Autologous bone marrow Th cells can support multiple myeloma cell proliferation *in vitro* and in xenografted mice

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The authors declare no conflict of interest.

Running title: Th CELLS STIMULATE MM CELL PROLIFERATION

Keywords: Multiple myeloma, Th cell, proliferation, Bone marrow microenvironment.

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Count: main text words (3975), abstract words (195), figures/tables (<8), and references (45).

Abstract (195)

Multiple myeloma (MM) is a plasma cell malignancy where MM cell growth is supported by the bone marrow (BM) microenvironment with poorly defined cellular and molecular mechanisms. MM cells express CD40, a receptor known to activate autocrine secretion of cytokines and elicit proliferation. Activated T helper (Th) cells express CD40 ligand (CD40L) and BM Th cells are significantly increased in MM patients. We hypothesized that activated BM Th cells could support MM cell growth. We here found that activated autologous BM Th cells supported MM cell growth in a contact- and CD40L-dependent manner *in vitro.* MM cells had retained the ability to activate Th cells that reciprocated and stimulated MM cell proliferation. Autologous BM Th cells supported MM cell growth in xenografted mice and were found in close contact with MM cells. MM cells secreted chemokines that attracted Th cells, secretion was augmented by CD40-stimulation. Within 14 days of culture of whole bone marrow aspirates in autologous serum, MM cells and Th cells mutually stimulated each other, and MM cells required Th cells for further expansion *in vitro* and in mice. The results suggest that Th cells may support the expansion of MM cells in patients.

Introduction

Patients with multiple myeloma (MM) and monoclonal gammopathy of undetermined significance (MGUS) have significantly increased numbers of T cells in the bone marrow (BM) (1), a microenvironment that also includes cell types such as endothelial cells, fibroblasts, osteoclasts, myeloid-derived suppressor cells (MDSC), macrophages and dendritic cells (2). This complex niche is still poorly understood and the mechanisms that induce MM cell proliferation remain unclear.

Conceptually, cellular elements may either be detrimental, beneficial or indifferent for MM cell proliferation. T cells can counteract the development of cancer in the act of immunosurveillance. However, cancer cells may edit the immune response (3) and tumors can subvert immunity (4). Experiments in mice have demonstrated that T cells can kill murine myeloma cell lines (5). Nevertheless, MM cells may eventually escape T cytotoxicity (6, 7). Recently, a new mechanism of escape was demonstrated with human primary MM cells: MM cells deactivated the proteasomal pathway in dendritic cells via binding of CD28 (MM cells) to CD80/86 (dendritic cells), and protected themselves from $CD8^+$ T cell killing (8). Concerning T helper (Th) cells, tumor cells including MM cells may evade cytotoxic immune responses by facilitating a skewed immune response, promoting a protective cytokine environment and by favoring non-cytotoxic Th cell subtypes as well as other immune cells (6, 9, 10). On the other hand, Th cells also played an active role in both lymphomagenesis in mice (11) and supported malignant B cells in humans (12-14). In fact, others have speculated that myeloma-specific Th cells can accommodate MM cells by secreting MM-supportive cytokines (15). Similarly, Th17 cell cytokines promoted the growth of MM cell lines *in vitro* and *in vivo* and supported colony formation of primary human MM cells (16).

The BM is an important niche for memory Th cells both in healthy individuals and MM patients (1, 2, 17, 18) and activated Th cells with high surface CD40L expression have been found in the human

BM (2, 17-22). Interestingly, MM cells express high levels of CD40; CD40 signaling triggers the NF-κB pathway and secretion of several pro-survival factors (23-25). It is therefore plausible that CD40 activation could play a role for primary MM cell proliferation, but it has yet to be resolved whether these activated Th cells can in fact support MM cell proliferation *in vitro* and *in vivo*.

To investigate the potential effect of activated Th cells on primary MM cells, we polyclonally stimulated BM Th cells and analyzed the net effect of the Th subsets present. Activated autologous Th cells were tested for their ability to support MM cells *in vitro* and *in vivo.* We also performed assays where primary MM cells were tested for their ability to activate and collaborate with allogeneic Th cells. The latter experiments were feasible because primary human MM cells are antigen presenting cells and can present antigen to Th cells (26, 27).

Materials and methods

MM patients and healthy controls

The study was approved by The Regional Committee for Medical and Health Research Ethics of South-East Norway; MM patients were recruited at Oslo University Hospital and Drammen Hospital. BM and blood samples from MM patients were collected from patients that were untreated (32 patients) or had relapsed after one or more treatments (22 patients), see Supplementary Information for details. Blood and BM samples from five healthy individuals were used in the experiments as indicated. Functional studies were performed on IgG^+ or IgA^+ patients as indicated in the figure legends.

