TACI expression is associated with a mature bone marrow plasma cell signature and C-MAF overexpression in human myeloma cell lines

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ABSTRACT

Background and Objectives

BAFF and APRIL stimulate the growth of multiple myeloma (MM) cells. 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 MM cells, the level of TACI expression correlates with a characteristic phenotypic pattern: $TACI^{high}$ MM cells resemble bone marrow plasma cells and $TACI^{low}$ resemble plasmablasts. The aim of this study was to further characterize the role of TACI expression in MM

Design and Methods

Using gene expression profiling, we investigated whether these patterns are kept in $TACI^+$ or $TACI^-$ HMCL.

Results

Eighty genes/EST interrogated by Affymetrix microarrays were differentially expressed between $TACI^+$ and $TACI^-$ HMCL, particularly c-maf, cyclin D2, and integrin β7. Triggered by the finding that TACI and *c-maf* expressions correlate in $TACI^+$ HMCL, we demonstrated that TACI activation influences *c-maf* expression: (i) activation of TACI by BAFF or APRIL increases *c-maf*, *cyclin D2*, and *integrin β7* gene expressions in $TACI^+$ HMCL, (ii) blocking of autocrine BAFF/APRIL stimulation in some $TACI^+$ HMCL by the TACI-Fc fusion protein reduces *c-maf*, *cyclin D2*, and *integrin β7* gene expression, (iii) nucleofection of siRNA to *c-maf* decreases *c-maf* mRNA levels and reduces the expression of *cyclin D2* and integrin β7 gene expressions, without affecting TACI expression

Interpretation and Conclusions

We conclude that TACI activation can upregulate *c-maf* expression which, in turn, controls *cyclin D2*, and *integrin β7* gene expression.

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

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Multiple myeloma (MM) is an incurable plasma cell 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 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 the 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 depends on the autocrine and paracrine stimulation by growth factors, such as interleukin-6 (IL-6),[5] interferon α,[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 three receptors - BAFF-R, BCMA and TACI -β 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 found that BAFF and APRIL activate MAPK, PI3K/AKT and NFκB pathways in MMC leading to an upregulation of Mcl-1 and Bcl-2 anti-apoptotic proteins.[16] Recently Tai *et al.* showed that MMC express BCMA and TACI but very low levels of BAFF-R.[24] They demonstrated that BAFF induces activation of NFκB and PI3K/AKT pathways confirming our previous results. Furthermore, they showed that 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 BMPC dependent 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]

Design 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 HMCL were obtained and characterized in our laboratory.[26-29] SKMM, OPM2, LP1 and RPMI8226 were purchased from ATTC (Rockville, MD, USA). Normal bone marrow specimens 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 previously.[25] Polyclonal plasmablasts were generated by differentiating peripheral blood CD19^{+} B cells *in vitro*.[30]

Flow cytometry analysis

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

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, USA) and APRIL (R&D Systems, Abington, UK) (200 ng/mL each) were added in one culture group for 12 hours in RPMI1640/10% fetal calf serum (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 was extracted for gene expression profiling or real-time polymerase chain reaction (PCR) analysis.

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

To investigate the modulation of gene expression by NF-κB pathway inhibition, RPMI8226 and LP1 cells were cultured for 12 hours with an inhibitory peptide of the NF-κB pathway (100 μg/mL, SN50) or the corresponding inactive peptide (BIOMOL, Plymouth Meeting, PA, USA), 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 double *in vitro* transcription and hybridized to the Affymetrix HG U133 set microarrays, according to the manufacturer's instructions (Affymetrix, Santa Clara, CA, USA). 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 $ZnCl_2$, 100 μM Na_3VO_4, 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 monoclonal antibody (Abnova, Taiwan, China) 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, USA) peroxidase-conjugated antibodies using an enhanced chemiluminescence detection system. As a control for protein loading, we used anti-β actin (1:2000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) 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 reverse transcriptase (RT)-PCR

