Clinical and biological aspects of Multiple Myeloma
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CD27 is heterogeneously expressed in multiple myeloma; low CD27 expression in patients with high risk disease

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Summary
Expression of CD27 on malignant plasma cells (PC) (CD138+ CD38++) was analyzed in a cross-sectional study of bone marrow (BM) samples from multiple myeloma (MM) patients (n=28), Monoclonal Gammapathy of Undetermined Significance (MGUS) (n=6) and BM PC from healthy donors (n=4). MM PC expressed CD27 with a variable, lower intensity pattern compared with the consistent high expression in MGUS and healthy donors. MM patients in complete clinical remission display a higher percentage of CD27+ PC compared with patients at diagnosis, relapse or in partial remission. In MM, loss of CD27 correlated with loss of CD19 (R²=0.4, P<0.0001). Human MM cell lines (n=9) did not express CD27 whereas de novo Plasma Cell Leukemia (PCL) (n=3) had a high expression. Reanalysis of a cDNA micro array data set, generated from newly diagnosed MM patients (n=74), demonstrated that the MM subgroup with the highest prevalence of poor prognostic factors had the lowest CD27 mRNA expression. Fluorescence activated cell sorting and allele-specific oligonucleotide polymerase chain reaction showed that both CD27+ and CD27- PC subpopulations in MM can belong to the clonal disorder. In conclusion, CD27 is heterogeneously expressed on MM PC and loss of CD27 expression might have prognostic value in MM.

Introduction
Multiple Myeloma (MM) is characterized by a clonal accumulation of malignant plasma cells in the bone marrow. The plasma cells are phenotypically characterized by a strong expression of CD38 and CD138 (syndecan-1)\textsuperscript{18}, but can display an aberrant phenotype compared to normal plasma cells. Asynchronous expression has been reported for CD56 (NCAM)\textsuperscript{78}, which is expressed in the majority of MM patients; CD117 (c-kit), which is expressed in approximately one third of the myeloma cases\textsuperscript{219} and CD28 and CD86, which are associated with extramedullary expansion of the tumor clone.\textsuperscript{30} Mature myeloma plasma cells usually lack expression of CD19.\textsuperscript{220} The importance of these phenotypic changes in relation to progression is largely unclear. CD27 is a 110 kDa homodimeric transmembrane glycoprotein of the Tumor Necrosis Factor Receptor (TNFR) family.\textsuperscript{221} CD27 is expressed by a subset of B-cells and by the majority of peripheral T-cells.\textsuperscript{222,224} CD27 expressing peripheral blood B-cells express somatically mutated surface immunoglobulin (Ig) receptors,
identifying them as memory B-cells.\textsuperscript{225-227} The natural ligand for CD27 is CD70, a member of the TNF family. CD70 is expressed on activated B and T-cells\textsuperscript{222,228-231} and the CD27/CD70 interaction is implicated in B-cell differentiation and survival.\textsuperscript{232-235} Normal plasma cells isolated from tonsils and the gut lamina propria and \textit{in vitro} generated plasma cells strongly express CD27.\textsuperscript{236,237} In the malignant counterpart it has been demonstrated that CD27 is expressed and being released as soluble CD27 by B-cell malignancies representing mature B-cells including B-cell chronic lymphatic leukaemia (B-CLL). In two previous studies it was suggested that CD27 is not expressed on MM plasma cells.\textsuperscript{232,238} However, both studies analysed only a limited number of MM patients. In this study, we present a comprehensive analysis of CD27 expression in a cross-sectional MM patient group (n=28). We show that CD27 can be expressed on malignant plasma cells in MM but with a variable expression pattern. Importantly, plasma cells from MM patients in complete clinical remission display a significantly higher CD27 expression compared to plasma cells obtained from newly diagnosed, relapsed and refractory MM patients. In a previous study it was shown that gene profiling of newly diagnosed MM patients identified hierarchical clustered MM subgroups, which are associated with the prevalence of established adverse prognostic factors.\textsuperscript{33} By re-analysis of this data set, we demonstrate that CD27 is differentially expressed in hierarchical clustered MM subgroups. MM patients displaying the lowest CD27 expression were all clustered in the high-risk patients groups, of which the mRNA expression profile closely resembles that of human MM cell lines. Additionally, CD27 expression was studied in plasma cells from healthy donors, from Monoclonal Gammopathy of Undetermined Significance (MGUS) patients and patients suffering from \textit{de novo} Plasma Cell Leukemia (PCL) and it was demonstrated that in contrast to MM, plasma cells from healthy donors, MGUS and PCL patients show a homogeneous high CD27 expression.