Cell purification, culture and microscopy

MM cells were purified from BM aspirates of MM patients using MACS $CD138^+$ microbeads (Miltenyi). Purified MM cells were >98% $CD138^+$ and >95% of cells expressed the clonal light (L) chain (κ or λ); non-clonal L chain (λ or κ respectively) were not detected on the MM cells. Further purity analyses were also performed by staining for CD56 and/or CD28, differentiating MM cells from normal plasma cells (28). As appropriate, these stains were included in the gating strategy for subsequent assays as indicated in Figure Legends. FlowComp Human CD4 kit (Life Technologies) was used to purify $CD4^+$ Th cells (>97%) from the BM and blood of healthy individuals as well as MM patients. Th cell lines from healthy individuals as well as patients were generated by expanding Th cells using Human T-Activator CD3/CD28 Dynabeads (Life Technologies, abbreviated as anti-CD3/28 beads herein) with 20U/ml interleukin-2 (IL-2, Roche) for short term stimulation lasting 5-7 days. Bright field images were taken with a Nikon Eclipse Ti fluorescent microscope equipped with a high-sensitivity CCD camera (Cascade 512, Photometrics) and a 40x objective lens. Culture of bulk bone marrow cultures was performed as described in the Supplementary Information by depleting T cell subsets (Dynabeads) and culturing cells in autologous serum and IL-2.

Cell proliferation assays

MM cells (0.5-1 x 10^6) were co-cultured with rested allogeneic Th cells in a 1:1 ratio. Alternatively, MM cells were co-cultured with either the allogeneic Th cells or autologous BM Th cells directly in the presence of anti-CD3/28 beads. In other settings, MM cells were stimulated with plate coated agonistic anti-human CD40 (1μg/ml, clone #5C3, BD Biosciences) or were co-cultured with 100 Gy irradiated murine L cell fibroblasts expressing human CD40L (herein abbreviated $CD40L^+$ L cells) (29). Cells were cultured in 6.5mm well Transwell culture plates (inserts with 0.4 μm pores; Corning). MM cells (5 x 10^5) were added to inserts and separated from Th cells, MM cells and anti-CD3/28 beads in the lower wells. Cells were co-cultured for 5 days and tested for DNA replication by BrdU-incorporation, BrdU (BrdU stain kit, BD) was added to cell cultures in the final 48h of assays; cells were stained with anti-BrdU as per manufacturer's protocol. MM cells were stimulated by BM Th cells in the presence of 20U/ml IL-2 and 10ng/ml IL-15 (PeproTech) and then were imaged with the Incucyte Zoom microscope with standardized manufacturer's protocol as detailed in the Supplementary Information. Inhibition of MM cell proliferation was performed in the co-culture system with Th cells in the presence of 20μg/ml anti-CD40L (clone #24-31, eBioscience) from day 3-5 or with matched isotype (eBioscience).

Th cell migration assay

Purified MM cells were stimulated without/with immobilized anti-CD40 (#5C3, BD). Supernatants were collected on day 3 for migration assays. The Transwell system (5 μm pore size, Corning) was used to test the migration of freshly purified autologous Th cells (2.5 x 10^5) from respective MM patients. Average background migration towards fresh medium was 6%. The enhanced Th cell migration (Δ migration) towards MM supernatant was calculated by subtracting the 6% background.

Flow cytometry

Cells were stained with combinations of the following fluorochrome labeled mAbs: From eBioscience, mAbs were specific for λ L chain (4C2), κ L chain (A8B5), CD4 (OKT4), CD28 (37.51), HLA-DR (LN3), CCR5 (T21/8), CCR6 (R6H1), PD-1 (J105), Bcl-6 (BCL-UP), T-bet (4B10), GATA3 (TWAY), IL-2 (MQ1-17H12), IL-4 (8D4-8), IL-17 (eBio64DEC17), IL-21 (3A3-N2) and TNFα (MAb11). mAbs from Biolegend were specific for: CXCR3 (TG1) and IL-13 (PVM13-1). mAbs from BD Bioscience were specific for: CXCR5 (RF8B2), CD56 (B159), CD138 (MI15), CRTH2 (BM16), RORγt (Q21-559), IFN-γ (25723) and BrdU (552598). Surface staining and intracellular staining were performed as described (12). Cells were analyzed with FACSCalibur or Fortessa (BD) and FlowJo X software (Tree Star Inc). Cells were stained for cytoplasmic Igκ or Igλ L chains as indicated after permbealization with cytoperm/cytofix buffer (BD).

