay for *c-maf*, *cyclin D, integrin beta7* and *TACI* expressions in RPMI8226 and LP1 HMCL 24 hours after being transduced with a *c-maf* siRNA oligonucleotide. Data are mean values of 5 independent experiments. * Mean value is statistically significantly different from that obtained without siRNA *c-maf* using a Student *t* test ($P \leq .05$).

(E) Real time RT-PCR assay for *c-maf*, *cyclin D2* and *integrin beta7* expressions in LP1 HMCL 24 hours after being transduced with a *c-maf* siRNA oligonucleotide and cultured with or without BAFF/APRIL. Data are mean values of 5 independent experiments. * Mean value is statistically significantly different from that obtained without siRNA *c-maf* using a Student *t* test ($P \leq .05$).

Figure 5. *C-maf* regulation by TACI is nor linked to NF-kappaB pathway.

Real time RT-PCR assay for *c-maf* expression in RPMI8226 and LP1 cells cultured with the TACI-Fc inhibitor, the SN50 NF-kappaB inhibitor (100 µg/ml), or the SN50 inactive peptide control (IC) (100 µg/ml). Expression values were normalized using those of *GAPDH*. For each experiment, the expression of the studied gene in IL-6 stimulated myeloma cells was compared to that of untreated myeloma cells that was assigned the 1 arbitrary value. Data are mean values of 5 independent experiments. * Mean value is statistically significantly different from that obtained without inhibitor using a Student t test ($P \leq .05$).

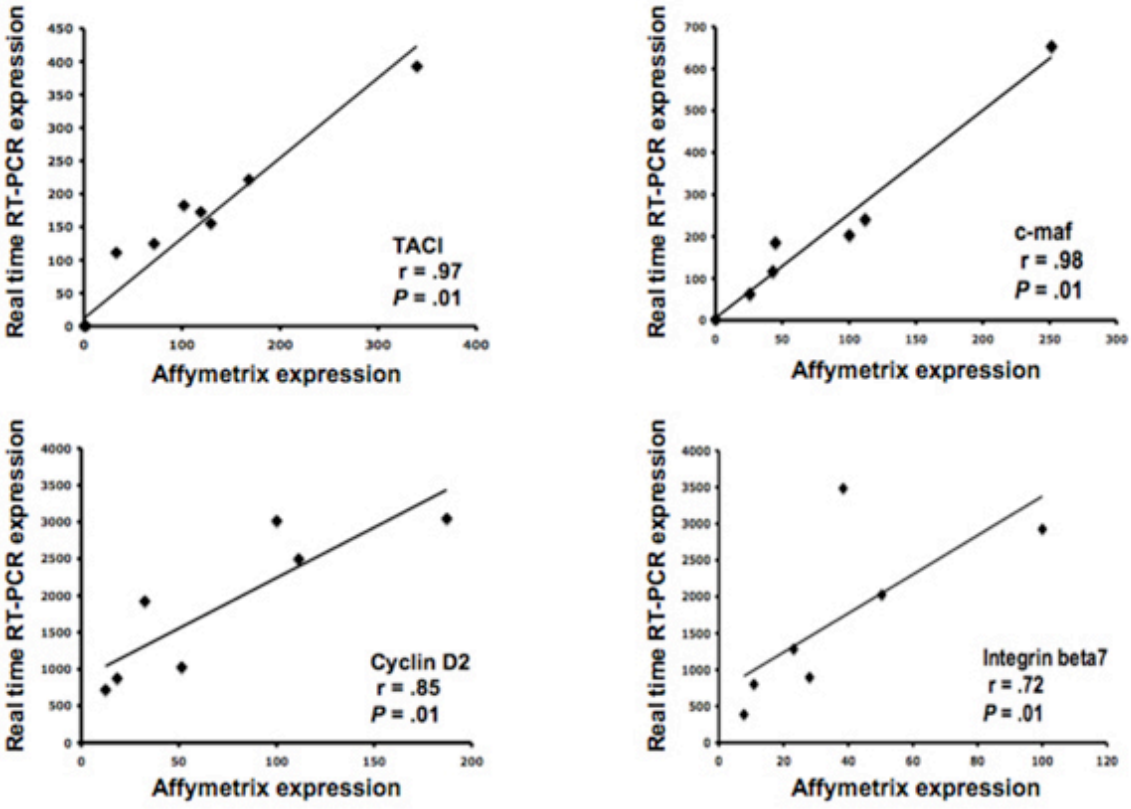
Table 1 : Cell communication signature in TACI$^+$ HMCL and plasmablastic signature in TACI$^-$ HMCL

	TACI$^+$	TACI$^-$	
Genes coding for protein implicated in:			
Cell communication signals (N = 8)	28.6 %	0 %	*P* < .01
Cytosqueleton (N = 3)	10.6 %	0 %	NS
Transduction signals (N = 10)	35.7 %	0 %	*P* < .01
Protein synthesis and regulation (N =2)	3.6 %	5.5 %	NS
Cell cycle (N = 2)	3.6 %	5.5 %	NS
Metabolism (N = 6)	14.3 %	11 %	NS
Cancer-testis antigens (N = 3)	0 %	16.5 %	NS
Nuclear functions (N = 12)	3.6 %	61.1 %	*P* < .01
Total of classified genes (N= 46)	100% (n = 28)	100% (n = 18)	

Of the 80 genes/EST differentially expressed between the TACI$^+$ and TACI$^-$ HMCL, 46 could be assigned to 8 functional categories using Gene Ontology terms. Data are the percentage of genes of a given category compared with the total number of TACI$^+$ (28 genes) or TACI$^-$ (18 genes) genes.

