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THE LEVEL OF TACI GENE EXPRESSION IN MYELOMA CELLS IS ASSOCIATED WITH A SIGNATURE OF MICROENVIRONMENT DEPENDENCE VERSUS A PLASMABLASTIC SIGNATURE

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Abstract

BAFF and APRIL have been shown to promote multiple myeloma (MM) cell growth. We show that the main site of production for BAFF and APRIL is the bone marrow (BM) environment, mainly by monocytes and neutrophils. In addition, osteoclasts produce very high levels of APRIL, unlike BM stromal cells. Myeloma cells (MMC) express TACI, the receptor of BAFF/APRIL at varying levels. *TACI* expression is a good indicator of a BAFF-binding receptor. Expression data of purified MMC from 65 newly-diagnosed patients have been generated using Affymetrix microarrays and were analyzed by supervised clustering of groups with higher ($TACI^{high}$) vs. lower *TACI* ($TACI^{low}$) expression levels. Patients in the $TACI^{low}$ group had clinical parameters associated with bad prognosis. A set of 659 genes was differentially expressed between $TACI^{high}$ and $TACI^{low}$ MMC. This set makes it possible to efficiently classify $TACI^{high}$ and $TACI^{low}$ MMC in an independent cohort of 40 patients. $TACI^{high}$ MMC displayed a mature plasma cell gene signature, indicating dependence on the BM environment. In contrast, the $TACI^{low}$ group had a gene signature of plasmablasts suggesting an attenuated dependence on the BM environment. Taken together, our findings suggest using gene expression profiling to identify the group of patients which might benefit most from treatment with BAFF/APRIL inhibitors.

Introduction

Multiple myeloma (MM) is a universally fatal neoplasia characterized by the accumulation of malignant plasma cells (MMC) in the BM ¹. There is increasing evidence for a major role of interactions between MMC and BM stroma for the support of survival and proliferation of the MMC ².

Patients with MM can be grouped in accordance to cytogenetic aberrations identified by conventional metaphase cytogenetics or (interphase) fluorescence in situ hybridization (iFISH) in MMC ^{3,4}. Patients can also be grouped in accordance to gene expression profiling (GEP) with a link between cytogenetic and GEP data ^{4,5}. A good example is the correlation between the translocation t(4;14) and the overexpression of the multiple myeloma set domain (MMSET), or between the translocation t(11;14) and a high CCND1 overexpression ^{6,7}. GEP has the potential to identify new pathways involved in the biology of MMC, in particular regarding the communication with the tumor environment ⁸⁻¹⁰. DNA arrays permitted to show that some HMCLs expressed a gene coding for heparin binding epidermal growth factor-like growth factor (HB-EGF), a member of the epidermal growth factor family able to bind syndecan-1 ¹¹. Further studies demonstrated that HB-EGF is a potent myeloma cell growth factor ^{12,13}. Using Affymetrix microarrays, we also found that *TACI* (transmembrane activator and calcium modulator and cyclophilin ligand interactor) and *BCMA* (B-cell maturation antigen), each gene coding for a receptor of B-cell activating factor (BAFF) ^{14,15} and A Proliferation-Inducing Ligand (APRIL) ¹⁶⁻¹⁸ were overexpressed in malignant plasma cells compared to their normal counterparts ^{8,9}. BAFF is a tumor necrosis factor (TNF)-family member involved in the survival of normal and malignant B cells as well as normal plasmablasts ^{18,19}. APRIL is highly expressed in several tumor tissues and stimulates the growth of tumor cells ¹⁶. A third

receptor specific for BAFF, BAFF-receptor (BAFF-R) was also identified ²⁰. We and others showed that BAFF and APRIL serum levels are elevated in patients with MM and that BAFF and APRIL contribute to the survival of some HMCLs and of primary MMC ^{21,22}.

In this study, we have shown that the tumor environment is the main source of BAFF and APRIL in patients with MM and that *TACI* expression yields a functional receptor. Using Affymetrix microarrays, we demonstrated that TACI^{high} MMC displayed a mature plasma cell gene signature, indicating dependence on the BM environment. In contrast, the TACI^{low} group had a gene signature of plasmablasts suggesting an attenuated dependence on interactions with cells in the BM environment.

Materials and methods

Cell samples

XG-1, 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 (HMCLs) were obtained as described²³. SKMM, OPM2, LP1 and RPMI8226 HMCLs were purchased from ATTC (LGC Promochem, France). MMC were purified from a first series of 65 myeloma patients at diagnosis (median age, 59 years) and a second series of 40 newly-diagnosed patients after written informed consent was given. In the first series, according to Durie-Salmon classification, 12 patients were of stage IA, 12 of stage IIA, 38 of stage IIIA, and 3 of stage IIIB. 11 patients had IgA κ MM, 7 IgA λ MM, 26 IgG κ MM, 9 IgG λ MM, 7 Bence-Jones κ MM, 3 Bence-Jones λ MM, and 2 non-secreting MM. In the second series, 6 patients were of stage IA, 5 of stage IIA, 27 of stage IIIA, and 2 of stage IIIB. 4 patients had IgA κ MM, 5 IgA λ MM, 18 IgG κ MM, 7 IgG λ MM, 4 Bence-Jones κ MM, 1 Bence-Jones λ MM, and 1 non-secreting MM. Normal BM plasma cells (BMPCs) and whole BM cells (WBMCs) were obtained from healthy donors after informed consent was given. WBMCs were collected after lysis of red blood cells with NH₄Cl. After Ficoll-density gradient centrifugation, plasma cells were purified using anti-CD138 MACS microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). BM environment cells from 7 newly diagnosed patients were obtained after depletion of MMC with anti-CD138 MACS microbeads (Miltenyi Biotec). BM culture supernatants were prepared culturing 5×10^5 BM cells depleted of MMC for 3 days in RPMI1640 and 10% FCS. Supernatants were frozen at -20°C until use. For 3 newly diagnosed patients, BM T cells, monocytes and polymorphonuclear neutrophils (PMN) were purified. BM cells were labeled with a phycoerythrin (PE)-conjugated anti-CD3 MoAb, Allophycocyanin (APC)-conjugated anti-CD14 MoAb, and a fluorescein

isothiocyanate (FITC)-conjugated anti-CD15 MoAb (all from Becton Dickinson, San Jose, CA). CD3⁺, CD14⁺ and CD15⁺ cells were sorted with a FACSAria cell sorter (Becton Dickinson). Memory B cells, polyclonal plasmablasts (PPCs), immature dendritic cells (DCs) and BM stromal cell lines (BMSCs) were generated as described previously^{9,24,25}. BMSC culture supernatants were prepared from 3-day culture before reaching confluence and were stored at -20°C until use.

Osteoclasts

Peripheral blood mononuclear cells were obtained from 7 patients with MM after informed consent. Cells were cultured at 2.5×10^6 cells/ml in α MEM-10% FCS. After 12 hours of culture, non-adherent cells were eliminated and adherent cells were cultured in α MEM-10% FCS, RANKL (50 ng/ml, PeproTech, EC Ltd, London, UK), M-CSF (25 ng/ml, Peprotech), and 10 nM dexamethasone for 14 days. Before use, osteoclasts were phenotyped by RT-PCR (TRAP and cathepsin K expression), by cytometry (integrin α v β 3 expression) and bone resorbing activity (OsteoLyse assay kit, Cambrex, Emerainville, France). Culture supernatants were prepared from mature osteoclasts cultured for 3 days with M-CSF, RANKL and DEX and were frozen at -20°C until use.

BAFF or APRIL ELISA

The concentration of BAFF was assayed using the ELISA as described previously²¹, and APRIL with an ELISA purchased from Bender MedSystems (Burlingame, CA) in accordance with the manufacturer's instructions.

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-13 HMCL²¹. XG-13 cells were starved of IL-6 for 2 hours, washed and

cultured for 12 hours in RPMI1640/10% FCS. Then BAFF and APRIL (200 ng/mL each) were added in one culture group for 10 hours. We also investigated the modulation of gene expression profile in primary MMC by BAFF and APRIL.²⁶ BM cells of a newly-diagnosed patient with TACI^{high} MMC were harvested and whole BM cells (5×10^5 cells/mL) were cultured for 24 hours in RPMI1640-10%FCS. Then, 20 μ g/ml of TACI-Fc (R&D Systems, Abington, United Kingdom) were added for 10 hours in one culture group. Cells were harvested, labeled with CD138 MACS microbeads at 4°C and primary MMC were purified. RNA were extracted for gene expression profiling.

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 human HG U133 A and B or U133 2:0 plus GeneChips, according to the manufacturer's instructions (Affymetrix, Santa Clara, CA). Fluorescence intensities were quantified and analyzed using the GECOS software (Affymetrix).

Conventional and real-time RT-PCR

BCMA, TACI and BAFF-R PCR analysis was done as previously described²¹. 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). Real-time RT-PCR was performed using the ABI Prism 7000 Sequence Detection System and normalized to GAPDH for each sample.

Binding of BAFF to MMC

The binding of BAFF to HMCLs and primary MMC was determined using a human BAFF-murine CD8 biotinylated fusion protein (Ansell, Bayport, MN) and FACS analysis as previously described²¹.

Fluorescence in-situ hybridization (FISH)

Interphase FISH was performed according to our previously reported standard protocol ²⁷. Metaphase spreads and interphase cells were evaluated using a DM RXA2 fluorescence microscope (Leica, Bensheim, Germany).