Total RNA was converted to cDNA using Superscript II reverse transcriptase (Invitrogen, Cergy Pontoise, France). The assays-on-demand primers and probes and the TaqMan Universal Master Mix were used according to the manufacturer's instructions (Applied Biosystems, Courtaboeuf, France). Gene expression was measured 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 the log phase of the cycle. The levels of genes interest 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 δδCt = 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 SAM (significance analysis of microarrays) software.[31] Statistical comparisons were done with Mann-Whitney, χ^2, or Student's t-tests. Probe sets differentially expressed between $TACI^+$ and $TACI^-$ HMCL were picked by two methods. First, we selected 109 probes ets that were differentially expressed between $TACI^+$ and $TACI^-$ HMCL with a Mann-Whitney test ($p \geq 0.01$) 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 probe sets with the three-presence and two-ratio filters. This SAM selection yielded 330 probe sets with a false discovery rate of 25.5% using 100 permutations. Crossing the two gene lists yielded 86 probe sets, corresponding to 80 genes/EST, which 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 seven $TACI^+$ HMCL and of 11 $TACI^-$ HMCL. One hundred and nine probe sets out of the 49,000 interrogated with U133 set Affymetrix microarrays were differentially expressed between $TACI^+$ and $TACI^-$ HMCL ($p \leq 0.01$ with a Mann Whitney test; ratio of the mean expressions ≥2 or ≤0.5). The analysis performed on the same samples using the SAM software with three-presence and a two-ratio filters on probe sets and 1000 permutations led to a larger 330 probe set list with a higher false discovery rate of 25.5%. This high false discovery rate is due to the number of samples. For the further analysis, we considered the probe sets picked up by the two methods, i.e. 86 probe sets corresponding to 80 genes/EST. This gene/EST list is available in supplementary Tables A (TACI+ probesets) and B (TACI- probe sets).

Some genes are noteworthy, particularly *c-maf*, *cyclin D2*, *integrin β7*, *MAGE-A3*, κ and λ immunoglobulin (Ig)-light

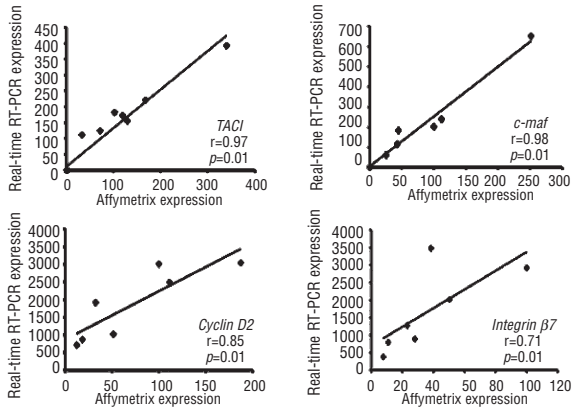

Figure 1. Validation of Affymetrix data. Gene expressions of *TACI*, *c-maf*, *cyclin D2* and *integrin β7* in TACI$^+$ HMCL were assayed with real time RT-PCR and normalized to *GAPDH* expression. The correlation between Affymetrix and real-time RT-PCR values was determined with a Spearman's test and the coefficient correlations and *p* value are provided in the panels.

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

	TACI$^+$ (%)	*TACI*$^-$ (%)	*p*
Genes coding for protein implicated in:			
Cell communication signals (N=8)	28.6	0	<0.01
Cytoskeleton (N=3)	10.6	0	NS
Transduction signals (N=10)	35.7	0	<0.01
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	<0.01
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 eight 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.