***In vivo* experiments**

Busulfan (Busilvex, Pierre Fabre Pharma) pre-conditioned NOD SCID gamma null mice (NSG, The Jackson Laboratory) were used in xenotransplantation with MM and Th cells (12). Mice were injected *i.v.* with purified MM cells (8 x 10^6) or MM cells and autologous BM Th cells (4 x 10^6). Alternatively, whole bone marrow cultures were cultured as described in figure legends and cells were injected into NSG mice, see Supplementary Methods for details. Engrafted cells were analyzed by flow cytometry. Immunofluorescence staining was performed on lungs, livers and spleens. All animal experiments were approved by the Norwegian Animal Research Authority. Further details of *in vivo* experiments are presented in the Supplementary Information.

ELISA and measurement of cytokines

Serum samples were used to determine IgGκ and IgGλ concentration by ELISA (12); IgG was captured with anti-human IgG (I2136) and detected with alkaline phosphatase conjugated anti-κ L chain (A3813), or anti-λ L chain (A2904, Sigma-Aldrich). Cytokines from supernatants were

157 collected on day 3 for Bio-Plex cytokine assay (Bio-Rad) according to the manufacturer's
158 instructions. ELISA kits were used to measure IL-23 (eBioscience) and RANKL (PeproTech).
159
160 **Statistical analysis**
161 Statistical analysis was calculated using two tailed Student's t-test or two tailed Mann-Whitney Test
162 with GraphPad Prism 5.02 (GraphPad Software, San Diego, CA). A P value < 0.05 was considered
163 statistically significant.

Results

Polyclonally activated allogeneic and autologous Th cells stimulated the proliferation of MM cells

Sorted $CD138^+$ MM cells were cultured for 5 days with polyclonally stimulated allogeneic Th (allo Th) cell lines from healthy individuals in the presence of anti-CD3/28 beads. Th cells activated the proliferation and blastogenesis of MM cells (Figure 1a). When CD138-depleted autologous BM cells were added back to $CD138^+$ MM cells in the presence of anti-CD3/28 beads, we found a similar proliferation and blastogenesis (Figure 1a). This suggested that autologous BM Th cells could stimulate MM cells. To confirm this, we purified autologous BM Th cells (Th_{MYEL}) that were added to MM cells in the presence of anti-CD3/28 beads. Autologous Th_{MYEL} cells from patients activated MM cells with similar efficiency to Th cells from healthy individuals (Figure 1a and b, Supplementary Figure S1a-c). These Th_{MYEL} cells significantly increased MM cell proliferation (MM cells with Th cells and beads vs. MM cells alone, P=0.0003, N=4, Figure 1b). MM cells increased cell size, became more granular, reduced their cell surface immunoglobulin (Ig) expression, and up-regulated expression of HLA-DR. No expansion was seen of $CD138^+$ cells with the uninvolved L chain (Figure 1b, and data not shown). Anti-CD3/28 beads did not significantly stimulate the proliferation of MM cells in the absence of Th cells, (P=0.873, N=6).

We next imaged the activated Th_{MYEL} cell – MM cell interaction in a 1:1 ratio in the presence of anti-CD3/28 beads and the Th cell growth factors IL-2 and IL-15. With this strategy, we found that MM cells responded by proliferating from 30 h, allowing a 5-10x expansion within 98 h (Figure 1c, d and Supplementary Figure S2).

Cytokine secretion of activated patient BM Th cells

A majority of BM Th cells from MM patients (Th_{MYEL}) expressed the chemokine receptors CXCR3 in combination with CCR6 after short term anti-CD3/28 bead stimulation, significantly different and increased compared to BM Th cells from healthy controls (Th_{NORM}) (Figure 2a). A similar but

less pronounced increase of $CXCR3^+CCR6^+$ Th cells was found in the BM of patients, Supplementary Figure S3). Th_{MYEL} cell lines were mostly $CXCR3^+CCR6^+CCR5^+CXCR5^-ICOS^-T\text{-}bet^+IFN\text{-}\gamma^+$. Th_{MYEL} cells also had a low/intermediate ROR-γt expression (Figure 2a). The CXCR3/CCR6/$T\text{-}bet^+ROR\text{-}\gamma t^{LOW}$ pattern has previously been described; this cell type has been labeled Th1/17 or Th1* (30, 31). The classical Th2 (GATA3/ CRTh2/IL-4) and Th17 (ROR-γt/IL-17) subsets each represented only about 2% of Th_{MYEL} cells (data not shown). We detected less than 1% of Th_{MYEL} expressing IL-21, BCL6, PD-1, CXCR5 and ICOS (the follicular Th subset, data not shown). A lower frequency of expanded Th_{MYEL} cells was $IFN\text{-}\gamma^+$ and $TNF\text{-}\alpha^+$ compared to expanded Th_{NORM} cells (Figure 2a, right histogram). In these expanded BM Th cells, we found that the Th_{MYEL} produced lower amounts of cytokines such as IL-4, IL-10, IL-13, GM-CSF, IFN-γ and TNF-α, and more of IL-6, IL-1β, IL-2, IL-17, CCL2 (MCP-1) and CCL4 (MIP-1β), Figure 2b. Other cytokines included CCL5 (RANTES), CCL3 (MIP-1α), CXCL10 (IP-10), VEGF and IL-9 (Supplementary Figure S3). Despite these variations, the allogeneic normal Th cells and the activated autologous Th_{MYEL} cells had a very similar capacity to stimulate MM cells (Figure 1a and b). Next, we tested whether Th cytokines were sufficient for MM cell proliferation.