Statistical analysis and TACI^{low} and TACI^{high} group definition

Gene expression data were normalized with the MAS5 algorithm and analyzed with our bioinformatics platform (RAGE, Remote Analysis of microarray Gene Expression) designed by Dr T Reme (INSERM U475, Montpellier, France). Hierarchical clustering was performed with the Cluster and Treeview softwares from Eisen ²⁸. We performed a two-sided supervised clustering between a group of patients with a high (TACI^{high}) and one with low (TACI^{low}) expression of *TACI*. To account for the log-normal distribution of *TACI* expression, we performed this analysis with decreasing proportions of patients in the TACI^{high} and TACI^{low} group: 40%, 25%, and 15%, respectively, of patients with the highest vs. lowest TACI expression. Genes were interpreted as differentially expressed if the p-value (Mann-Whitney test) was $\leq .01$ and the ratio ≥ 1.5 or ≤ 0.67 . A permutation test ²⁹ was performed to generate 100 times two arbitrary groups comprising 40%, 25% or 15%, respectively, of patients in order to get an evaluation for the number of genes differentially expressed between two arbitrary groups ($P \leq .01$ and ratio ≥ 1.5 or ≤ 0.67) by chance. Statistical comparisons were done with Mann-Whitney, Chi-square, or Student t-tests.

Results

***BAFF* and *APRIL* genes are mainly expressed by the BM environment**

BAFF and *APRIL* gene expression levels were analyzed by real time RT-PCR in 4 samples of memory B cells, 7 of normal plasmablasts, 10 of HMCLs, 7 of primary MMC, 7 of BM environment cells depleted from MMC and 5 of BM cells of healthy individuals. These data were compared with those of dendritic cells to which an arbitrary value of 100 was assigned (Figures 1A). *BAFF* and *APRIL* were highly expressed in MM BM environment as well as in normal BM. No significant differences were found between MM and normal BM samples ($P = .26$ for *BAFF* expression and $P = .88$ for *APRIL*). Concerning MM BM environment samples, median expression values were 66 for *BAFF* and 46 for *APRIL*, about half of the expression in dendritic cells known to highly produce *BAFF* or *APRIL* ³⁰. Primary MMC weakly expressed *BAFF* or *APRIL*. The median expressions were 102-fold and 45-fold lower than those in the tumor environment ($P = .005$) respectively. The majority of HMCLs and normal plasmablasts also expressed *BAFF* or *APRIL* but at a rather low level as compared to the BM environment. The two cell lines (XG-5 and XG-20) with the highest *APRIL* expression have been previously identified with conventional RT-PCR ²¹. *BAFF* and *APRIL* expressions in plasmablasts were 29- and 31-fold lower, respectively, than those in memory B cells from which they arose ($P = .002$ and $P = .007$) ⁹. Thus, *BAFF* and *APRIL* genes are mainly expressed by the BM environment and only occasionally by MMC.

In order to identify the cell type expressing *BAFF* or *APRIL* in the MM environment, BM CD3, CD14 and CD15 cells of 3 patients were purified. *BAFF* was mainly expressed by BM CD14 and CD15 cells and *APRIL* by CD14 cells. CD3 cells

expressed *BAFF* and *APRIL* only at a low level (Figure 1B). To assess a broad range of populations present in the bone microenvironment, BMSCs and osteoclasts were also studied. BMSCs from 7 patients with MM expressed weakly *BAFF* and *APRIL* (Figure 1C). Of major interest, osteoclasts largely expressed *APRIL* gene. Median *APRIL* expression was 80-fold higher than that in BM environment ($P = .001$). Osteoclasts also express *BAFF* gene but in the same extent as the BM environment.

BAFF and APRIL production by BM cells

The production of *BAFF* and *APRIL* was investigated in 3-day culture supernatants of BM cells of 10 newly-diagnosed patients, of 7 osteoclasts, and of 7 BMSCs. In agreement with real time RT-PCR data, low amount of *BAFF* and no *APRIL* could be found in BMSC supernatants. Median concentrations of 12.5 ng/ml of *BAFF* and 24.6 ng/mL of *APRIL* were measured in supernatants of BM cells. Again, in agreement with RT-PCR data, osteoclasts produced large amounts of *APRIL* (a concentration of 206 ng/ml), *i.e.* 8-fold higher than BM cells ($P \leq .001$). The median production of *BAFF* by osteoclasts was 3-fold higher than that of BM cells ($P \leq .001$).

TACI gene expression yields a functional BAFF-binding receptor in HMCLs and primary MMC.

Gene expression profiling (GEP) was performed on 17 HMCL using Affymetrix U133 A+B microarrays. On these, no probeset for *BAFF-R* was available. The Affymetrix data for *TACI* expression perfectly matched the real time RT-PCR and previous RT-PCR data²¹ and were consistent with the HMCL ability to bind *BAFF*-murine CD8. Indeed *TACI* had an Affymetrix “absent” call (as decided by the GCOS-software) in these HMCLs that showed a lack of *TACI* mRNA expression by real time or conventional RT-PCR and a lack of binding of *BAFF*-murine CD8. *TACI* had an Affymetrix “present” call in the *TACI* RT-PCR⁺ HMCLs which also bound *BAFF*-

murine CD8 (Table 1). The *BCMA* gene had a “present” call in 17/17 HMCLs, as had been assumed after *BCMA* was found in RT-PCR experiments (Table 1). These data suggest that although *BCMA* is expressed, the BCMA protein is not functional, possibly due to retention in the Golgi apparatus as shown for plasma cells³¹. This observation holds true for purified primary MMC. MMC (# 1-5) that highly expressed *BCMA* but weakly *TACI* according to RT-PCR results, failed to bind BAFF-murine CD8 (Figure 2). In contrast, primary MMC (# 6-10) that highly expressed *TACI* bound human BAFF-murine CD8 (Figure 2). Thus, *TACI* gene expression yields a BAFF-binding receptor in HMCLs or primary MMC.

Clinical and genetic data of the *TACI*^{low} and *TACI*^{high} patients

Gene expression profiles of the 65 primary myeloma cell samples were determined with Affymetrix microarrays. *TACI* had a “present” Affymetrix call in all primary MMC investigated, displaying a lognormal distribution. We did not find the clear-cut difference (absent or present Affymetrix call) for primary MMC which was observed for HMCLs. One possible explanation is the presence of MMC subclones with different levels of *TACI* expressions in the same patient. We looked for differences in the major clinical, biological or genetic markers in patients with a low or high *TACI* expression in MMC (*TACI*^{low} or *TACI*^{high} patients). To find the best way to delimit *TACI*^{low} and *TACI*^{high} patients, we considered subgroups defined by 50%, 40%, 25% or 15%, respectively, of the patients with the highest or lowest *TACI* expression in MMC. The clinical and biological differences observed showed the clearest distinction when the 15%-group was considered. Here, the *TACI*^{high} group included a higher frequency of patients within stage I MM, with lambda light-chain isotype, with t(4;14) translocations, with hemoglobin > 10 g/dL, and a lower frequency of patients with bone lesions (Table 2). The percentage of patients with MMC harboring a t(4;14)

translocations was 14% in agreement with published data ^{3,32}. Most of these differences were kept when considering TACI groups representing 25% and 40% of the patients (distribution of bone lesions, t(4;14) translocations, lambda light chain isotype). However, they were lost when considering TACI subgroups delimited by the median. Whatever method used to define TACI^{low} and TACI^{high} subgroups, no differences in age, β 2m, CRP, albumin, LDH levels, IgG subtypes, or chromosome 13 deletion were found (Table 2).

Gene expression signature of microenvironment dependence in TACI^{high} MMC and plasmablastic signature in TACI^{low} MMC with Affymetrix microarrays

In order to investigate if different gene signatures could be identified comparing two groups with different TACI expression, we performed a supervised clustering. We considered the three groups with significant clinical differences (40%, 25% and 15% of the patients). Of 49.000 probesets present on the U133 A+B DNA-microarrays, we found 1612, 2709 and 1805 probesets, respectively, to be differentially expressed between the two groups ($P \leq .01$ with Mann Whitney test and ratio of the mean expressions ≥ 1.5 or ≤ 0.67). To get an impression about the number of genes differentially expressed by chance between the groups, we performed 100 permutations with two subgroups comprising 40%, 25% or 15% of the MM patients randomly sorted out of the 65 samples. Only 5% of the genes appeared to be differentially expressed, if the groups were selected by chance. A common set of 548 distinct genes and 111 distinct expressed sequence tags (EST) were differentially expressed between TACI^{high} and TACI^{low} MMC independently of the definition of TACI^{high} and TACI^{low} groups. This list of 659 genes/EST is available on our web site as supplementary Tables A and B.

We performed an unsupervised hierarchical clustering using these 659 genes/EST (Figure 3 A). $TACI^{high}$ and $TACI^{low}$ MMC appear in different branches of the cluster analysis. The $TACI_{40}^{low}$, $TACI_{25}^{low}$ and $TACI_{15}^{low}$ groups were represented by graded blue color (more intense in $TACI_{15}^{low}$) and were classified in one branch with limited misclassifications in each category. For example, the 40%-groups were significantly classified ($P \leq .01$) with 5 misclassifications. The $TACI_{40}^{high}$, $TACI_{25}^{high}$, and $TACI_{15}^{high}$ were represented with graded red color (more intense in $TACI_{15}^{high}$) and classified in another branch. Patients outside the subgroups (white color) were interdispersed in the two clusters. The ability of this 659 gene/EST list to classify $TACI^{high}$ and $TACI^{low}$ MMC was validated with another series of GEP from 40 purified MMC, unrelated to the 65 previous ones, that was obtained with Affymetrix U133 2.0 plus microarrays (Figure 3 B). Although this new series of primary MMC was smaller and that a different microarray was used, the U133 2.0 plus probesets corresponding to the 659 U133 A+B genes/EST were able to efficiently classify $TACI^{high}$ and $TACI^{low}$ MMC. In particular, the 40%-groups were significantly classified ($P \leq .01$) with 6 misclassifications. This differential gene expression in $TACI^{high}$ and $TACI^{low}$ MMC observed with Affymetrix microarrays was validated for *TACI* and 3 other genes (CDC2, TYMS and c-met) with real time RT-PCR. ³³³⁴Real time RT-PCR and Affymetrix data showed a good correlation ($r \geq .85$, $P \leq .01$) (Figure 4).