**Material and methods**

**Patient material**

For flow-cytometry analysis, bone marrow cells from iliac crest were obtained from MM patients (n=28) at various stages of treatment and disease.
Six samples were obtained at presentation, 12 samples from relapsed patients, 4 samples from patients in partial remission and 6 samples from patients that achieved a complete remission based on cytological criteria. Furthermore, bone marrow cells were obtained from newly diagnosed plasma cell leukemia (PCL) patients (n=3), monoclonal gammopathy of undetermined significance (MGUS) patients (n=6), solitary plasmacytoma patients (n=2), cold agglutination IgM syndrome patient (n=1), amyloidosis patient (n=1) and from healthy donors (n=4). For microarray mRNA expression analysis, anti-CD138 (Miltenyi-Biotec, CA, USA) plasma cell purification from 32 normal healthy donors and 74 cases of untreated newly diagnosed MM have been described. The mononuclear cell fraction from aspirates was separated by a standard Ficoll-Hypaque gradient centrifugation (Amersham-Pharmacia Biotech). Tonsils were obtained from 11 patients undergoing tonsillectomy for chronic tonsillitis. B-cell isolation from 7 tonsil preparations was performed using directly conjugated monoclonal mouse anti-human CD19 antibodies, plasma cells were purified as described above (Miltenyi-Biotec). All samples were obtained after informed consent. MM and PCL patients were staged according to the Salmon & Durie criteria.

**Cell lines**

The human MM cell lines JJN3, OPM-1, U266, RPMI8226, LB84-1, LP-1, EJM, MM-S1, Karpas 707 (courtesy of Dr. MHC Bakkus) were maintained in supplemented RPMI1640 with 10% Fetal Calf Serum (FCS, Life Technologies, Gaitherburg, MD, USA).

**Monoclonal antibodies**

The following mouse-anti-human monoclonal antibodies were used in this study: Peridine chlorophyll protein (PerCP)-conjugated anti-CD19 (SJ25-C1, Becton & Dickinson, Mountain View, CA, USA), allophycocyanin (APC)-conjugated anti-CD38 (T16, ImmunoQuality Products, Groningen, the Netherlands), fluorescein isothiocyanate (FITC)-conjugated anti--CD138, phycoerythrine (PE)-conjugated anti-CD138, APC-conjugated anti-CD138 (BB4, ImmunoQuality Products), PE-conjugated anti-CD27 (MT-271, BD Pharmingen, San Diego, CA, USA), FITC-conjugated anti-CD27
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(CLB27/1, Central laboratory of the Netherlands Red Cross, Amsterdam, the Netherlands), biotinylated anti-CD27 (O323, E-Bioscience, San Diego, CA, USA). FITC-conjugated anti-Kappa and anti-Lambda light chain (DAKO patts, Glostrup, Denmark). Isotype- & fluorochrome-matched controls were included in all experiments.

**Flow cytometry analysis**

Four-color staining procedures were performed on freshly obtained bone marrow cells; red cells were lysed using FACS-brand™ lysing solution (Becton & Dickinson, CA, USA). Intracellular staining of Ig-light chains was performed using the Fix & Perm intracellular staining kit according to the manufacturer instructions (an der Grub, Kaumberg, Austria). Cryopreserved mononuclear cells were thawed in newborn calf serum (NCS, Life Technologies). Thawed bone marrow cells were treated with DNase I (0.25 mg/ml), MgSO₄ and heparin (125 U/ml), cells were washed prior to staining. Staining of nucleated cells was determined by gating based on forward and side scatter (FSC/SSC) properties. Cell debris and red blood cells were excluded by FSC/SSC gating. A minimum of 40,000 events was measured using a FACS-Calibur apparatus (Becton & Dickinson, CA, USA). Four-color staining was analyzed using the Winlist software package (Verity Software house Inc, ME, USA) and the FlowJo package (TreeStar, Stanford, CA, USA). CD27 expression was determined within the CD138⁺ CD38++ plasma cell gate. The percentage of positive cells was calculated by subtracting isotype-control histograms from the CD27 histograms using the Enhanced Normalization Subtraction (ENS) protocol from the Winlist software-package. Mean fluorescence intensities were calculated for CD27 and CD19. Normalization was performed by calibration of the flow-cytometer using Calibrite™ beads (Becton & Dickinson).