Th cell dependent stimulation of MM cells was dependent on cell contact and CD40L stimulation

Using Transwell assays, we found that the presence of Th cytokines was not sufficient to reconstitute full MM cell proliferation. MM cells that were in contact with activated Th cells responded much stronger (Figure 3a). Proliferation of MM cells could also be induced by co-culture with murine L cells, L cells expressing human CD40L ($CD40L^+$ L cells) or by providing agonistic anti-CD40 mAb. The CD40-related responses were seen without addition of exogenous cytokines (Figure 3b). We next repeated Th cell-MM cell experiments as shown in Figure 1, but added antagonistic anti-CD40L from day 3-5: Blocking CD40L reduced the ability of activated Th_{MYEL} to support the proliferation of MM cells (Figure 3c).

CD40 stimulated MM cells secreted Th cell attracting cytokines & chemokines

We assayed supernatants (SN) from MM cells stimulated by agonistic anti-CD40, and found that the secretion of some cytokines/chemokines was significantly increased in comparison to resting MM cells (N=6, P<0.05; Figure 4a). This included sRANKL, a TNF ligand family member involved in MM pathogenesis and bone resorption (32, 33). We also found IL-9 and IL-23, which are not previously associated with MM cells. It was of interest whether the inflammatory mediators in MM cell supernatants could recruit Th cells. We found that these supernatants attracted Th cells 4 times as efficiently as supernatants from unstimulated MM cells, suggesting that CD40L activation of MM cells could further recruit Th cells to MM niches in the BM, Mann-Whitney test, P=0.0003, N=6 (Figure 4b).

MM cells activated and collaborated with alloreactive Th cells

Others have described that a variable but consistent subset of MM cells express HLA-DR and co-stimulatory molecules and that MM cells retain antigen presentation function (26, 27). We confirmed the finding of HLA-DR expression on the current MM cells (IgG^+ and IgA^+ subsets, Figure 5a). Similar results were found in light chain MM (data not shown). The light chain MM patients were not included in functional experiments as MM cell purity could not be easily accessed by κ/λ surface Ig staining. No significant differences in HLA-DR expression were found between these groups. On average, HLA-DR expression was found on 17% of MM cells (Figure 5a). We thereafter performed mixed leukocyte reactions with MM cells (N=7) and Th cell lines derived from healthy individuals. When mixed in culture, MM cells could stimulate allogeneic Th cells and obtain help from Th cells to proliferate (Figure 5b and Supplementary Figure S1b). This was also the case for MM cells with BM Th cells from other patients (Supplementary Figure S1c). Although HLA-DR expression varied within these samples, all MM cells activated alloreactive Th responses and received help to proliferate, P=0.004 (Figure 5c).

MM cells were supported in vivo by autologous BM Th cells

We next purified Th_{MYEL} cells from the BM of 4 MM patients and activated cells in wells coated with anti-CD3 and anti-CD28 antibodies (α-CD3/28) for 24 h. Activated autologous Th_{MYEL} cells were co-injected with MM cells, or MM cells were injected alone into conditioned NSG mice (Figure 6a). Low levels of serum M-component were obtained when MM cells were transferred in the absence of autologous Th cells. In mice that received both MM cells and Th cells, Th cells supported significant secretion of M-component from 30 days (N=4, P=0.004; Figure 6b) and MM cell proliferation in the BM (N=4, Mann-Whitney test, P=0.004, Figure 6b). Some variation in MM cell proliferation was seen between patients; high and low responders are shown, less variation was seen in serum titers of M-component (Figure 6b), suggesting that our results could have been influenced by varying distribution of cells in BMs as compared to other tissues of mice. In fact, we found that both MM cells and Th cells had localized into the splenic niche where Th cell blasts and MM cells were in close contact, suggesting active collaboration between these 2 cell types *in vivo* in this extramedullary niche (Supplementary Figure S4).