339 of the 659 genes and ESTs mentioned above (52% of the 479 genes/EST overexpressed in $TACI^{high}$ patients and 50% of the 180 genes/EST overexpressed in $TACI^{low}$ patients) could be assigned to 8 functional categories listed in Table 3 using Gene Ontology terms. $TACI^{high}$ MMC expressed a higher percentage of genes coding for intercellular communication signals, cytoskeleton-associated proteins and signal

transduction (Table 3). Conversely, $TACI^{low}$ MMC overexpressed genes coding for proteins involved in cell cycle and nuclear functions (Table 3).

$TACI^{high}$ MMC have a BMPC signature and $TACI^{low}$ a plasmablastic signature

We investigated the association of plasmablasts and normal BMPCs with MMC in the $TACI^{high}$ and $TACI^{low}$ groups. Using an unsupervised clustering with the above mentioned 659 genes, the samples of patients in the $TACI^{low}$ group clustered together with plasmablasts (correlation coefficient, 0.33; $P = .001$) whereas $TACI^{high}$ samples clustered with BMPC (correlation coefficient, 0.35; $P = .001$) (Figure 5). When comparing plasmablasts with normal BMPC, of the 659 genes/EST differentially expressed between the $TACI^{high}$ and $TACI^{low}$ groups, 195 also appeared as differentially expressed between the plasmablasts and BMPC. Of these, 115 were overexpressed in BMPC compared to plasmablasts and 80 overexpressed in plasmablasts compared to BMPC (ratio of the mean expressions ≥ 1.5 and ≤ 0.67 , and $P \leq .01$). 111 out of 115 BMPC genes were also upregulated in $TACI^{high}$ compared to $TACI^{low}$ MMC. Conversely, 77 out of the 80 PPC genes were overexpressed in $TACI^{low}$ MMC ($P \leq .01$). BMPC and plasmablast genes are indicated in supplementary Tables C and D.

Taken together, these results suggest that grouping of patients in accordance with “high” and “low” *TACI* expression delineates a group of patients whose MMC share gene expression characteristics with plasmablasts ($TACI^{low}$) and a group in which the gene expression profiling resembles normal mature BM plasma cells ($TACI^{high}$). $TACI^{high}$ MMC overexpress many genes coding for potentially interesting intercellular communication or transduction signals whose biology is discussed below. In contrast, $TACI^{low}$ MMC mainly overexpress cell cycle genes.

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

In order to look whether the TACI^{high} and TACI^{low} gene signature could be induced by BAFF/APRIL, we first used the XG-13 HMCL which is stimulated by BAFF and APRIL²¹. After growth factor starvation, addition of BAFF and APRIL for 10 hours induced an increase of 196 genes/EST and a decrease of 348 genes/EST ($P \leq .01$ and ratio ≥ 1.5 or ≤ 0.67). However, only 19 genes were common with the TACI-related gene signature (supplementary data in Table E). We then²⁶ decided to investigate the effect of BAFF/APRIL deprivation using a whole BM culture system in which BAFF and APRIL were blocked by TACI-Fc inhibitor for 10 hours. Subsequently, MMC were purified for gene expression profiling with Affymetrix U133 2.0 plus microarrays. 188 genes and ESTs were down regulated in BAFF/APRIL deprived MMC (treated with TACI-Fc) and 259 were upregulated ($P \leq .01$ and ratio ≥ 1.5 or ≤ 0.67). However, only some of these genes were common with the TACI^{high} or TACI^{low} gene signature. This list is available as supplementary data in Table F.

Discussion

Two recent studies have shown that BAFF and APRIL are growth factors for human MMC and that serum levels of BAFF and APRIL are increased in these patients ^{21,22}. Using real time PCR, we show that *BAFF* and *APRIL* genes are poorly expressed by MMC and mainly by the BM environment of MM patients. BM cells from healthy individuals exhibit a similar expression. The MM bone environment comprises mainly of PMN (45%), monocytes (5%) and T cells (10%). We show here that *BAFF* was largely expressed by PMN and monocytes whereas *APRIL* was expressed by monocytes only. A majority of the studies about MM use BM mononuclear cells after removal of PMN with centrifugation on a ficoll hypaque cushion. Thus, the contribution of PMN is in many cases ignored whereas they are the main BM cell components in vivo and here shown to be the main source of BAFF. Another cell component in the BM environment important for myeloma cell biology is bone cells. Primary MMC can survive in contact with BMSCs ³⁵ or osteoclasts ³⁶. In addition, fetal bone is critical to grow MMC in SCID mice ^{37,38}. As these bone cells cannot be harvested by BM aspiration, they are usually produced in vitro. We show here that cultured osteoclasts display a huge *APRIL* expression, about 80-fold higher than those found in the MM BM environment or in dendritic cells. The BAFF expression was in the same order of magnitude in both cases. Using ELISA, we confirmed that osteoclasts produced very large amounts of APRIL. Thus APRIL could likely play an important role in the MMC growth stimulation by osteoclasts ³⁶. BM stromal cells produced only a low amount of BAFF and no APRIL.

Purified MMC also express *BAFF* or *APRIL* but in the median about 100 fold weaker than the BM environment. This BAFF or APRIL expression pattern and distribution resembles the one of IL-6. A current consensus is that IL-6 is a paracrine growth

factor in multiple myeloma and that some MMC may produce autocrine IL-6 and uses it to escape from their dependency on the tumor environment.^{39,40} In particular, several HMCLs use IL-6 as an autocrine growth factor^{41,42}. Similarly, we have previously found that two HMCLs - L363 and RPMI 8226 - use BAFF or APRIL as autocrine growth factors, while we found here that BAFF and APRIL are mainly paracrine growth factors if assessing primary MM cells. The autocrine production may have contributed to the escape of these MMC from the tumor environment.

Given that BAFF and APRIL are paracrine growth factors, our aim was to look for a difference in the GEP of patients' MMC expressing a high or a low level of BAFF and APRIL receptors. An important point is that *TACI* expression (assayed with Affymetrix microarrays or RT-PCR) is a good indicator for the presence of a receptor, able to bind BAFF. There are three receptors for BAFF and APRIL, BCMA, BAFF-R and TACI. Probesets for *BCMA* and *TACI* were available on the Affymetrix U133 A+B chips, unlike BAFF-R. We could not identify an EST with *BAFF-R* sequence homology in any Affymetrix probesets. BCMA was probably not functional in MMC and *TACI* expression was a good indicator of a functional BAFF receptor. Indeed only the 6 out of the 17 HMCLs with a "present" call for *TACI* expression with Affymetrix microarrays were able to bind a BAFF-murine fusion protein, independent of *BCMA* expression. BCMA was already shown to be not functional in several cell types, in particular in human plasma cells and the U266 myeloma cell line due to retention in golgi apparatus³¹. However, it is not likely the case for murine plasma cells since *BCMA*^{-/-} mice have normal B cell function but fail to develop mature plasma cells⁴³.

When grouping the patients in accordance with the *TACI*-expression levels as described above, the MMC in the *TACI*^{high} group show a signature resembling mature

BMPCs with an emphasis on the expression of genes with products functioning in intercellular communication and signal transduction. In contrast, the TACI^{low} MMC showed a signature sharing similarities with plasmablasts, especially as regards to the expression of cell cycle genes. The consistency of this signature was validated on an independent group of 40 MMC using Affymetrix U133 2.0 plus microarrays. We looked for the possibility that this gene signature could be due in part to signal transduction induced by TACI activation. We used first the XG-13 HMCL whose growth is stimulated by BAFF or APRIL ²¹. We also used primary MMC from a newly-diagnosed patient. In this case, as primary MMC rapidly apoptosed as soon as they are purified ²⁶, we looked for the effect of BAFF/APRIL deprivation on the gene expression profile of MMC cultured together with their BM environment. In the two cases investigated, a large number of genes were up- or downregulated but only a few genes belonged to the TACI signature.

This indicated that this gene signature is not linked to TACI stimulation but to the degree of dependency of myeloma cell growth on the interaction with the BM environment. As BAFF or APRIL are mainly produced by the BM environment and are potent growth factors for normal and malignant plasma cells, it seems logical that MMC that depend more on BAFF/APRIL for their growth, overexpress TACI and molecules involved in the interaction with their environment.

Of interest, clinical and genetic differences were found between patients with TACI^{low} or TACI^{high} MMC. TACI^{low} patients had a plasmablast signature, an increase in the percentage of stage III MM, a decrease in hemoglobin level, an increase in the percentage of bone lesions. It is of note that clinical parameters and risk factors (β 2m, CRP, LDH) were not different between TACI groups suggesting that TACI might be an independent prognostic factor, which has to be evaluated in further

studies. No significant difference in event-free and overall survivals were found between TACI^{low} and TACI^{high} patients. This is understandable because the median follow-up of this series of patients was short at the time of the study (18 months). Recently, *DKK* gene upregulation was associated with increased bone lesions¹⁰. Tian et al. pointed out that *DKK1* was not expressed in plasmablastic MMC¹⁰, one of the major characteristic features of TACI^{low} MMC. This may explain why we found no upregulation of *DKK1* in TACI^{low} MMC although increased bone lesions are found in TACI^{low} patients. Regarding genetic alterations, we found a higher percentage of patients with a t(4;14) translocation within the TACI^{high} group and no increase of chromosome 13 deletion in the TACI^{high} group although t(4;14) was strongly associated with deletion 13³. Actually about half of the MMC have deletion 13 and only 14% a t(4;14) translocation, which may explain this discrepancy.