**Reanalysis of cDNA microarray dataset**

Detailed protocols for cell purification, cDNA synthesis, cRNA preparation and hybridization to the Affymetrix HuGeneFL GeneChip microarray have been described in a previous study. The natural log of the average difference of the *TNFRSF7* (CD27) gene expression was determined in this data set.
Chapter 6

The natural log of the average difference was used in a one-way ANOVA analysis of variability using separate means for each of the groups, B-cells (BC), Tonsillar Plasma Cells (TPC), Bone marrow Plasma Cells (BPC) and MM subgroups and compared individually.

Flow-cytometry sorting

CD138+ CD38++ CD27+ and CD138+ CD38++ CD27− plasma cell populations were sorted from the bone marrow from a MM patient using a MoFlo high-speed sorting device (Cytomation, Heidelberg, Germany). Purity of sorted populations was >98% as assessed by reanalysis.

Reverse transcripting polymerase chain reaction (RT-PCR) and cloning of patient-specific Ig VH genes

Total RNA was isolated from 1x10⁶ bone marrow mononuclear cells, sorted plasma cells and human MM cell lines using Trizol™ reagent (Life Technologies) according to the manufactures instructions. Approximately 1 μg of RNA was primed with 165 ng random hexamers (Amersham-Pharmacia Biotech) and Murine Moloney Leukemia Virus reverse transcriptase (SuperscriptII™, Life Technologies) in a volume of 20 μl, containing 50 mmol/l KCl, 20 mmol/l Tris-HCl (pH 8.4), 2.5 mmol/l MgCl₂, 1 mmol/l dithiothreitol and 0.5 mmol/l of each deoxynucleotide triphosphate, for 50 minutes at 42 °C. The reaction was stopped by enzyme inactivation for 5 minutes at 95 °C. cDNA integrity was confirmed by β-Actin RT-PCR. β-Actin forward: 5’-TCA CCC ACA CTG TGC CCA TCT ACG A-3’, β-Actin backward: 5’-CAG CGG AAC CGC TCA TTG CCA ATG G-3’. Ig VH genes expressed by the malignant plasma cells were amplified using VH family-specific Framework 1 (FR1) and Ig-constant region-specific oligonucleotides as described. 0.5 μl cDNA was amplified in a 25 μl PCR reaction containing 20 mmol/l Tris-HCl, pH 8.4; 50 mmol/l KCl, 1.5 mmol/l MgCl₂, with 15 pmol/l of each primer and 1.25 U Taq DNA polymerase (Life Technologies); all PCR reactions were performed at 60 °C annealing temperature, 30 amplification cycles. PCR-products were separated on LE agarose gels (Seakem, FMC Bioproducts, Rockland, MN, USA) and visualized by ethidium bromide staining and UV-illumination.
PCR-products were cloned directly into the pCR2.1TOPO™ vector (Invitrogen, Leek, the Netherlands) according to the manufacturers instructions. Positive colonies were identified by X-gal blue/white screening and EcoRI restriction enzyme analysis. Plasmids were isolated with the High-Pure plasmid isolation kit (Roche Molecular Biochemicals, Mannheim, Germany). At least 8 clones were partially sequenced using only the dideoxy thymidine triphosphates (ddTTP) (‘T-tracks’) to identify clones in which the dominant tumor-derived IgH sequence were present. Dominant clones were then cycle-sequenced with the complete set of ddNTPs using FITC-labeled plasmid-specific oligonucleotides (M13forward and M13reverse). Sequencing gels were analyzed using the ALFwin automated sequencing device (Amersham-Pharmacia Biotech). DNA-sequences were compared to the IMGT-sequence database using DNAplot software (http://imgt.cines.fr:8104) and submitted to the EMBL database (available under accession number AJ316217). Allele specific oligonucleotides (ASO) were designed for the hypervariable CDR1 and CDR3 regions. The CDR1 oligonucleotide was designed to bind a somatic mutation at the 3’ end. The CDR3 oligonucleotide was chosen in the N-region from the D-JH junction. Oligonucleotide specificity was confirmed by RT-PCR on bone marrow from healthy donors and other MM patients. CD27 RT-PCR was performed at the above mentioned conditions. CD27 forward: 5’-CCA GCT TGG AGG TGC TAA CT-3’, CD27 backward: 5’-TGC AGG CTC CAC AGG ACT T-3’.