Collaborating MM cells and Th cells from whole bone marrow cultures

The above results suggested that Th cells could stimulate MM cells and potentially play a role in the expansion of MM cells in patients. We therefore cultured cells from the whole bone marrow compartment in culture medium supplemented with autologous serum and IL-2. This approach allowed prolonged expansion of MM cells from 13 consecutive patient samples. The cells appeared quiescent for the first 5-8 days. Thereafter, clusters of activated and proliferating MM cells and Th cells were observed, Supplementary Figure S5 and data not shown. After 3-4 weeks, Th cells and MM cells were expanded (>x4-10). The expansion of MM cells was dependent on Th cells, as removal of Th cells abrogated growth and MM cell proliferation could be inhibited by providing anti class II mAbs (anti-pan HLA DP resulted in ≈ 30% inhibition, anti-pan DQ ≈ 50% and anti-pan DR ≈ 60% inhibition, data not shown). After this expansion phase, we also found activated $CD8^+$ T

cells that appeared to inhibit MM cell growth (from week 3-4, data not shown). We therefore repeated bulk culture experiments with depletion of $CD8^+$ T cells. This was compared to combined depletion of both CD4+ Th cells and $CD8^+$T cells. Within 14 days, Th cells and MM cells had expanded in cultures that lacked $CD8^+$ T cells, but not in cultures containing no T cells (Fig 6c, Suppl. Fig S6). Further, when bulk-cultured bone marrow cells were transferred into NSG mice, we found that MM cells expressing the appropriate L chain (but not normal plasma cells with uninvolved L chain) were activated in the presence of $CXCR3^+$ Th cells and that bone marrow stroma cells without T cells could not support the growth of MM cells in the spleen and bone marrow of NSG mice (Fig 6 d, Supplementary Fig S6).

Discussion

We found that polyclonally activated Th cells potently stimulated the proliferation of MM cells in a contact dependent and CD40L dependent manner. MM cells stimulated alloreactive Th-cells and MM cells thereby elicited help for their own proliferation. Autologous BM Th cells also supported MM cell proliferation and M component secretion in xenografted NSG mice. We found juxtaposed Th cells and MM cells in clusters at the extramedullary sites of xenografted mice. Similarly, *in vitro*, CD40-activated MM cells secreted chemokines and cytokines that attracted Th cells, suggesting that MM cells recruited Th cells into the MM microenvironment. Activated MM cells also secreted cytokines involved in Th cell subset differentiation (IL-9 and IL-23), bone resorption (sRANKL) and angiogenesis (TNFα and VEGF). MM cells in *in vitro* cultures of whole bone marrows expanded in the presence of Th cells in an HLA class II-dependent manner, removal of Th cells abolished MM proliferation. The BM stroma could only support the expansion of MM cells in NSG mice when Th cells were included in the graft. Taken together, our results suggest that Th cells may play an important role to support MM cell proliferation in the BM niche.

It has become clear that the BM is a niche for activated memory T cells in a microenvironment with increased levels of cytokines including IL-15 and IL-6 (18, 34). CD40L is predominantly expressed by activated Th cells; activated Th cells further up-regulate CD40L surface expression 10 fold within 2-8 hours of stimulation (19-21). Reciprocally, MM cells retain the ability to stimulate Th cells since a subset of MM cells express MHC class II and costimulatory molecules (26, 27). MM cells could therefore potentially present mutated self-epitopes to Th cells and elicit support for proliferation. After a week of culture in autologous serum, bulk bone marrow cultures gradually expanded as both Th cells and MM cells were spontaneously activated, and MM cell expansion (*in vitro* and *in vivo*) was abrogated by depletion of Th cells. This suggests that the bone marrow compartment contains Th cells specific for MM cell antigens, a suggestion that is compatible with previous findings on oligoclonal expansions of Th with unknown significance in the MM patients

(1, 35). Clonotypic determinants in the V regions of the BCR (Idiotypes) (6, 11) are plausible antigenic drivers for such Th cell partners. In fact, 0.7-10% of BM Th cells were previously found to respond to monoclonal Ig derived from MM cells. Many of these Ig-specific Th cells secreted Th2-type cytokines. Therefore, Ostad et al. speculated that such Th cells could provide support for MM cell growth (15).

It has previously been demonstrated that human primary MM cells very poorly engraft into NSG mice without provision of other human BM constituents (36). When MM cells very rarely engrafted in NSG mice, a multi-lineage reconstitution including T cells and human $CD13^+$ myeloid stromal cells was found (36). The current results demonstrate that pre-activated autologous Th cells (in Fig 6a) were sufficient for engraftment and that Th cells from bulk cultures (Fig 6d) were required when transferring unfractioned mononuclear cells from the bone marrow compartment. MM cells and Th cells were found in the same anatomical compartment; cell-cell contacts were clearly evident suggesting active recruitment of Th cells by MM cells. Further, the current results demonstrated that stromal elements other than Th cells could not support MM cell growth as Th depleted stroma failed to sustain MM cell proliferation. In the mouse, plasma cells and resting memory Th cells have two separate BM niches (17, 37). For interactions between MM cells and Th cells to occur, cells must contact the other type. In this regard, an inflammatory environment could drive chemokine secretion. A recent study demonstrated significantly increased pro-inflammatory cytokines (TNF-α, IFN-γ, IL-6 and IL-8) in the BM of MM patients (38); this adds to earlier studies that have pointed to increased inflammatory cytokines in MM (39) and other studies showing that MM cells themselves can secrete inflammatory mediators such as IL-1β, IL-6 and TNF-α (2, 40).