It was not in the scope of our work to comment on all the genes differentially expressed between TACI^{low} and TACI^{high} MMC. Therefore, in figure 6, we have selected for genes that appeared to us the most promising for myeloma biology understanding. First, we found an increased proportion of MMC secreting lambda light chain in TACI^{high} MMC. Given that immunoglobulin light chain rearrangement occurs in pre-B cells, we have no explanation for this observation. Focusing on growth factor related molecules, we found that MMC in the TACI^{high} group overexpress two neurotrophic tyrosine kinase receptors, NTRK3 which binds neurotrophin 3 and NTRK4. They also overexpress Platelet derived growth factor, CCR6, TNF, interleukin-22 receptor, two ephrins (B4 and B6) and the somatostatin receptor 2 (SSTR2). The IL-22 receptor complex consists of IL-10R β and IL-22R⁴⁴. The IL-10R β serves as a common receptor chain for both IL-10 and IL-22. As we previously demonstrated IL-10 to be a growth factor for human MMC⁴⁵, the role of IL-

22 in multiple myeloma biology needs to be investigated. The somatostatin receptor family comprises several members and SSTR2 is expressed in different cancers ^{46,47}. Of interest, the somatostatin analog octeotide binds SSRT2 and has been shown to induce apoptosis in MMC ⁴⁸. MMC in the TACI^{high} group overexpress the carcinoembryonic antigen-related cell adhesion molecule (CEACAM)1, CEACAM5 and CEACAM6 adhesion molecules. CEACAM1 is necessary for VEGF activity ⁴⁹. Given the likely role of VEGF in multiple myeloma, a role of CEACAM1 in MM demands further investigation. Furthermore, we found an overexpression of two ephrin receptors: EphB4 and EphB6. Ephrin receptors are overexpressed in various tumor types, suggesting that they play a role in cancer progression ⁵⁰. Recently, a study demonstrated that EphB4 acts pro-angiogenic in breast cancer ⁵¹. It proposes that the interplay between the EphB4 ectodomain on tumor cells and the ephrin-B2 in the vasculature promotes blood vessel formation and remodeling. TACI^{high} MMC overexpress the chemokine CXCL1. This is of interest because CXCL1 was recently found to bind soluble syndecan-1 ⁵², a proteoglycan that is a hallmark of plasma cell differentiation. Actually, MMP7 deficient mice have a major defect in neutrophil attraction due to a defect in cleavage of syndecan-1 by MMP7, of binding of CXCL-1 to syndecan-1 and thus of a chemoattracting role of CXCL-1 ⁵². MMC produce soluble syndecan-1, the serum level of which is increased in patients with MM ⁵³. MMP7 is expressed in all myeloma cell samples investigated by Affymetrix microarrays. CXCL1 produced by MMC in the TACI^{high} group could play an important role in the biology of MMC by recruiting cells of the microenvironment, in particular PMN that largely express *BAFF*.

Regarding adhesion molecules, MMC in the TACI^{high} group overexpress several of these, in particular integrin alpha 2B, also overexpressed in BMPCs compared to plasmablasts⁵⁴.

Regarding the transduction elements that may be associated with TACI activation, we found an overexpression of mitogen-activated kinase kinase 3 (*MKK3*), mitogen-activated protein kinase (ERK2), mitogen-activated protein kinase 10 (JNK3), mitogen-activated protein kinase 14 (P38 MAP Kinase), and mitogen-activated protein kinase 8-interacting protein 2 (*JIP2*) in MMC belonging to the TACI^{high} group. The substrate of MKK3 is P38 MAP kinase, which is known to be induced by BAFF/APRIL in various cells⁵⁵. JIP2 forms complexes with MAP kinases, in particular with MKK3 and P38^{56,57}. The overexpression of ERK2 in MMC of the TACI^{high} group is in good agreement with our previous results demonstrating that the MAPK pathway is activated, in MMC, by BAFF and APRIL. P38 kinase is also activated in MMC and an inhibitor of P38 induces apoptosis in MMC⁵⁸. BAFF/APRIL trigger PI-3 kinase in MMC²¹, and in good agreement, the *P85 subunit* of PI-3K is overexpressed in TACI^{high} MMC. *PIK3CD (P110 delta polypeptide)* is also overexpressed in TACI^{high} MMC. P110 delta is expressed at high levels in lymphocytes of lymphoid tissues and play a role in phosphatidylinositol 3-kinase-mediated signaling in the immune system⁵⁹. Promotion of survival of BAFF and APRIL in B cells and MMC are consistent with NF-kappaB activation. In agreement with these data, the NF-kappaB inducing kinase (NIK) is overexpressed in TACI^{high} patients. In T1 B cells, BAFF stimulation promote B cell survival, causing the processing of the p100 form of NfκB2 to p52 which required BAFF-R and NIK⁶⁰. Previously, we demonstrated that BAFF and APRIL induce the upregulation of the

anti-apoptotic protein Bcl-2²¹. Confirming these results, we show here that TACI^{high} patients overexpress *Bcl-2*.

MMC from patients belonging to the TACI^{low} group overexpress genes coding for proteins implicated in cell-cycle and in nuclear functions. These cells overexpress genes coding for protein involved in G1/S transition and mitosis and contributing to plasmablastic signature. Among them, we validated two genes: thymidilate synthetase (TYMS) and cell cycle controller CDC2.⁶¹ Their overexpression is associated with chemotherapy resistance in cancer^{62,63} and these two genes are also differentially expressed between patients with or without chromosomal abnormalities³³.⁶⁴ MMC in the TACI^{low} group overexpress two genes coding for proteasome subunits and could also be an indicator of increased sensitivity to proteasome inhibitors⁶⁵. MMC from patients belonging to the TACI^{low} group overexpress c-met, the receptor of HGF. HGF is a myeloma growth factor^{66,67}.⁶⁸ These results suggest that c-met could be a therapeutic target in TACI^{low} MMC.

In conclusion, TACI is overexpressed in patients whose MMC have a gene signature indicative of a dependence on the tumor environment. This fits well with the production of the TACI ligands, BAFF and APRIL, by the tumor environment. TACI^{low} MMC overexpress in turn cell cycle genes indicative of a plasmablastic signature. Taken together, our findings foster a clinical use of inhibitors interfering with the BAFF/APRIL – TACI interaction like the TACI-Fc fusion protein, and at the same time suggest to use gene expression profiling to identify the group of patients which might benefit most from this kind of treatment.

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HMCLs	PCR			Binding of BAFF-muCD8	Real-time RT-PCR TACI	Affymetrix TACI	Affymetrix BCMA
	BCMA	BAFF-R	TACI				
XG-12	+	-	+	+	+	P	P
XG-13	+*	+*	+*	+*	+	P	P
XG-19	+	+	+	+	+	P	P
XG-20	+*	+*	+*	+*	+	P	P
RPMI8226	+*	+*	+*	+*	+	P	P
LP1	+*	+*	+*	+*	+	P	P
XG-1	+*	+*	-*	-*	-	A	P
XG-3	+	-	-	-	-	A	P
XG-4	+	+	-	-	-	A	P
XG-5	+*	-*	-*	-*	-	A	P
XG-6	+*	-*	-*	-*	-	A	P
XG-7	+*	+*	-*	-*	-	A	P
XG-10	+	+	-	-	-	A	P
XG-11	+*	-*	-*	-*	-	A	P
XG-16	+	-	-	-	-	A	P
OPM2	+	+	-	-	-	A	P
SKMM	+	-	-	-	-	A	P

Table 1. TACI gene expression yields a BAFF/APRIL receptor with functional binding capacity in HMCLs.

Expression of *BCMA*, *BAFF-R* and *TACI* mRNA were determined by RT-PCR in 17 HMCLs. *TACI* and *BCMA* expressions were also determined with Affymetrix microarrays. The probeset for *BAFF-R* was not available on the Affymetrix HG U133 A+B DNA microarrays. The expression of BAFF-binding was determined by flow cytometry using biotinylated human BAFF-murine CD8 fusion protein and phycoerythrin-conjugated streptavidin.

* Indicates that these data were from our previous published data ²¹.