chains. The differential expression of these genes was validated with real-time RT-PCR for *TACI*, *c-maf*, *cyclin D2* and *integrin β7* (Figure 1) and with flow-cytometry for κ/λ Ig (*data not shown*). Interestingly, 7/7 TACI$^+$ HMCL expressed λ Ig light chains, whereas among the 11 TACI$^-$ HMCL, six expressed κ chains and five expressed λ chains. Forty-six 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 eight functional categories using gene ontology terms (Table 1). TACI+ HMCL express a higher percentage of genes coding for cell communication signals or signal transduction ($p<0.05$, 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 BMPC, whereas TACI^{low} MMC have a plasmablastic gene signature, we investigated whether TACI$^+$/TACI$^-$ HMCL keep these properties. The gene expression profile of seven normal BMPC and 7 normal plasmablasts were determined with U133 Affymetrix microarrays. Using unsupervised clustering with the above-mentioned 80 genes/EST, the TACI$^+$ HMCL clustered together with BMPC whereas six out of seven 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. Nineteen 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$^-$ have a plasmablast signature. These *BMPC* and *plasmablast "genes"* are indicated in supplementary Tables C and D. Figure 2B shows the expression of some remarkable TACI$^+$ HMCL or TACI$^-$ HMCL genes in BMPC and PPC. TACI$^+$ HMCL overexpressed integrin β8, which 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 in 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 in TACI$^-$ HMCL, ratio=8.4, $p<0.01$). In the TACI$^+$ HMCL, TACI and *c-maf* expressions correlated well (r =0.94, $p<0.01$) (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 three TACI$^+$ HMCLs and three TACI$^-$ HMCL (Figure 3B). C-maf Affymetrix expression was significantly correlated with c-maf protein (r=0.8, $p<0.05$). TACI$^+$ HMCL also showed higher expressions of cyclin D2 (mean expression in TACI$^+$ of 2059.4 versus 588.2 TACI$^-$ HMCL, ratio = 3.5, $p<0.01$) and integrin β7 (mean expression in TACI$^+$ of 1842.4 versus 458.5 in TACI$^-$ HMCL, ratio=4, $p<0.01$) (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 to BAFF and APRIL. For XG-13 and XG-20 HMCL, BAFF/APRIL stimulation induced a significant upregulation of *c-maf*, *cyclin D2* and *integrin β7* expressions in five separate experiments (Figure 4A, p=0.01, p=0.03 and p=0.02, 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).

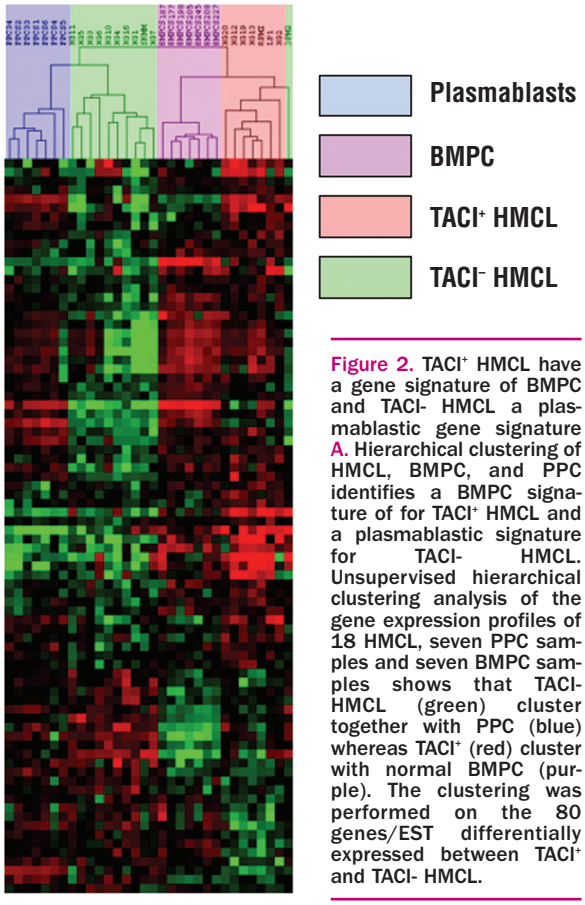

Figure 2. TACI⁺ HMCL have a gene signature of BMPC and TACI- HMCL a plasmablastic gene signature A. Hierarchical clustering of HMCL, BMPC, and PPC identifies a BMPC signature of for TACI⁺ HMCL and a plasmablastic signature for TACI- HMCL. Unsupervised hierarchical clustering analysis of the gene expression profiles of 18 HMCL, seven PPC samples and seven BMPC samples shows that TACI- HMCL (green) cluster together with PPC (blue) whereas TACI⁺ (red) cluster with normal BMPC (purple). The clustering was performed on the 80 genes/EST differentially expressed between TACI⁺ and TACI- HMCL.