For MM cells and Th cells to interact, cells must co-localize. MM cells were previously found to secrete pro-inflammatory Th cell attracting chemokines such as CCL3 (MIP-1α) that can attract $CCR5^+/CCR1^+$ Th1 cells, CCL5 (RANTES) that can attract $CCR3^+$ or $CCR5^+/CCR1^+$ Th1 and Th2

cells, CCL2 (MCP-1), and CCL3 for $CCR4^+$ Th2 and Th17 cells (2, 41). In our assays these results were confirmed. Moreover, chemokine secretion was significantly up-regulated after CD40-activation. The greatest change was in CXCL10 (IP-10), a chemokine that can attract $CXCR3^+$ Th cells (Th1 and Th1/17 or Th1*). We found that an increased proportion of the BM Th cells were $CXCR3^+$ in MM patients. In addition, the BM Th cells often expressed CCR6, a receptor usually found on Th17 cells. It is increasingly clear that Th cells can develop into functional variants, as has also been described for $CXCR3^+CCR6^+$ Th1/17 or Th1* cells in humans (30, 31). Thus, inflammatory stimuli e.g. IL-1β can skew Th cell phenotypes (30, 31). The release of such mediators in the MM cell microenvironment by MM cells, Th cells and other cells may have caused the unusual chemokine receptor profile that we observed.

MM cells can also migrate in response to chemokines. MM cells express CCR1, CCR2, CCR5 and CXCR3 and migrate towards CCL3 and CCL5 (42, 43). The pro-inflammatory CCL3 and CCL5 can be secreted by activated Th cells as also seen in our data, suggesting active migration of both Th cells and MM cells towards a Th-MM cell interaction.

The current results and previous findings suggest that Th cells may drive MM growth. Previous findings support this conjecture including descriptions of 1) an inflammatory microenvironment in the BM of patients (2, 38-40), 2) chemokines supporting chemotaxis between Th cell and MM cell (42, 43), 3) the functionality of MM cells as antigen presenting cells (26, 27), 4) a high frequency of Idiotype-specific Th cells in the BM (15), 5) a high level of CD40 on MM cells and previous reports of MM cell responsiveness to CD40L (23-25, 44, 45), and 6) the responsiveness of MM cells to Th cell derived cytokines (15, 16). We suspect that MM cells have shaped and co-evolved with their microenvironment, co-opting favorable supportive Th cell subtypes, escaping cytotoxic T cell responses and sculpting other elements such as MDSC. Hence, a better understanding of the interplay between T cells and myeloma cells may be of special relevance to immunotherapy.

Acknowledgements

This work was supported by grants from the Norwegian South East Health region to L.A.M., the Research Council of Norway through its Centres of Excellence funding scheme, (project number 179573/V40) to L.A.M., the Torsteds fund, the Raagholt fund and Unifor to D.W.

Authorship

Contribution: D.W., Y.F., C.M., P.O.H., S.B., A.P.R., A.T., and L.A.M. performed experiments. J.D., Y.F., F.S. and G.E.T. provided patient samples and clinical information. D.W., Y.F., C.M., S.B., A.P.R., P.O.H., B.B., A.T. and L.A.M. analyzed data. All co-authors contributed to data interpretation, manuscript preparation. D.W. and L.A.M designed the research and wrote the paper. Conflict-of-interest disclosure: The authors declare no competing financial interests. Correspondence: Dong Wang (Dong.Wang@rr-research.no), Anders Tveita (anders.tveita@medisin.uio.no) and Ludvig A Munthe (l.a.munthe@medisin.uio.no), Centre for Immune Regulation, Institute of Clinical Medicine, University of Oslo.

References

1. Perez-Andres M, Almeida J, Martin-Ayuso M, Moro MJ, Martin-Nunez G, Galende J, et al. Characterization of bone marrow T cells in monoclonal gammopathy of undetermined significance, multiple myeloma, and plasma cell leukemia demonstrates increased infiltration by cytotoxic/Th1 T cells demonstrating a squed TCR-Vbeta repertoire. Cancer. 2006;106(6):1296-305.

2. Bianchi G, Munshi NC. Pathogenesis beyond the cancer clone(s) in multiple myeloma. Blood. 2015;125(20):3049-58.

3. Dunn GP, Old LJ, Schreiber RD. The Immunobiology of Cancer Immunosurveillance and Immunoediting. Immunity. 2004 8//;21(2):137-48.

4. Zitvogel L, Tesniere A, Kroemer G. Cancer despite immunosurveillance: immunoselection and immunosubversion. Nat Rev Immunol. 2006 10//print;6(10):715-27.