	Definition of TACI ^{low} and TACI ^{high} subgroups (% of all patients)											
	50%/50%			40%/40%			25%/25%			15%/15%		
Categories	TACI ^{high}			TACI ^{low}			TACI ^{high}			TACI ^{low}		
	% of patients in each subgroups											
Age ≥ 65 yr	30%	24%	28%	28%	20%	40%	20%	40%	20%	40%	20%	40%
Kappa light chain	65%	70%	64%	80%	60%	66%	50%	70%				
Lambda light chain	35%	26%	36%*	16%*	40%*	16%*	50%*	20%*				
Non-secreting	0%	4%	0%	4%	0%*	18%*	0%*	10%*				
IgA subtype	25%	31%	24%	24%	33%	27%	20%	30%				
Beta2-microglobulin ≥ 4 mg/liter	44%	37%	40%	40%	40%	60%	30%	60%				
C-reactive protein ≥ 4 mg/liter	44%	53%	40%	56%	47%	67%	50%	70%				
Lactate dehydrogenase ≥ 190 IU/liter	28%	16%	28%	20%	27%	20%	40%	30%				
Albumin < 35 g/liter	37%	37%	40%	36%	47%	33%	60%	40%				
Hemoglobin < 10 g/dl	28%	35%	36%	44%	20%*	47%*	20%*	60%*				
Bone lesions												
0 lesions	42%	20%	45%*	10%*	43%*	8%*	44%*	0%*				
≥ 1 lesions	58%	80%	55%*	90%*	57%*	92%*	56%*	100%*				
Cytogenetic abnormalities												
Chromosome 13 deletion	22%	31%	24%	32%	27%	33%	30%	40%				
t(4;14) translocation	22%*	6%*	20%*	8%*	27%*	6%*	30%*	0%*				
t(11;14) translocation	9%	9%	12%	12%	20%	6%	20%	10%				

	50%/50%						40%/40%						25%/25%						15%/15%								
	TACI ^{high}			TACI ^{low}			TACI ^{high}			TACI ^{low}			TACI ^{high}			TACI ^{low}			TACI ^{high}			TACI ^{low}					
Staging	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
	9	5	18	3	5	24	8	5	12	3	4	18	6	2	7	0	3	12	4	1	5	0	2	8			
	P = 0.1						P = 0.02						P = 0.02						P = 0.08								

Table 2 : Clinical data of the TACI^{high} and TACI^{low} patients. 65 newly-diagnosed patients with MM were separated in 2 subgroups according to TACI gene expression in MMC assayed with Affymetrix microarrays. The 2 subgroups represent either 50%

(n = 32), 40% (n = 25), 25% (n = 15), or 15% (n = 10) of the patients with highest (TACI^{high}) or lowest (TACI^{low}) TACI expression. Data are the percentages of patients within each subgroup with the indicated clinical or biological parameters. *When the percentages were different with the Chisquare test ($P \leq .05$), the data are shown in italic.

	TACI ^{low}	TACI ^{high}	
<u>Genes coding for protein implicated in:</u>			
Intercellular communication signals (N = 69)*	2.1 %	26.3 %	$P = 4.9.10^{-7**}$
Cytoskeleton (N = 20)	2.1 %	7.2 %	$P = .04**$
Transduction signals (N = 52)	11.2 %	17 %	$P = .05**$
Protein synthesis and regulation (N =19)	7.7 %	4.8 %	NS
Cell cycle (N = 11)	10 %	0.8 %	$P = 2.5.10^{-5**}$
Metabolism (N = 85)	22.1 %	26.6 %	NS
Apoptosis (N = 10)	4.2 %	2.8 %	NS
Nuclear functions (N =73)	40.6 %	15.2 %	$P = 1.10^{-6**}$
Total of classified genes (N= 339)	100% (n = 91)	100% (n = 248)	

Table 3: Intercellular communication signature in TACI^{high} MMC and plasmablastic signature in TACI^{low} MMC. A. 339 of the 659 genes differentially expressed between the TACI^{high} and TACI^{low} groups could be assigned to 8 functional categories using Gene Ontology terms. Data are the percentages of genes of a given category compared to the total number of TACI^{low} (91 genes) or TACI^{high} (248 genes) genes.

* in parenthesis are indicated the number of genes out of the 339 in one category.

** the statistical comparisons of the percentages of genes belonging to one GO-category between the TACI^{low} and TACI^{high} group genes were performed with Chisquare test.

Figure legends

Figure 1. Expression of *BAFF* and *APRIL* in the BM environment cells and MMC.

A. *BAFF* and *APRIL* expressions were determined by quantitative PCR in 4 samples of memory B cells, 7 of normal plasmablasts, 10 of HMCLs, 7 of primary MMC, 7 of BM environment cells depleted from MMC and 5 of BM cells of healthy individuals.

B. *BAFF* and *APRIL* expressions were determined by quantitative PCR in purified BM CD3, CD14, or CD15 positive cells. These cells were obtained from 3 newly-diagnosed patients with MM.

C. *BAFF* and *APRIL* expressions were determined by quantitative PCR in 7 samples of BM stromal cell lines and 7 samples of in-vitro-generated osteoclasts. Primary cells were obtained from newly-diagnosed patients with MM.

Dendritic cells (DC) were used as positive control and assigned the expression value of 100 arbitrary units.

D. *BAFF* and *APRIL* concentrations in culture supernatants of BM cells of 10 patients with MM, 7 different BM stromal cell line samples from MM patients, and 7 different samples of osteoclasts were determined by ELISA.

Figure 2. Expression of the receptors for *BAFF* and *APRIL* in MMC.

Expression of *BCMA*, *BAFF-R*, *TACI* and *GAPDH* mRNA were determined by RT-PCR in CD138+ purified primary MMC from 10 patients with intramedullary MM. The ability of MMC to bind *BAFF* was determined by flow cytometry using a biotinylated human *BAFF*-murine CD8 fusion protein and phycoerythrin-conjugated streptavidin.

Figure 3. Unsupervised hierarchical clustering of $TACI^{high}$ and $TACI^{low}$ MMC.

The 659 genes that statistically ($P \leq .01$) distinguished (ratio ≥ 1.5 or ≤ 0.67) $TACI^{high}$ patients and $TACI^{low}$ patients are presented graphically using hierarchical clustering. The color of each cell in the tabular image represents the expression level of each

gene (red, expression higher than the mean; green, expression lower than the mean; increasing color intensity represents a higher magnitude of deviation from the mean). The $TACI_{40}^{low}$, $TACI_{25}^{low}$ and $TACI_{15}^{low}$ subgroups were represented by graded blue color (more intense in $TACI_{15}^{low}$) and the $TACI_{40}^{high}$, $TACI_{25}^{high}$, and $TACI_{15}^{high}$ were represented with graded red color (more intense in $TACI_{15}^{high}$).

A. Initial set of 65 patients.

B. Validation set of 40 patients.

Figure 4. Validation of Affymetrix data.

Gene expressions of *TACI*, *MET*, *TYMS*, and *CDC2* in $TACI^{low}$ and $TACI^{high}$ patients were assayed with real time RT-PCR and normalized with GAPDH. The XG-13 HMCL was used as the standard. The coefficient of correlations between Affymetrix and real-time RT-PCR values were determined.

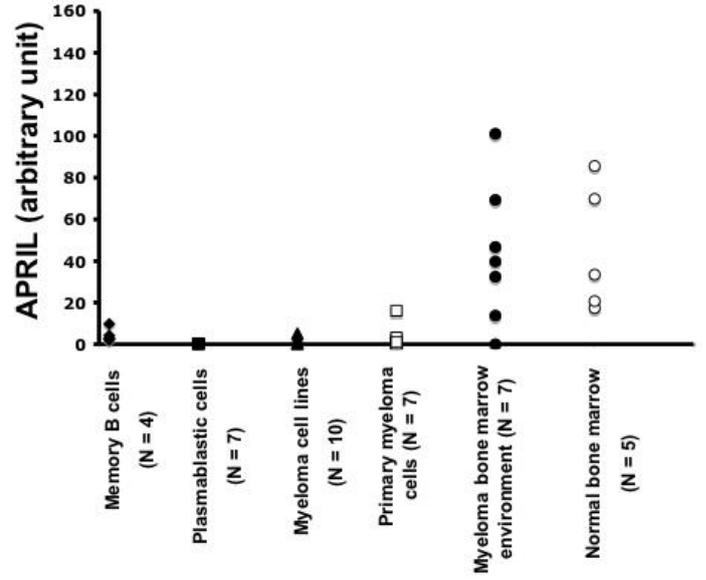
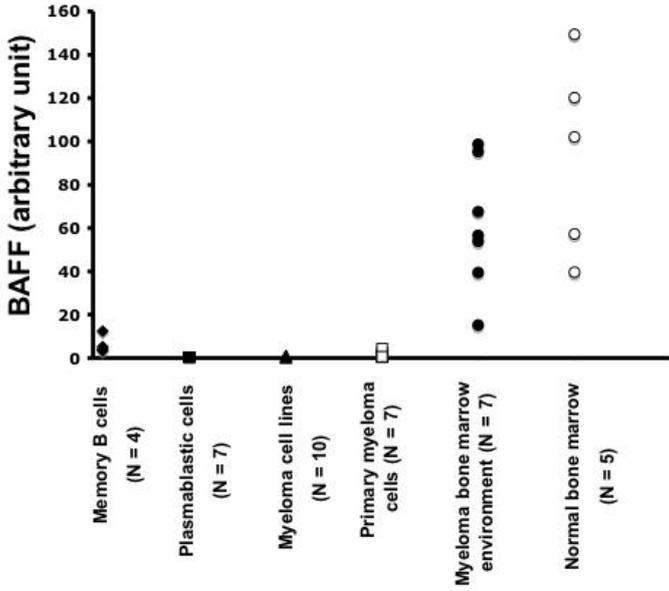
Figure 5. Hierarchical clustering of MMC, BMPC, and PPC identifies a signature of BM stroma interaction for $TACI^{high}$ patients and a plasmablastic signature for $TACI^{low}$ patients.

Unsupervised hierarchical clustering analysis of the expression profile of MMC of 65 patients at diagnosis, 7 PPC samples and 7 BMPC samples show that $TACI^{low}$ MMC cluster (blue) together with PPC (yellow) whereas $TACI^{high}$ cluster (red) with normal BMPC (purple). The clustering was performed on the 659 genes differentially expressed between $TACI^{high}$ and $TACI^{low}$ MMC. The $TACI_{40}^{low}$, $TACI_{25}^{low}$ and $TACI_{15}^{low}$ subgroups were represented by graded blue color (more intense in $TACI_{15}^{low}$) and the $TACI_{40}^{high}$, $TACI_{25}^{high}$, and $TACI_{15}^{high}$ with graded red color (more intense in $TACI_{15}^{high}$).