NS indicates not significant.

Figures



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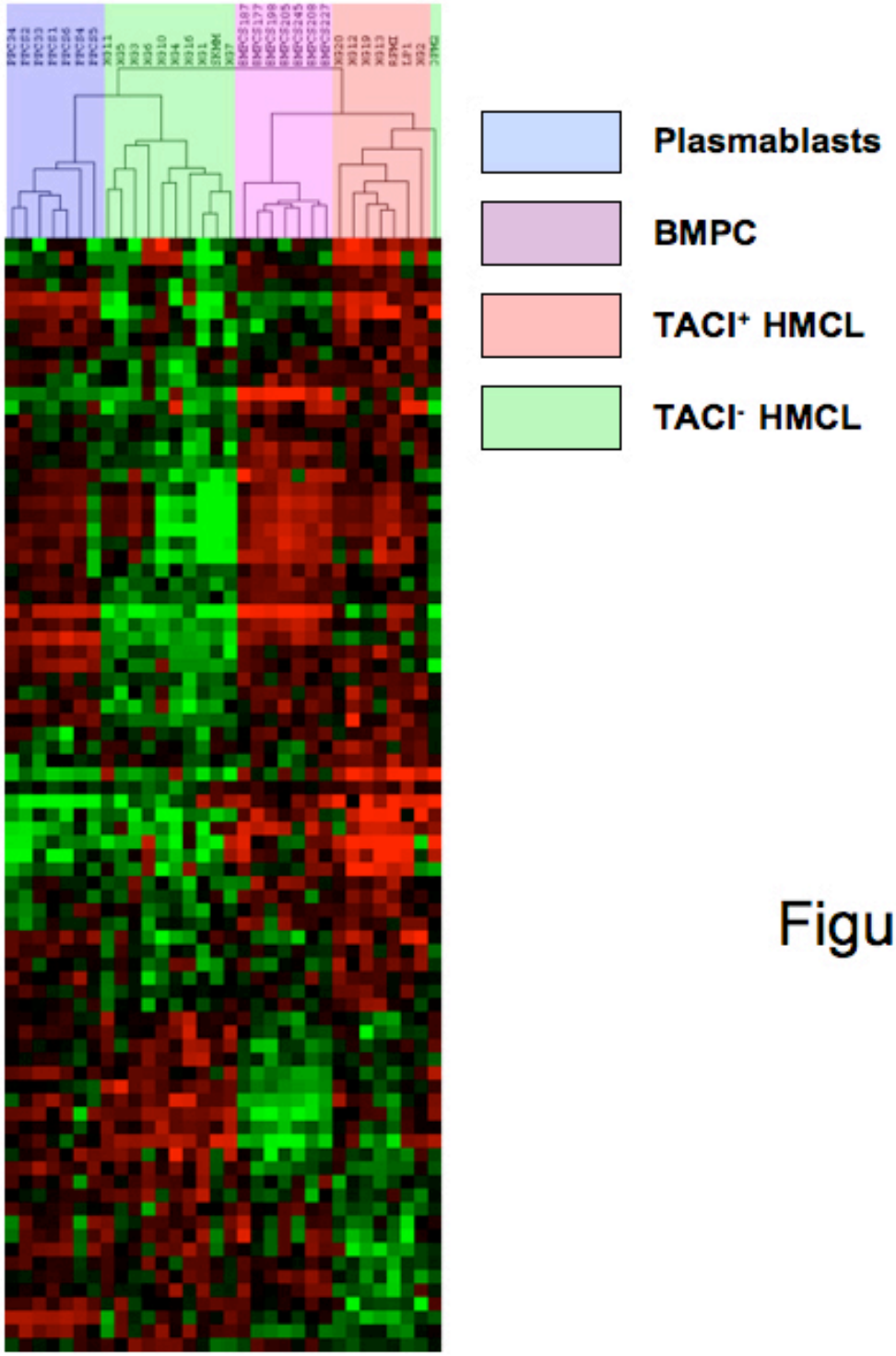