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 β7* gene expressions in 5 separate experiments (Figure 4B). *Cyclin 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 produce BAFF/APRIL as autocrine growth factors.[16] Blockade 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=0.01) and by 40% in LP1 cells (p=0.005). The expression of *cyclin D2* was also reduced by 35% (p=0.005) and 28% (p=0.01) in these two HMCL as was that of *integrin β7* (39% of inhibition in RPMI8226, p=0.006 and 27% of inhibition in LP1, p=0.01) (Figure 4C).

As RPMI8226 and LP1 could be nucleotransfected with siRNA, unlike XG HMCL, we used these two cell lines to investigate further the link between TACI activation and c-maf expression. The nucleofection with c-maf siRNA significantly (p=0.001) decreased 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 β7* (40 and 35% in RPMI8226 and LP1, respectively) ($p \leq 0.05$) (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 β7 expression induced by the c-maf siRNA (Figure 4E).

The NF-κB 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-κB pathway (SN50), unlike TACI-Fc (Figure 5). This SN50 peptide inhibitor efficiently inhibited NF-κB 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

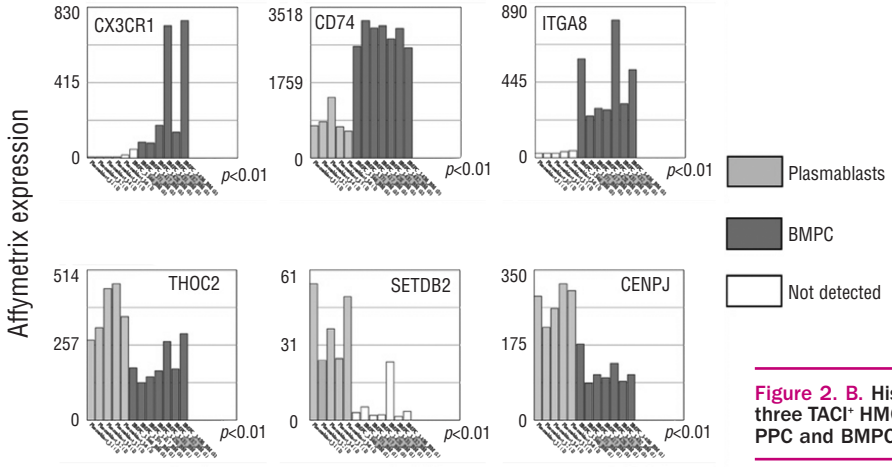

Figure 2. B. Histograms show the expression of three TACI⁺ HMCL and three TACI⁻ HMCL genes in PPC and BMPC.

shown that BAFF and APRIL are important growth factors for MMC, and that their 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 seem not to be able to bind BAFF/APRIL. Indeed, the ability of HMCL to bind BAFF is strictly restricted to TAC$^+$ HMCL.[16] Interestingly, the level of TACI expression in primary MMC correlated with a characteristic phenotypic pattern, namely, TACIhigh MMC with an expression pattern resembling BMPC, and TACIlow MMC with a plasmablastic expression pattern.[25]

First we showed that these expression patterns are maintained in HMCL. Using gene expression profiling 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, unsupervised clustering shows that TACI$^+$ HMCL clustered together with BMPC whereas six out of seven TACI$^-$ HMCL clustered with plasmablasts. Secondly, TACI$^+$ HMCL overexpressed genes coding for cell communication, noteworthy the adhesion molecules (integrin α8, integrin β2 and integrin β7), the CX3CR1 chemokine receptor and CD74. Integrin α8 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] The CD45 gene was not picked up in this study because there is only a trend (p=0.01) 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$^-$ HMCL and TACI^{high} and TACI^{low} primary MMC - see our previous report[25] - only 4 genes/EST were common to the two lists: TACI, λ 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 TACIhigh MMC and of plasmablasts 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 from 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*, possibly as the counterpart of normal plasma