Statistical analysis

Spearman’s nonparametric test was used to calculate correlation coefficients. The Mann-Whitney *U*-test was used to determine significance of differences between groups.

Results

*Multiple Myeloma plasma cells are characterized by a heterogeneous expression of CD27.*

The percentage of CD27 expressing CD138⁺ CD38++ plasma cells was analyzed in a cross-sectional study of bone marrow samples from MM patients in various
stages of the disease and treatment (n=28). The percentage of CD27 expressing plasma cells in the bone marrow from MM patients showed a marked heterogeneity (Figures 6.1A-C)*. MGUS patients (n=6) have a significant higher percentage of CD27 expressing plasma cells compared to MM patients (mean 93.48±8.49 vs 67.02±30.2, \( P=0.0154 \)) (Figures 6.1D and 6.2)*. Plasma cells obtained from healthy donors (n=4) (Figure 6.1F)*, PCL (n=3) (Figure 6.1E)*, solitary plasmacytoma (n=2), cold agglutination IgM syndrome (n=1) and AL-amyloidosis (n=1) patients all show homogeneous high CD27 expression (>98%). In a previous study it has been demonstrated that in contrast with plasma cells from healthy volunteers, plasma cells from MM patients show loss of CD19 expression. Analysis of mean fluorescence intensities showed that CD27 downmodulation was associated with CD19 downmodulation (\( R^2=0.4, P<0.0001 \)) (Figures 6.3A, B)*, indicating that CD27 is specifically lost from CD19- plasma cells in MM.

In addition, all human multiple myeloma cell lines tested (JJN3, OPM-1, U266, RPMI8226, LB84-1, LP-1, EJM, MM-S1 and Karpas 707) were negative for CD27 by flow-cytometry and CD27 specific RT-PCR (Figures 6.1G-H)*. These MM cell lines are mostly derived from patients with progressive disease and reflect in most cases extramedullary locations of myeloma. To exclude the possibility that the use of different monoclonal antibodies is responsible for the discrepancy of our results compared to earlier reports, several CD27 specific mouse-anti-human monoclonal antibody clones (M-T271, CLB27/1, O323) were tested on fresh bone marrow samples from 20 MM patients. All monoclonal antibodies yielded similar results (data not shown).

**Downmodulation of CD27 mRNA expression is specific for MM plasma cells and is associated with high risk disease.**

Patients who achieved a complete clinical remission upon treatment are characterized by a significant higher percentage of CD27 expressing plasma cells (98.5±1.8%) compared with newly diagnosed and relapsed patients (mean 67.0±30.2, \( P=0.0022 \)) (Figure 6.2)*. To further assess the significance of CD27 expression, 7 additional groups were analyzed by cDNA microarray; tonsil B-cells (BC; n=7), tonsil plasma cells (TPC; n=11),
bone marrow plasma cells (BPC, n=32) and gene expression profile MM patient subgroups (MM1, n=20; MM2, n=21; MM3, n=15 and MM4, n=18) defined by hierarchical clustering as described.\textsuperscript{33} By comparing 5,483 genes, patients were grouped according to gene expression profile in which MM1 expression pattern was similar to normal plasma cells and MGUS whereas MM4 was similar to MM cell lines. Clinical parameters have been compared among clustered MM subgroups. Clinical parameters linked to poor prognosis (abnormal cytogenetics, high serum β2-microglobulin, chromosome 13 deletions) were most prevalent in MM4.\textsuperscript{33} In this study, we compared TNFRS7 (CD27) gene expression in these hierarchical clustered MM subgroups and normal cells using this data set. All MM cases had lower TNFRSF7 (CD27) gene expression than their normal BPC counterparts. A gradual decrease of CD27 mRNA expression could be observed through MM subgroups 1 to 4, in which the MM4 subgroup demonstrated the lowest level of CD27 expression (Figure 6.4)*. Apparently, this is in contrast with the homogeneous high expression of CD27 on de novo PCL that is generally associated with a higher incidence of poor prognostic factors and consequently a more aggressive clinical course. The PCL patients presented in this study showed typical clinical behavior of poor prognosis PCL.