5. Haabeth OA, Lorvik KB, Hammarstrom C, Donaldson IM, Haraldsen G, Bogen B, et al. Inflammation driven by tumour-specific Th1 cells protects against B-cell cancer. Nature communications. 2011;2:240. PubMed PMID: 21407206. Pubmed Central PMCID: PMC3072106. Epub 2011/03/17. eng.

6. Haabeth OA, Tveita AA, Fauskanger M, Schjesvold F, Lorvik KB, Hofgaard PO, et al. How Do CD4(+) T Cells Detect and Eliminate Tumor Cells That Either Lack or Express MHC Class II Molecules? Front Immunol. 2014 2014;5:174.

7. Tveita AA, Schjesvold F, Haabeth OA, Fauskanger M, Bogen B. Tumors Escape CD4+ T-cell-Mediated Immunosurveillance by Impairing the Ability of Infiltrating Macrophages to Indirectly Present Tumor Antigens. Cancer Res. 2015 Aug 15;75(16):3268-78. PubMed PMID: 26038231. Epub 2015/06/04. eng.

8. Leone P, Berardi S, Frassanito MA, Ria R, De Re V, Cicco S, et al. Dendritic cells accumulate in the bone marrow of myeloma patients where they protect tumor plasma cells from CD8+ T cell killing. Blood. 2015 Jul 16. PubMed PMID: 26185130.

9. Flavell RA, Sanjabi S, Wrzesinski SH, Licona-Limon P. The polarization of immune cells in the tumour environment by TGFbeta. Nature reviewsImmunology. 2010;10(8):554-67.

10. Ruffell B, DeNardo DG, Affara NI, Coussens LM. Lymphocytes in cancer development: polarization towards pro-tumor immunity. Cytokine & growth factor reviews. 2010;21(1):3-10.

11. Zangani MM, Froyland M, Qiu GY, Meza-Zepeda LA, Kutok JL, Thompson KM, et al. Lymphomas can develop from B cells chronically helped by idiotype-specific T cells. JExpMed. 2007 5/14/2007;204(5):1181-91.

12. Os A, Burgler S, Ribes AP, Funderud A, Wang D, Thompson KM, et al. Chronic lymphocytic leukemia cells are activated and proliferate in response to specific T helper cells. Cell Rep. 2013 8/15/2013;4(3):566-77.

13. Kuppers R. Mechanisms of B-cell lymphoma pathogenesis. Nature reviews Cancer. 2005 Apr;5(4):251-62. PubMed PMID: 15803153. Epub 2005/04/02. eng.

14. Wein F, Kuppers R. The role of T cells in the microenvironment of Hodgkin lymphoma. J Leukoc Biol. 2016 Jan;99(1):45-50. PubMed PMID: 26320264. Pubmed Central PMCID: PMC4673485. Epub 2015/09/01. eng.

15. Ostad M, Andersson M, Gruber A, Sundblad A. Expansion of immunoglobulin autoreactive T-helper cells in multiple myeloma. Blood. 2008 3/1/2008;111(5):2725-32.

16. Prabhala RH, Pelluru D, Fulciniti M, Prabhala HK, Nanjappa P, Song W, et al. Elevated IL-17 produced by TH17 cells promotes myeloma cell growth and inhibits immune function in multiple myeloma. Blood. 2010 7/1/2010;115(26):5385-92.

17. Tokoyoda K, Hauser AE, Nakayama T, Radbruch A. Organization of immunological memory by bone marrow stroma. NatRevImmunol. 2010 3/2010;10(3):193-200.

18. Herndler-Brandstetter D, Landgraf K, Jenewein B, Tzankov A, Brunauer R, Brunner S, et al. Human bone marrow hosts polyfunctional memory CD4+ and CD8+ T cells with close contact to IL-15-producing cells. JImmunol. 2011 6/15/2011;186(12):6965-71.

19. Meers S, Kasran A, Boon L, Lemmens J, Ravoet C, Boogaerts M, et al. Monocytes are activated in patients with myelodysplastic syndromes and can contribute to bone marrow failure through CD40-CD40L interactions with T helper cells. Leukemia. 2007 12/2007;21(12):2411-9.

20. Lesley R, Kelly LM, Xu Y, Cyster JG. Naive CD4 T cells constitutively express CD40L and augment autoreactive B cell survival. ProcNatlAcadSciUSA. 2006 7/11/2006;103(28):10717-22.

21. Ford GS, Barnhart B, Shone S, Covey LR. Regulation of CD154 (CD40 ligand) mRNA stability during T cell activation. JImmunol. 1999 4/1/1999;162(7):4037-44.