Figure 2A

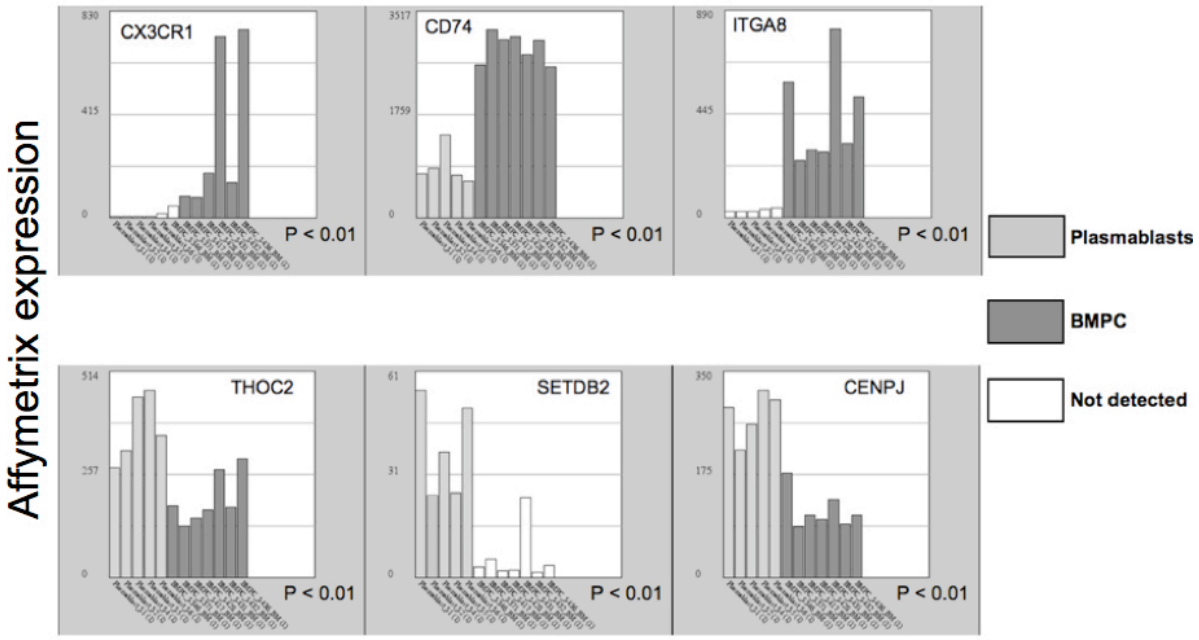


Figure 2B

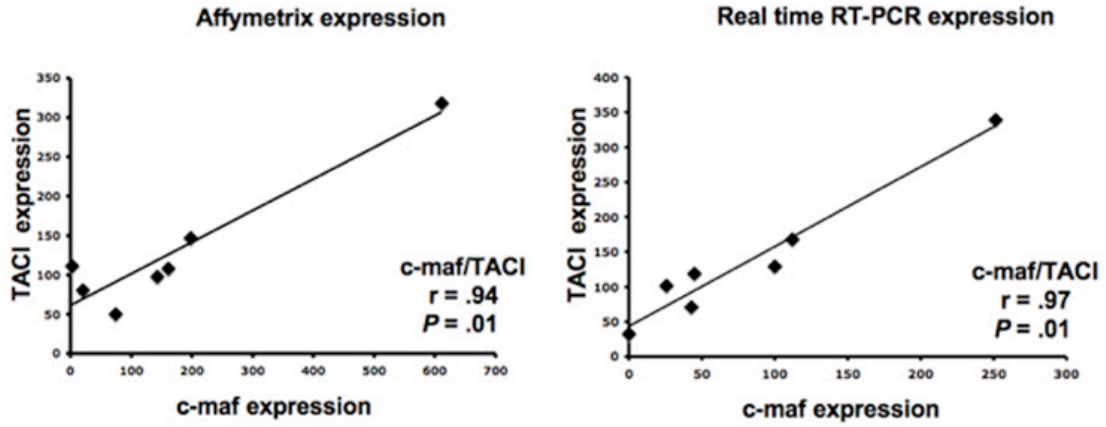
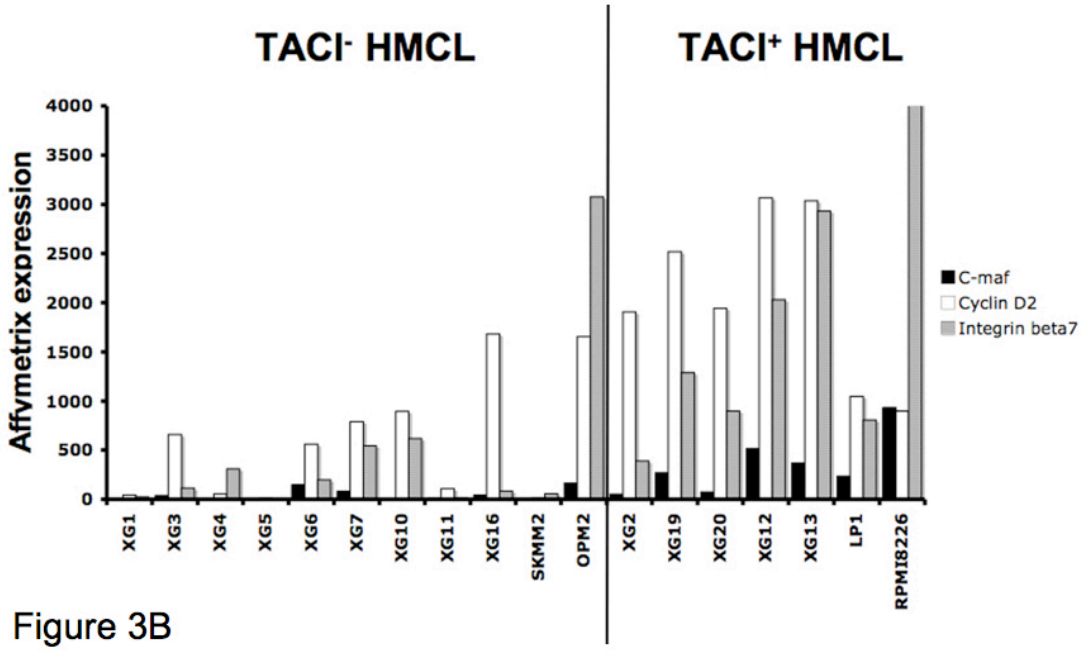