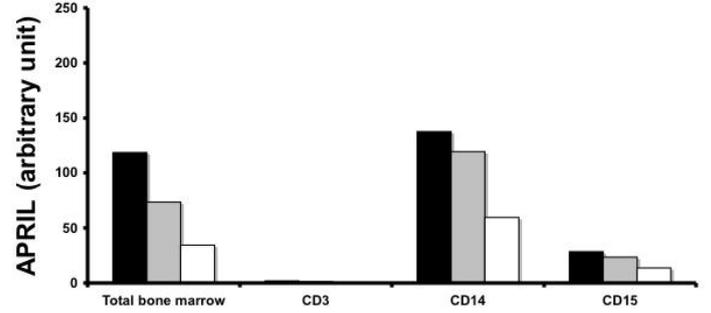
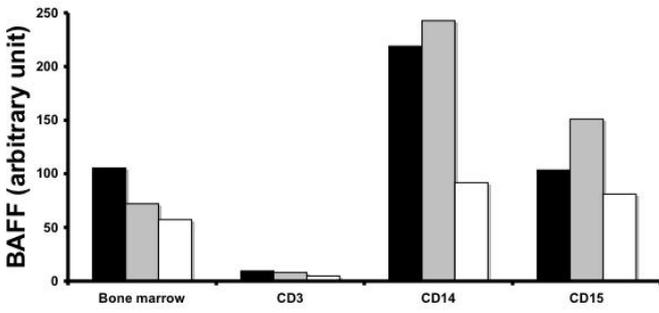
Figure 6. BM environment dependence signature of $TACI^{high}$ patients and plasmablastic signature of $TACI^{low}$ patients.

TACI^{high} MMC had a BM environment dependence signature with overexpression of intercellular communication and transduction genes. TACI^{low} MMC have a plasmablastic signature with an overexpression of genes involved in the cell cycle.

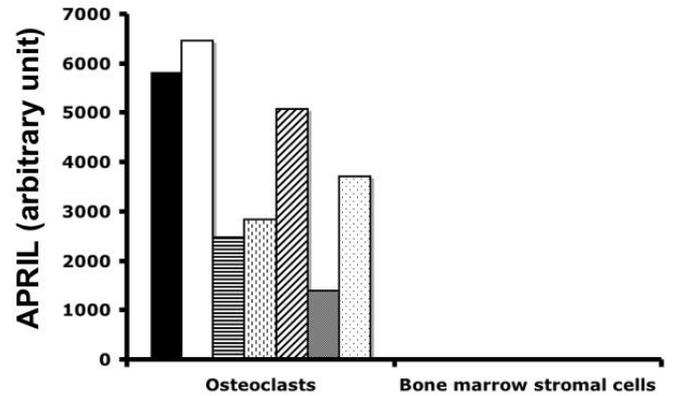
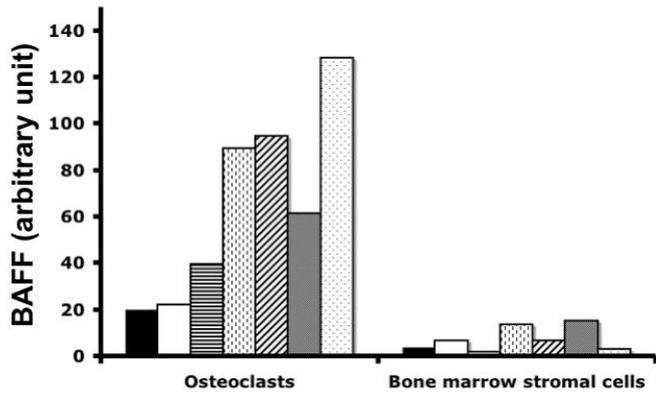
A Figure 1



B



C



D

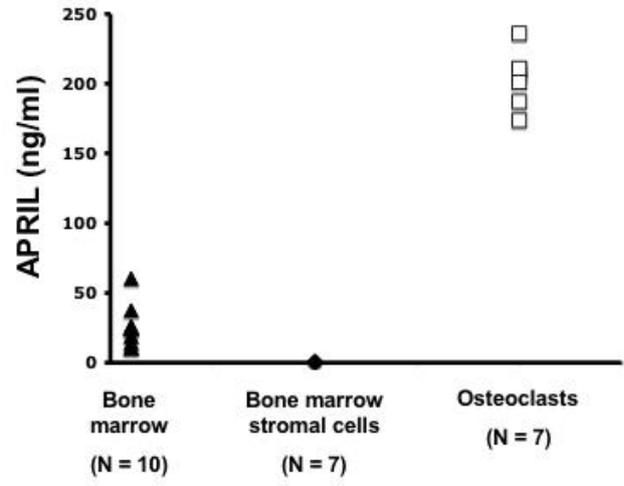
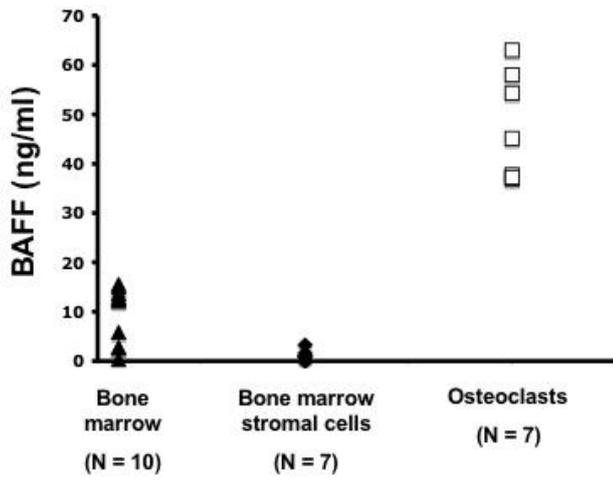