Both CD27\textsuperscript{+} and CD27\textsuperscript{-} plasma cells belong to the clonal disorder in MM.

In many patient samples (17/28), distinct CD27\textsuperscript{+} and CD27\textsuperscript{-} plasma cell subpopulations could be distinguished (figure 6.1B)*. Sorted CD27\textsuperscript{+} and CD27\textsuperscript{-} plasma cells (CD138\textsuperscript{+} CD38\textsuperscript{++}) from the bone marrow of a newly diagnosed MM patient (n=1) express the malignant plasma cell V(D)J rearrangement as determined by CDR1-CDR3 RT-PCR. Restricted Ig-κ light chain expression was observed in both CD27\textsuperscript{+} and CD27\textsuperscript{-} plasma cell populations (Figure 6.5)*.

* figures 6.4 and 6.5 see page 108
CD27 expression was determined within the CD138+ CD38++ plasma cell gate; two representative patients for each subgroup are shown: (A) multiple myeloma patients incomplete clinical remission, (B) newly diagnosed multiple myeloma patients, (C) multiple myeloma patients with progressive disease, (D) MGUS patients, (E) PCL patients, (F) normal donor bone marrow plasma cells, (G) human myeloma cell lines LB84-1 and JJN3, and (H) CD27-specific RT-PCR on human myeloma cell lines. Burkitt’s lymphoma cell line Daudi was used as a positive control. β-actin RT-PCR was performed to confirm cDNA integrity.

Figure 6.1. CD27 expression on plasma cells. CD27 expression was assessed by four-color flow cytometry and RT-PCR.
**Figure 6.2.** CD27 expression on plasma cells from multiple myeloma and MGUS patients.

The percentage of CD27-positive plasma cells (CD138⁺ CD38++ gate) was determined by enhanced normalization subtraction. Newly diagnosed, relapsed and patients with progressive MM were compared with patients who achieved complete clinical remission (MM CR), and MGUS patients. The Mann-Whitney U-test was used to determine significance of differences.

**Figure 6.3.** Loss of CD27 expression on plasma cells is associated with loss of CD19 expression.

(A) Mean fluorescence intensities (MFI) for CD27 and CD19 were determined within the CD138⁺ CD38++ plasma cell gate. MFI values for CD27 were plotted against MFI values for CD19. Each black square represents an individual patient; dashed lines represent 95% confidence intervals. (B) CD27 and CD19 expression on plasma cells (CD138⁺ CD38++) from MM patients was determined by four-color flow cytometry. Four patients are depicted, demonstrating the association between CD27 and CD19 expression.
Figure 6.4. Box plot of F-values. Expression of CD27 was evaluated in seven groups.

B cells (BC; n=7), tonsil plasma cells (TPC; n=11), bone marrow plasma cells (BPC; n=32), and gene expression-defined multiple myeloma subgroups (MM1, n=20; MM2, n=21; MM3, n=15; MM4, n=18) are distributed along the x-axis. The natural log transformed average difference (AD) is plotted on the y-axis. The one-way ANOVA F-value for the TNFRSF7 is presented. An F-value greater than 2.99 indicates a higher likelihood that a gene is variable among the groups (P<0.0001). Consistent with the AD, the absolute call (AC) indicated that the TNFRSF7 gene was present in all BC, TPC and BPC samples, but was absent in a significant portion of MM subgroups and showed highly significant loss in MM4 (P<0.01).

Figure 6.5. CD27-positive and CD27-negative plasma cells both belong to the clonal disorder as determined by Ig light chain expression and ASO-RT-PCR.