Figure 3A



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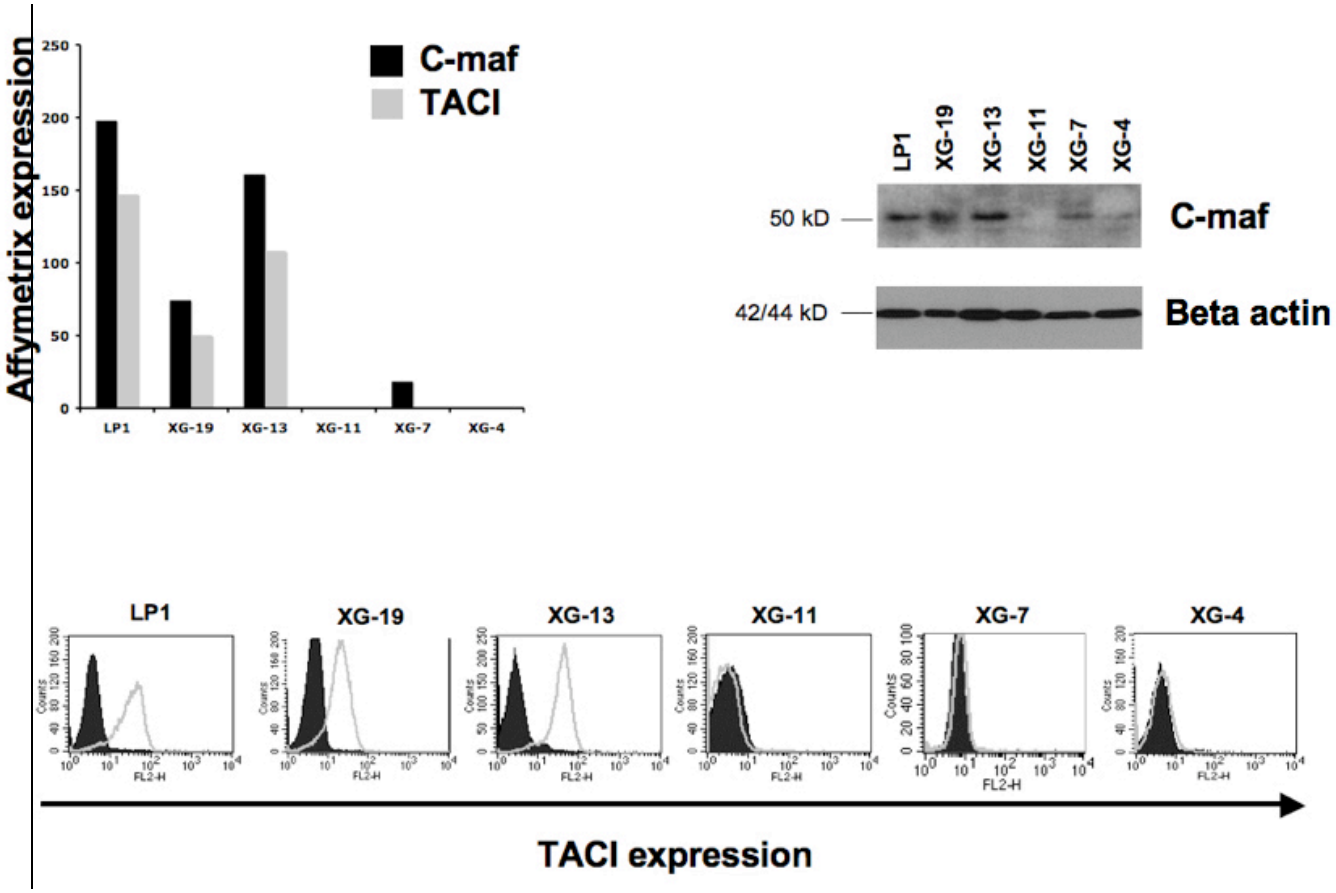