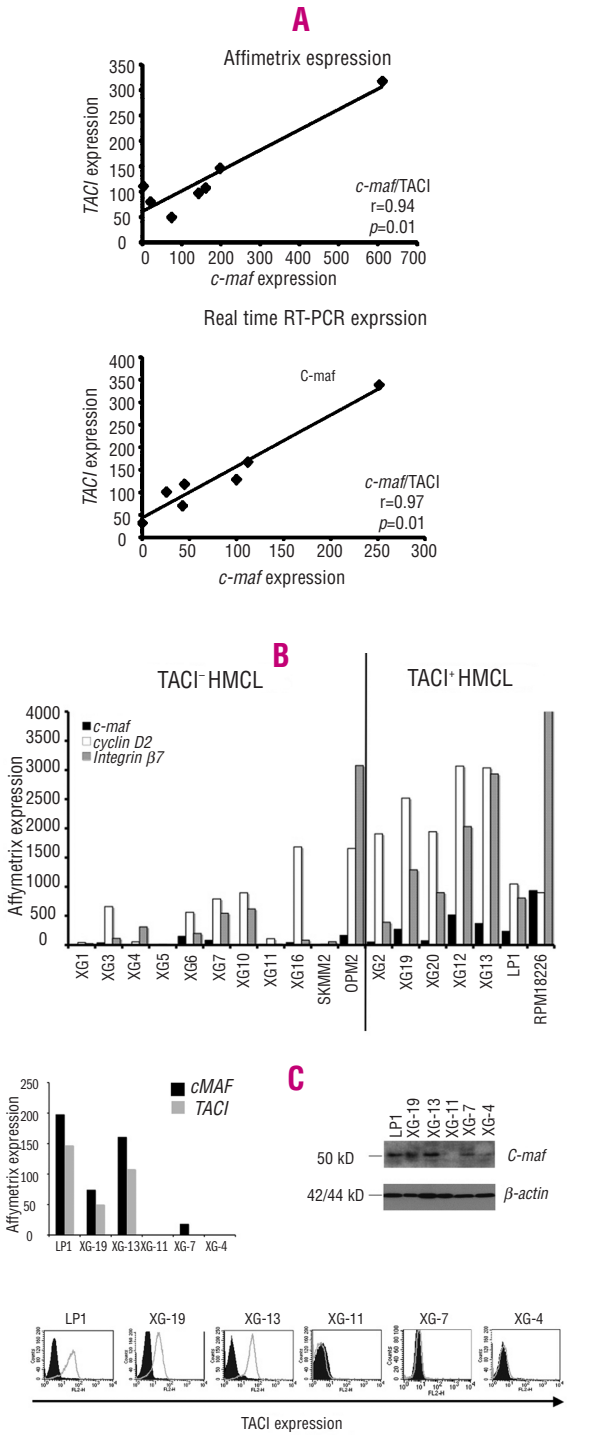

Figure 3. ***TACI*** **and** ***c-maf*** **expressions are correlated in HMCL. A. Correlation between TACI and c-maf expressions in TACI$^+$ HMCL using Affymetrix microarrays or real time RT-PCR. A. Expression levels of *c-maf*, *cyclin D2* and *integrin β7* in TACI$^+$ and TACI$^-$ HMCL using Affymetrix microarrays. C. Expression levels 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 β actin proteins were determined in order to compare *c-maf* protein expression between cell lines.**