Bone marrow mononuclear cells from an IgGκ-expressing MM patient at presentation were sorted to obtain CD138⁺CD38⁺CD27⁺ and D138⁺CD38⁺CD27⁻ subpopulations. The purity of sorted cells was >98% as assessed by re-analysis. Immunoglobulin κ and λ light chain expression was determined by intracellular staining. ASO-RT-PCR was performed on sorted populations using CDR1 and CDR3-specific oligonucleotides.
Discussion
The present study demonstrates that CD27 is expressed on malignant plasma cells in a heterogeneous fashion. MM patients in complete clinical remission show a significant higher percentage of CD27 expressing plasma cells compared to MM patients at diagnosis and at relapse. This heterogeneity might be responsible for the discrepancy between our results and earlier reports in which only a limited number of patients were analyzed.\(^{232,238}\) Our data suggest that progression/relapse of MM is associated with loss of CD27 expression and that clinical response, which is associated with reappearance of normal plasma cells, can be monitored by assessment of CD27 expression on plasma cells. Bone marrow plasma cells from MGUS patients show a homogeneous high expression of CD27, which is analogous with the CD27 expression on normal plasma cells. This is in agreement with the initial benign nature of this disease. Of interest is whether progression of MGUS to overt MM is associated with loss of CD27 expression.

Loss or aberrant expression of surface markers has been described for MM. For instance, normal plasma cells have a phenotype of CD19\(^+\)/CD56\(^-\) whereas malignant plasma cells are CD19\(^-\)/CD56\(^+\).\(^{220,248,249}\) A previous study suggests that loss of CD19 is involved in tumour progression, as enforced overexpression of CD19 in human myeloma cell lines leads to growth inhibition and reduced tumorigenicity \textit{in vivo}.\(^{250}\) Analysis of Mean Fluorescence Intensities (MFI) demonstrated a significant correlation between loss of CD27 expression and loss of CD19 expression. This is further supported by the lack of both CD19 and CD27 expression on the human myeloma cell lines we tested (data not shown). Furthermore, CD27\(^+\) plasma cells displayed a lower Forward Scattering (FSC) profile than CD27\(^-\) plasma cells (data not shown), which is characteristic for more immature plasma cells. Although the CD27\(^+\) plasma cell population apparently includes normal plasma cells, ASO-PCR analysis demonstrated that this population can also harbor clonotypic cells, suggesting that CD27\(^+\) and CD27\(^-\) plasma cell populations both can belong to the clonal disorder. In a previous study it was demonstrated by cDNA microarray analysis that \textit{TNFRS7} (CD27) gene expression was lower in MM plasma cells compared to normal plasma cells present in bone marrow and tonsil, again indicating that loss of CD27 is specific for MM. In this study we compared hierarchical clustered MM subgroups and show that the CD27\(^-\) expression was lowest in the MM4 subgroup.
This group includes patients with poor prognostic factors, (e.g. high β2-microglobulin and abnormal cytogenetics) supporting the hypothesis that loss of CD27 is associated with a more aggressive disease. However, this view is not in line with the results in de novo PCL where a homogeneous high expression CD27 is observed in spite of a more aggressive clinical behavior. It has been suggested that de novo PCL represents a different clinical entity that can be distinguished from MM on basis of immunophenotyping and chromosomal abnormalities. Expression of CD27 might be regarded as an additional parameter that differentiates PCL from MM.

In the bone marrow of many MM patients distinct CD27⁺ and CD27⁻ plasma cell populations were present. It is conceivable that the reduced expression of CD27 on a subpopulation of plasma cells might be related to clonal selection to a more aggressive subtype, since both the CD27⁺ and CD27⁻ populations belong to the malignant clone. This is supported by the results of the human MM cell lines. All tested cell lines lack CD27 at the mRNA and protein level. These cell lines are mostly derived from MM patients with progressive disease. In a recent study it has been demonstrated that CD27 expression can be induced from purified normal CD27⁻ naive peripheral blood B-cells by CD40 ligation. In contrast, CD40 ligation was not capable of inducing CD27 expression on the CD40 expressing myeloma cell line U266 and the EBV-transformed lymphoblastoid cell line ARH-77 (data not shown), indicating that loss of CD27 expression is an intrinsic feature of MM plasma cells and probably not due to the absence of appropriate co-stimulatory signals. Further study into the regulation of CD27 and its association with progression could provide insight into the role of CD27 in the biological and clinical aspects of MM.

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