Figure 3C

Figure 4A

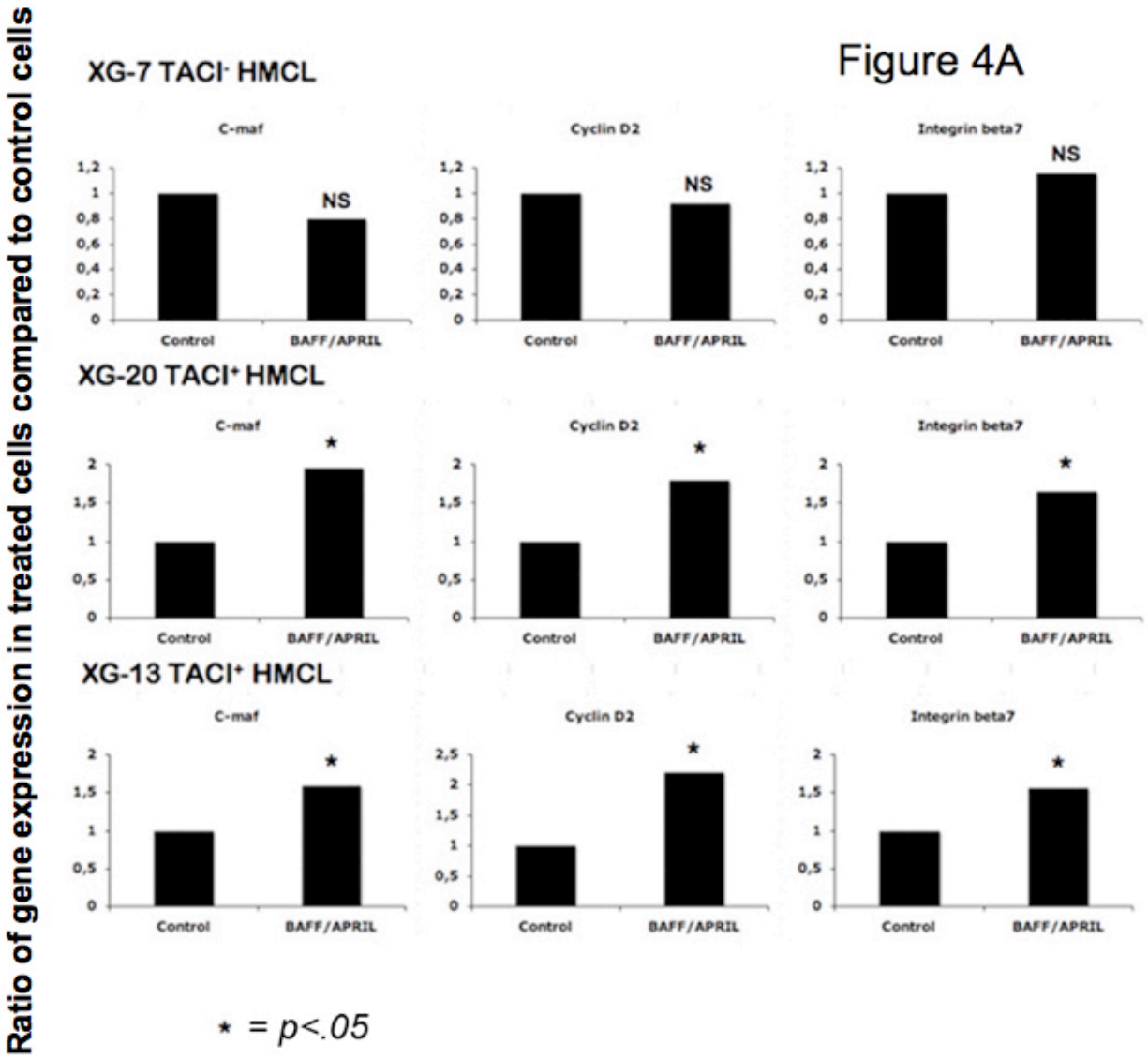
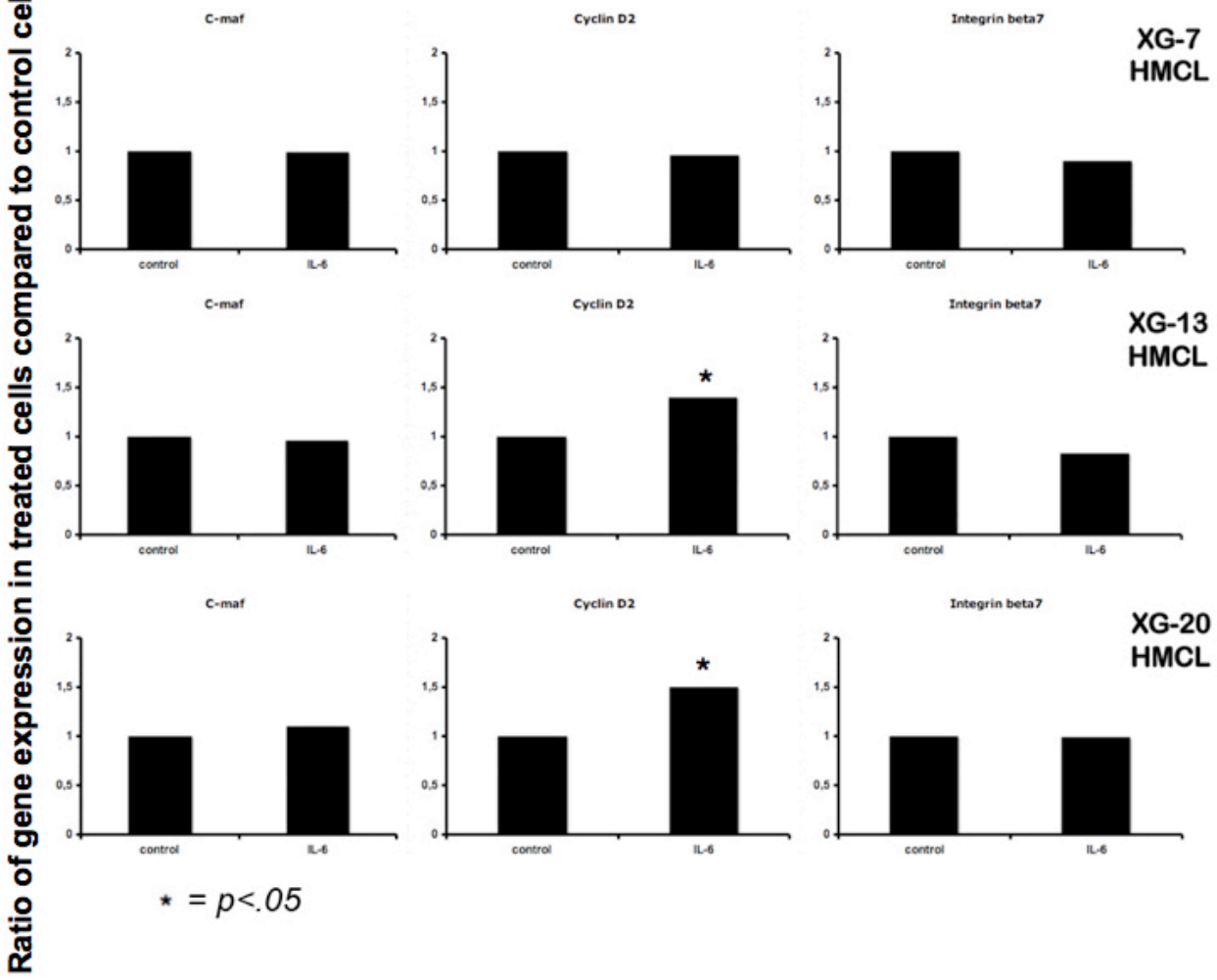