Figure 2

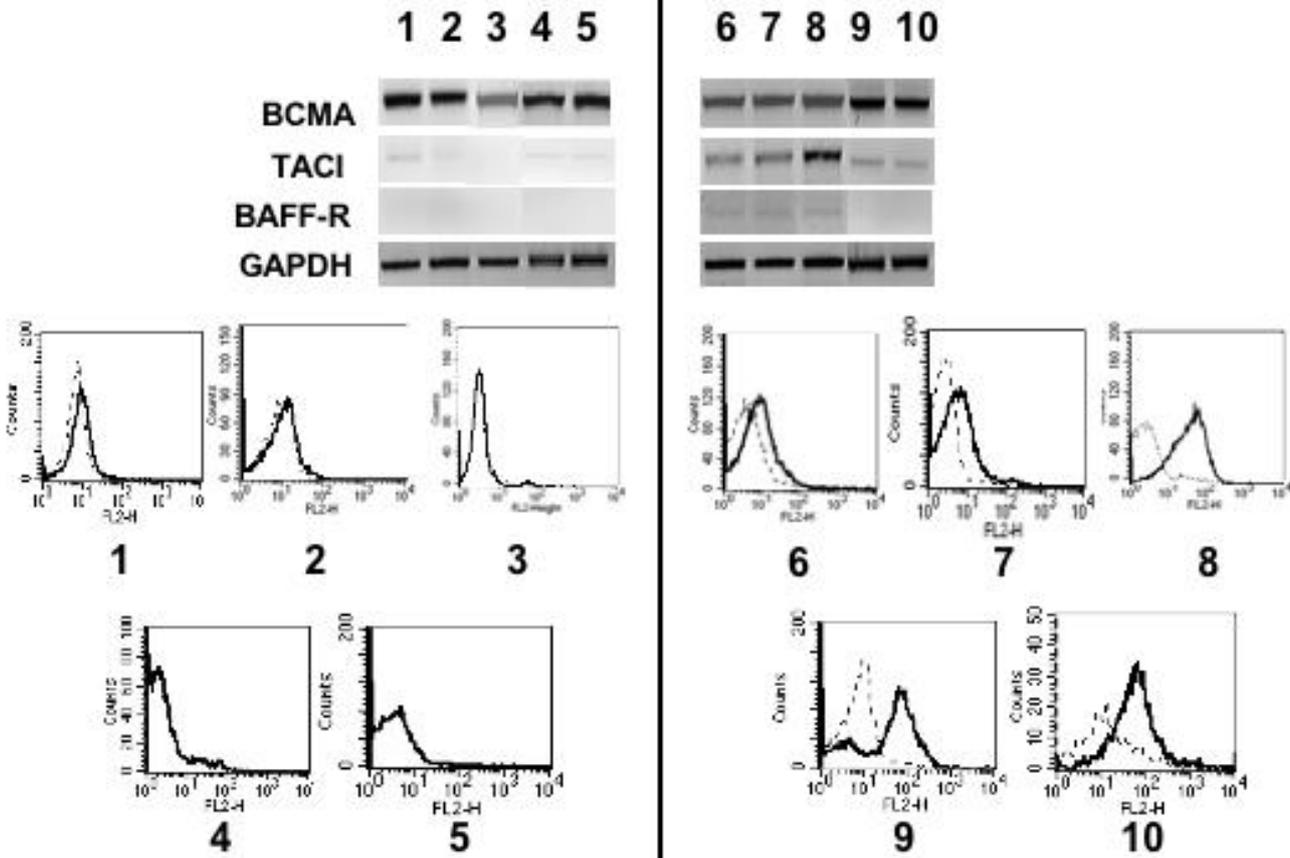


Figure 3 A

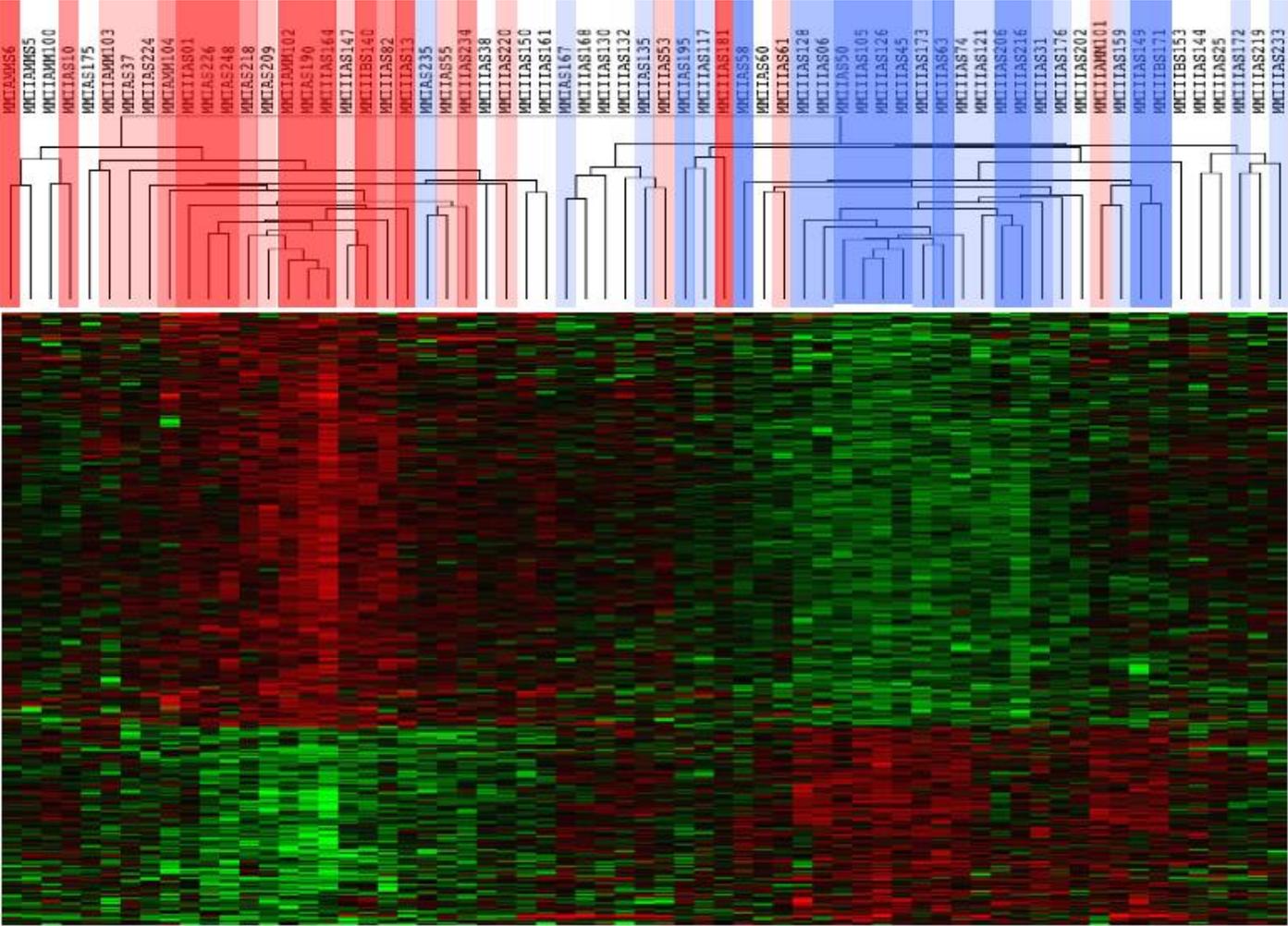


Figure 3 B

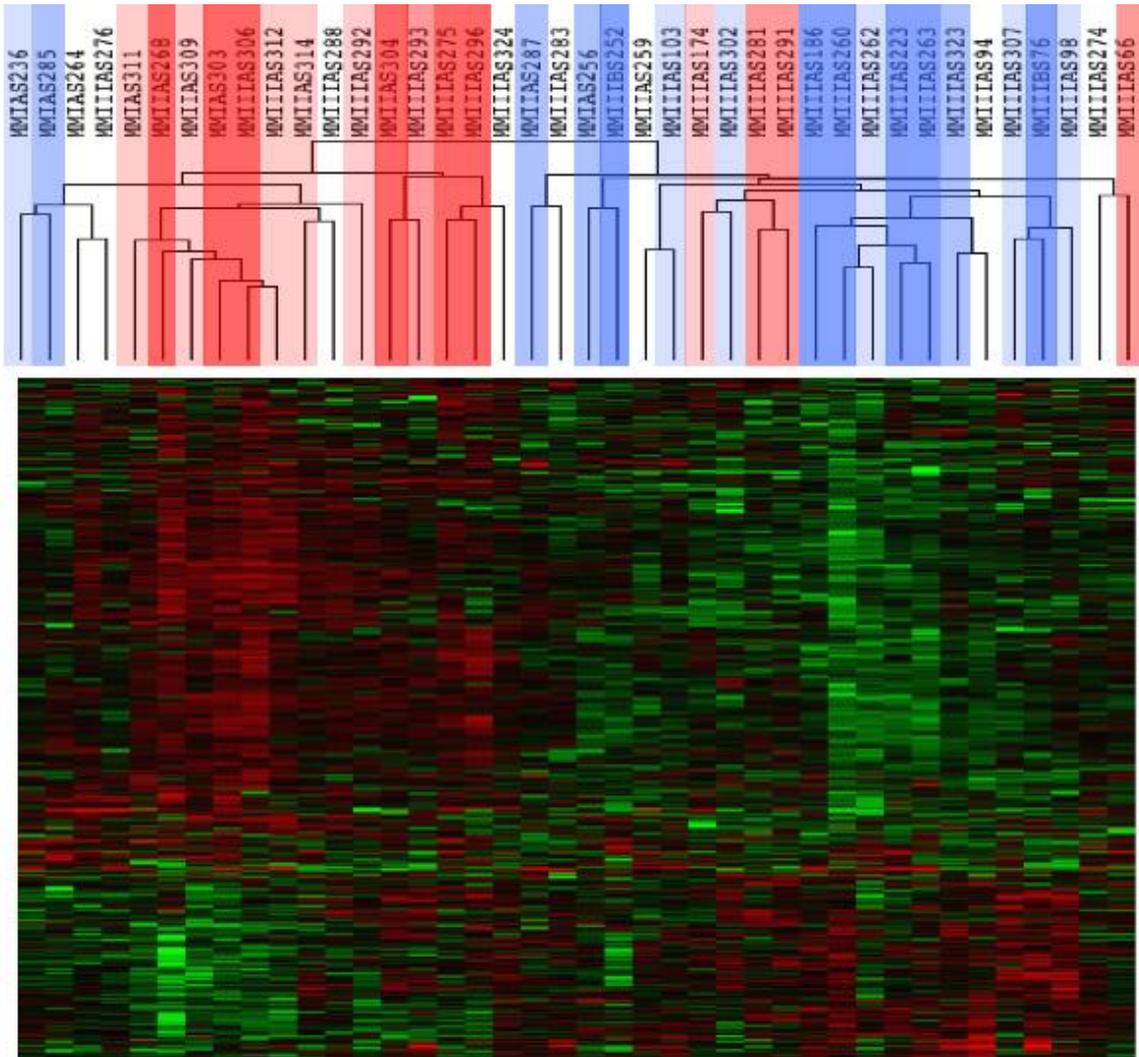


Figure 4