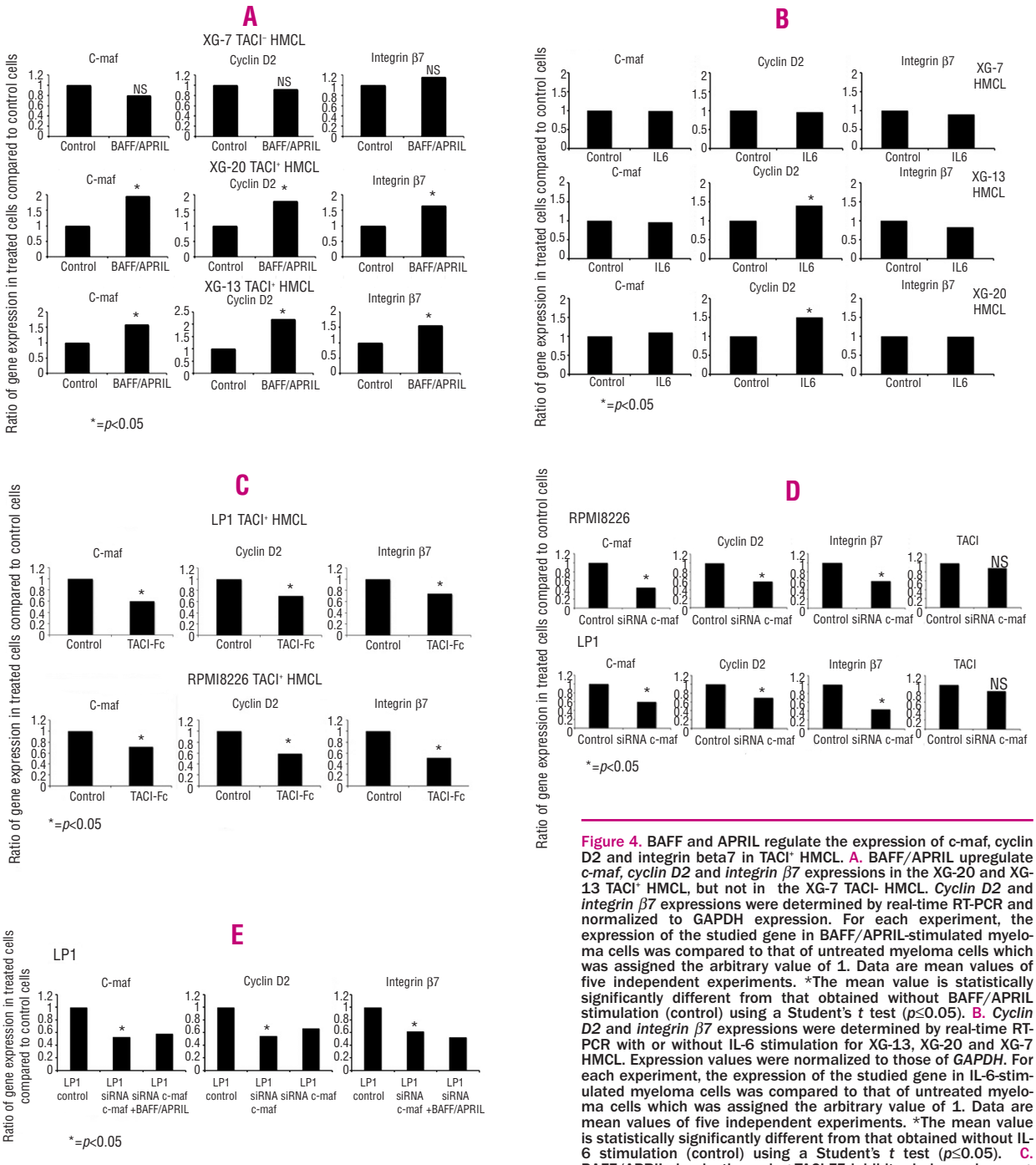

Figure 4. BAFF and APRIL regulate the expression of c-maf, cyclin D2 and integrin beta7 in TACI⁺ HMCL. **A.** BAFF/APRIL upregulate *c-maf*, *cyclin D2* and *integrin β7* expressions in the XG-20 and XG-13 TACI⁺ HMCL, but not in the XG-7 TACI- HMCL. *Cyclin D2* and *integrin β7* expressions were determined by real-time RT-PCR and normalized to 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 which was assigned the arbitrary value of 1. Data are mean values of five independent experiments. *The mean value is statistically significantly different from that obtained without BAFF/APRIL stimulation (control) using a Student's *t* test ($p \leq 0.05$). **B.** *Cyclin D2* and *integrin β7* 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 to 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 which was assigned the arbitrary value of 1. Data are mean values of five independent experiments. *The mean value is statistically significantly different from that obtained without IL-6 stimulation (control) using a Student's *t* test ($p \leq 0.05$). **C.** BAFF/APRIL deprivation using TACI-F5 inhibitor induces downregulation of *c-maf*, *cyclin D2* and *integrin β7* expressions in RPMI 8226 and LP1 HMCL. C-maf, cyclin D2 and integrin beta7 expressions were determined by real-time RT-PCR and normalized to *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 which was assigned the arbitrary value of 1. Data are mean values of five independent experiments. *The mean value is statistically significantly different from that obtained without TACI-F5 inhibitor (control) using a Student's *t* test ($p \leq 0.05$). **D.** Real time RT-PCR assay for *c-maf*, *cyclin D*, *integrin β7* 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's *t* test ($p \leq 0.05$). **E.** Real time RT-PCR assay for *c-maf*, *cyclin D2* and *integrin β7* 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. *The mean value is statistically significantly different from that obtained without siRNA *c-maf* using a Student t test ($p \leq 0.05$).