Figure 4B



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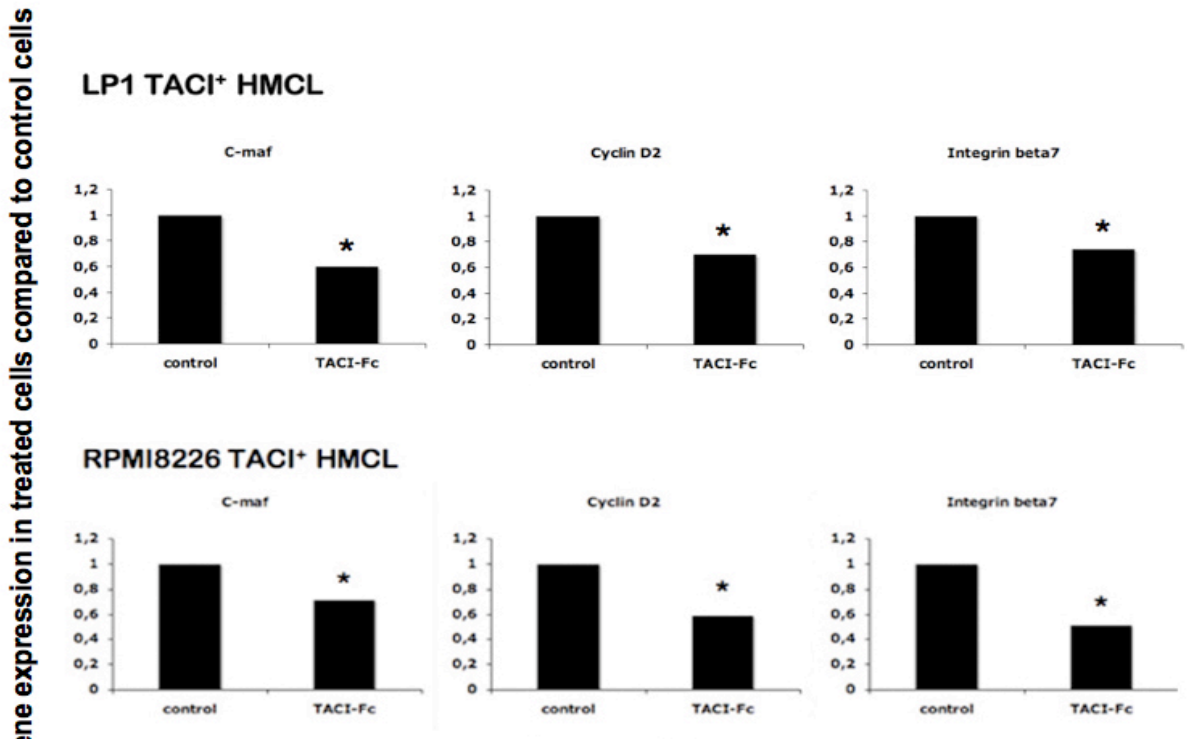