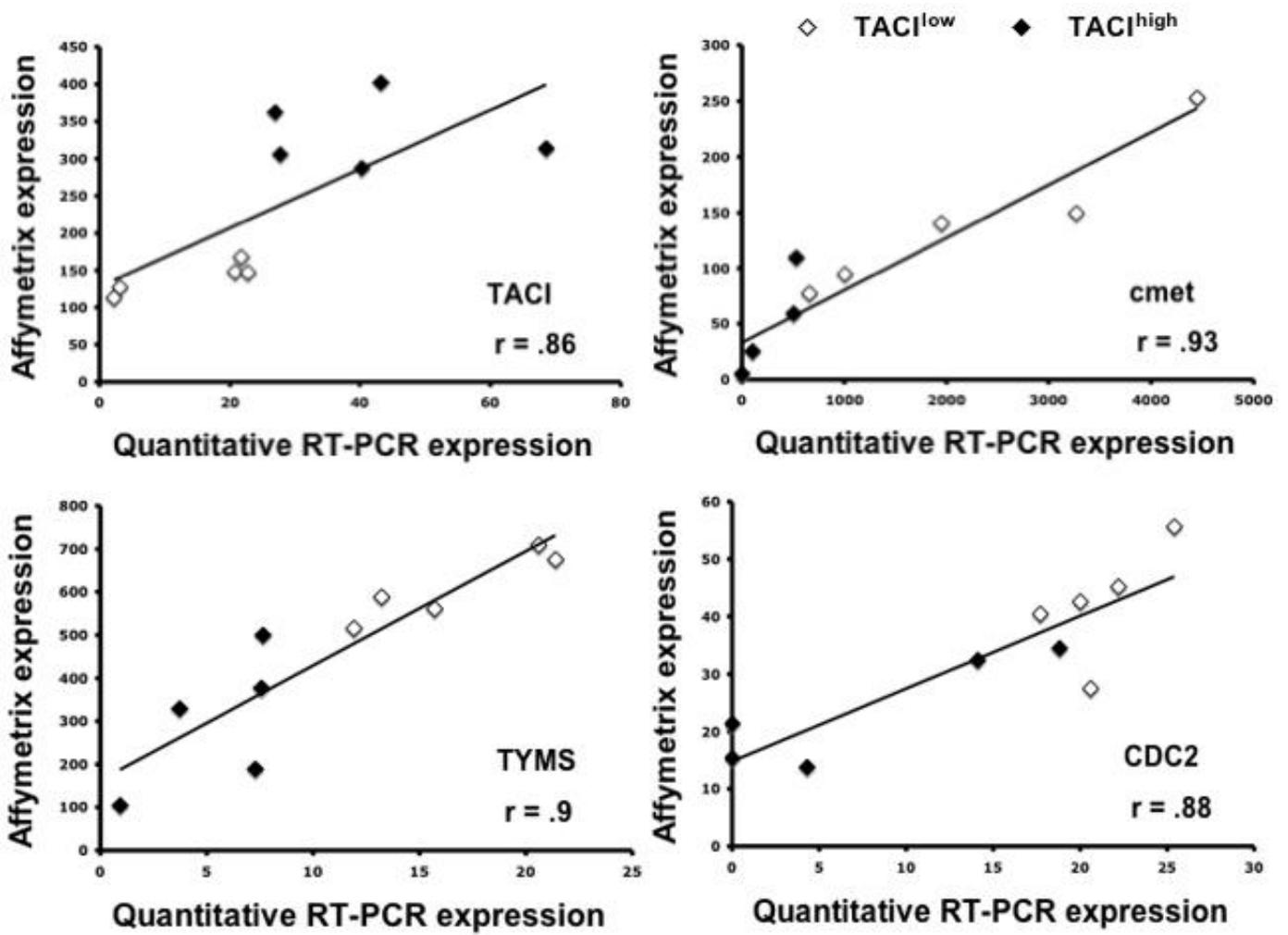


Figure 5

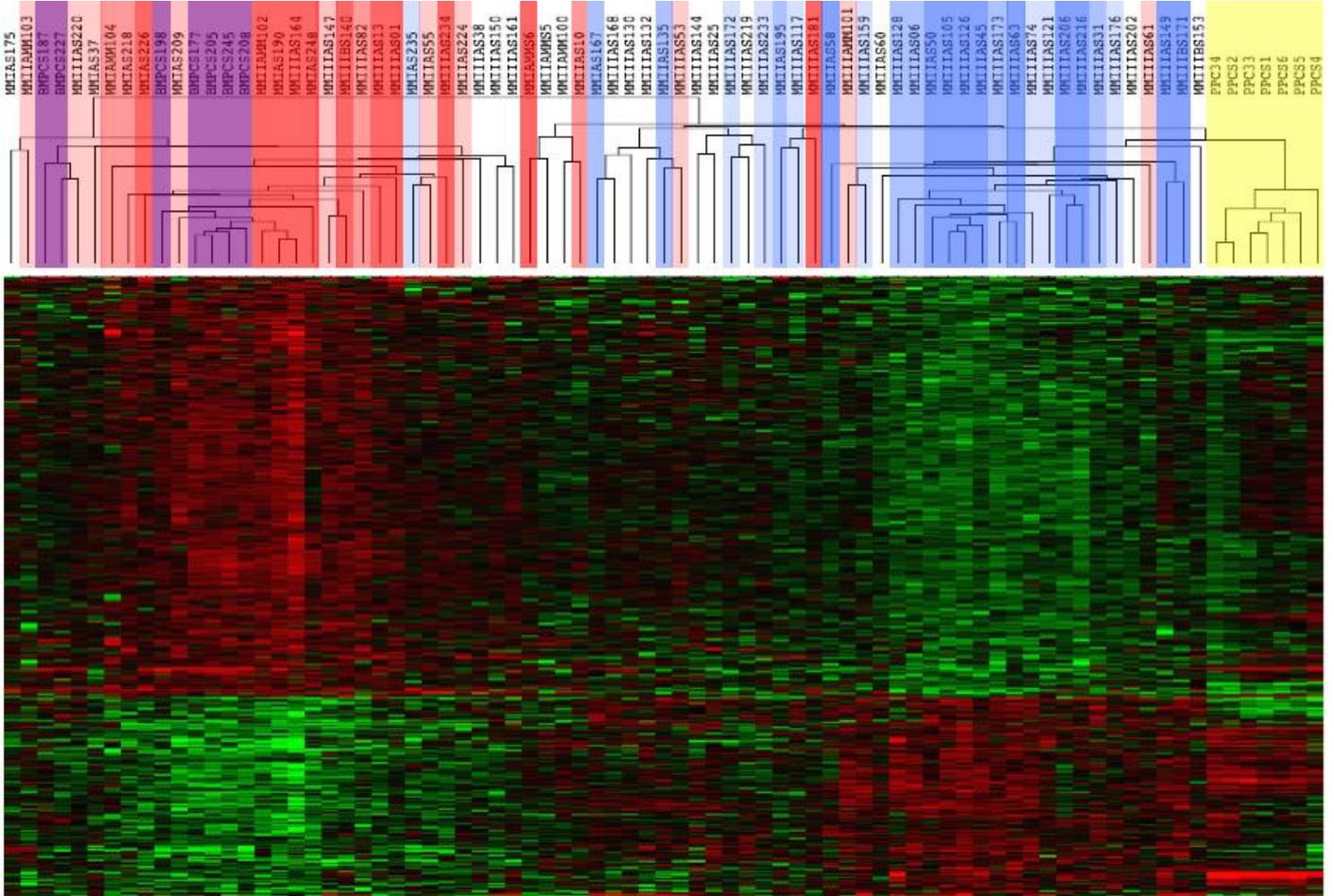


Figure 6 A

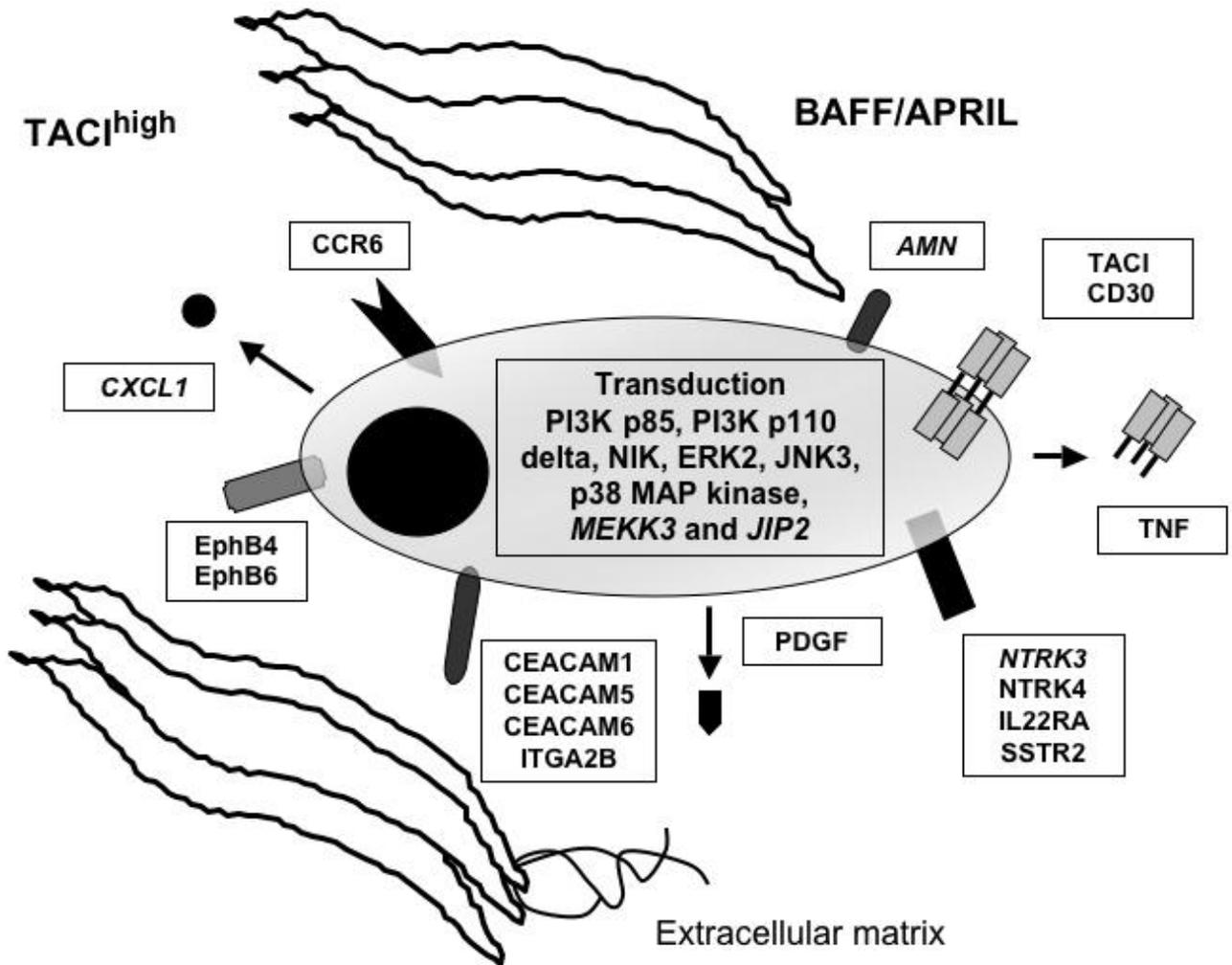


Figure 6 B

TACI^{low}