cell differentiation, which is poorly understood. This might also be due to a proceeding oncogenic process, rendering MMC less dependent 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 bone marrow 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 produces up- or a downregulation of *c-maf* expression whereas IL-6 did not affect the expression

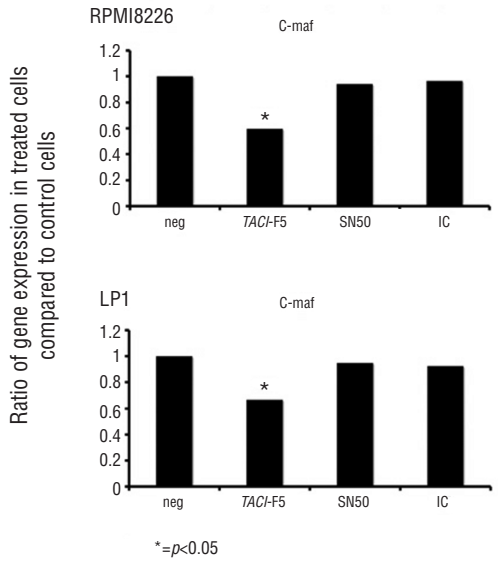

Figure 5. ***C-maf*** **regulation by TACI is not linked to the NF-κB pathway. Real-time RT-PCR assay for c-maf expression in RPMI8226 and LP1 cells cultured with the TACI-F5 inhibitor, the SN50 NF-κB inhibitor (100 μg/mL), or the SN50 inactive peptide control (IC) (100 μg/mL). Expression values were 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 which was assigned the arbitrary value of 1. Data are mean values of five independent experiments. *The mean value is statistically significantly different from that obtained without inhibitor using a Student's *t* test ($p \leq 0.05$).**

of *c-maf*. It also produce a concomitant increase or decrease of *cyclin D2* and *integrin β7* expressions. A recent study has shown that these two genes are upregulated in response to *c-maf*.[36] It has been suggested that *c-maf* could promote malignant transformation of plasma cells by enhanced proliferation and adhesion with bone marrow stromal cells known to provide survival signals to plasma cells.[36,37] Regulation of *cyclin D2* and *integrin β7* 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 β7*. Blocking *c-maf* RNA did not affect TACI expression and addition of BAFF/APRIL could not reverse the downregulation of cyclin D2 and integrin β7 expression induced by the *c-maf* siRNA. These results indicate that TACI activation can upregulate *c-maf* expression which, in turn, controls *cyclin D2*, and *integrin β7* gene expression as reported.[36]

The mechanisms of regulation of *c-maf* expression are poorly understood. TACI activates several transduction pathways in human myeloma cells, the ERK, PI-3-Kinase and NF-κ 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 found 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 which triggers *c-maf* expression.[40] However, 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 the 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] supporting a role for TACI/BAFF/APRIL signaling in BMPC survival. It will be 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 translate into differences in responsiveness to TACI-Fc5 treatment. In particular, it will be important to investigate whether, in some patients, TACI-Fc5 treatment may select for TACI- MMC subclones with a plasmablastic gene signature.

Authors' Contributions

JM: performed the experiments and wrote the paper; BK supervised the project and wrote the paper; DH, HG, MM and MJ provided with bone marrow plasma cells and/or revised the paper; JDV and TR developed the bioinformatics tools and revised the paper; NR and PM provided with technical assistance.

Conflict of Interest

The authors reported no potential conflicts of interest.

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