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Figure 4C

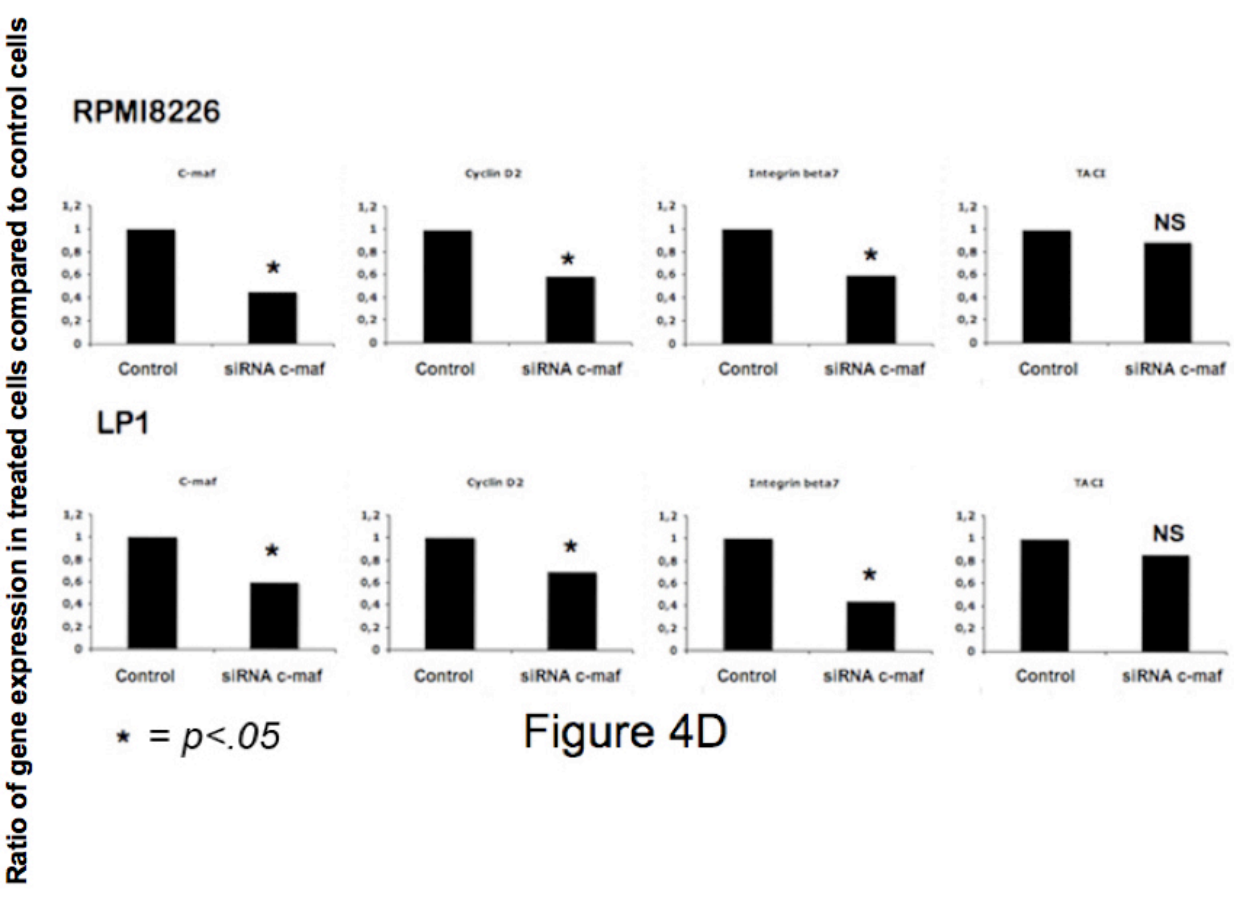

Figure 4D

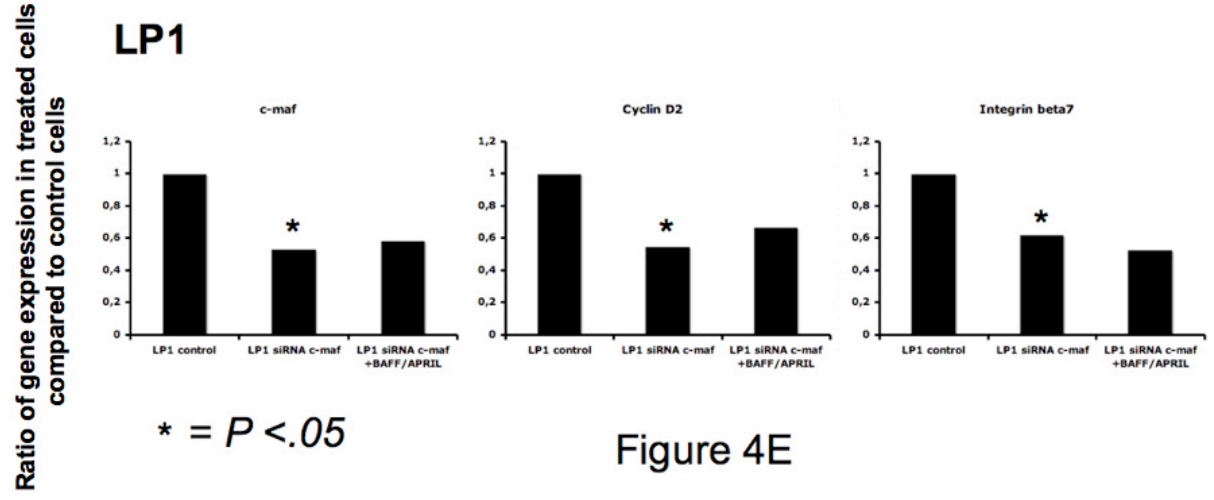

Figure 4E

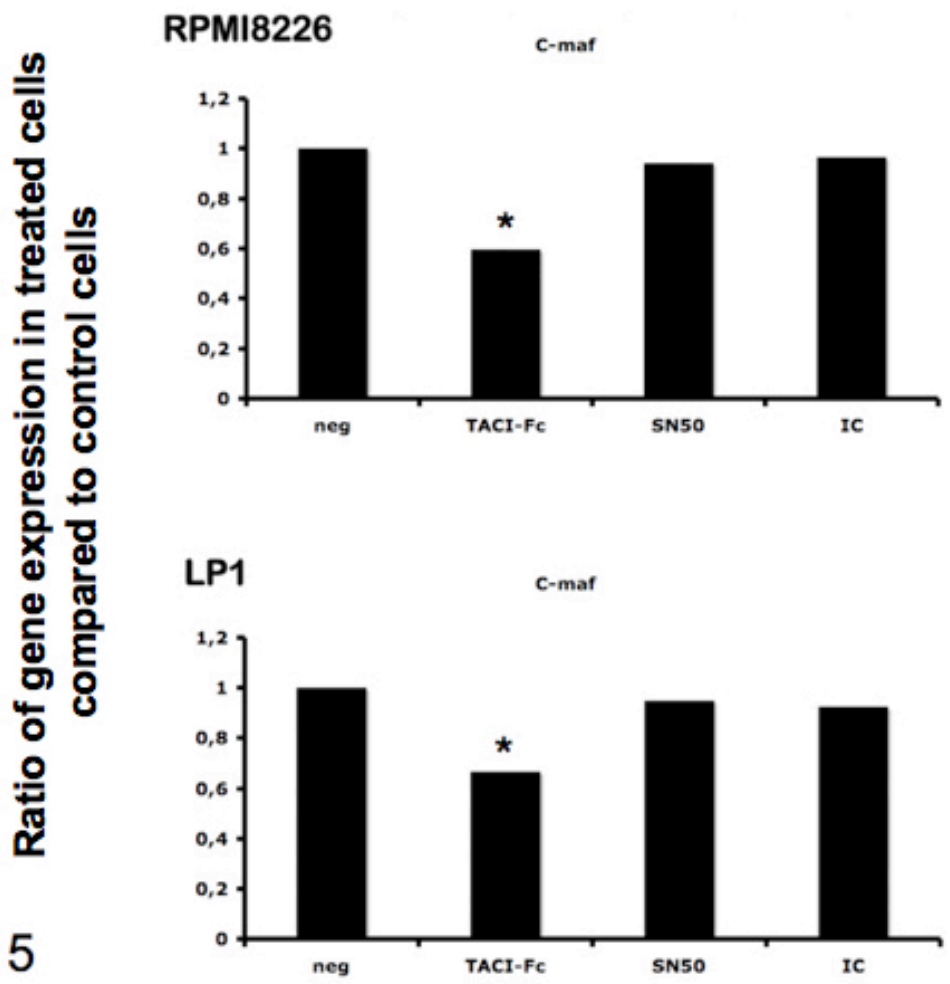

Figure 5

* = $p<.05$