22. Monteiro JP, Benjamin A, Costa ES, Barcinski MA, Bonomo A. Normal hematopoiesis is maintained by activated bone marrow CD4+ T cells. Blood. 2005 Feb 15;105(4):1484-91. PubMed PMID: 15514013. Epub 2004/10/30. eng.

23. Tai YT, Li X, Tong X, Santos D, Otsuki T, Catley L, et al. Human anti-CD40 antagonist antibody triggers significant antitumor activity against human multiple myeloma. Cancer Res. 2005 7/1/2005;65(13):5898-906.

24. Tai YT, Podar K, Mitsiades N, Lin B, Mitsiades C, Gupta D, et al. CD40 induces human multiple myeloma cell migration via phosphatidylinositol 3-kinase/AKT/NF-kappa B signaling. Blood. 2003 4/1/2003;101(7):2762-9.

25. Urashima M, Chauhan D, Uchiyama H, Freeman GJ, Anderson KC. CD40 ligand triggered interleukin-6 secretion in multiple myeloma. Blood. 1995 4/1/1995;85(7):1903-12.

26. Yi Q, Dabadghao S, Osterborg A, Bergenbrant S, Holm G. Myeloma bone marrow plasma cells: evidence for their capacity as antigen-presenting cells. Blood. 1997 9/1/1997;90(5):1960-7.

27. Walz S, Stickel JS, Kowalewski DJ, Schuster H, Weisel K, Backert L, et al. The antigenic landscape of multiple myeloma: mass spectrometry (re)defines targets for T-cell-based immunotherapy. Blood. 2015 Sep 3;126(10):1203-13. PubMed PMID: 26138685. Epub 2015/07/04. eng.

28. Harada H, Kawano MM, Huang N, Harada Y, Iwato K, Tanabe O, et al. Phenotypic difference of normal plasma cells from mature myeloma cells. Blood. 1993 May 15;81(10):2658-63. PubMed PMID: 8490175. Epub 1993/05/15. eng.

29. Garrone P, Neidhardt EM, Garcia E, Galibert L, van KC, Banchereau J. Fas ligation induces apoptosis of CD40-activated human B lymphocytes. JExpMed. 1995 11/1/1995;182(5):1265-73.

30. Duhen T, Campbell DJ. IL-1beta promotes the differentiation of polyfunctional human CCR6+CXCR3+ Th1/17 cells that are specific for pathogenic and commensal microbes. Journal of immunology (Baltimore, Md: 1950). 2014;193(1):120-9.

31. Becattini S, Latorre D, Mele F, Foglierini M, De Gregorio C, Cassotta A, et al. T cell immunity. Functional heterogeneity of human memory CD4(+) T cell clones primed by pathogens or vaccines. Science (New York, NY). 2015 Jan 23;347(6220):400-6. PubMed PMID: 25477212. Epub 2014/12/06. eng.

32. Buckle CH DLE, Lawson MA, Yong K, Rabin N, Perry M, Vanderkerken K, Croucher PI. Soluble rank ligand produced by myeloma cells causes generalised bone loss in multiple myeloma. PLoS One. 2012;7(8). Pubmed Central PMCID: PMC3430669. Epub 2012 Aug 29.

33. Schmiedel BJ, Scheible CA, Nuebling T, Kopp HG, Wirths S, Azuma M, et al. RANKL expression, function, and therapeutic targeting in multiple myeloma and chronic lymphocytic leukemia. Cancer research. 2013;73(2):683-94.

34. Herndler-Brandstetter D, Landgraf K, Tzankov A, Jenewein B, Brunauer R, Laschober GT, et al. The impact of aging on memory T cell phenotype and function in the human bone marrow. JLeukocBiol. 2012 2/2012;91(2):197-205.

35. Moss P, Gillespie G, Frodsham P, Bell J, Reyburn H. Clonal populations of CD4+ and CD8+ T cells in patients with multiple myeloma and paraproteinemia. Blood. 1996 Apr 15;87(8):3297-306. PubMed PMID: 8605346.

36. Kim D, Park CY, Medeiros BC, Weissman IL. CD19-CD45 low/- CD38 high/CD138+ plasma cells enrich for human tumorigenic myeloma cells. Leukemia. 2012 12/2012;26(12):2530-7.

37. Tokoyoda K, Zehentmeier S, Hegazy AN, Albrecht I, Grun JR, Lohning M, et al. Professional memory CD4+ T lymphocytes preferentially reside and rest in the bone marrow. Immunity. 2009 5/2009;30(5):721-30.

38. Zingone A, Wang W, Corrigan-Cummins M, Wu SP, Plyler R, Korde N, et al. Altered cytokine and chemokine profiles in multiple myeloma and its precursor disease. Cytokine. 2014 7/17/2014.

39. Ehrlich LA, Roodman GD. The role of immune cells and inflammatory cytokines in Paget's disease and multiple myeloma. Immunological reviews. 2005 Dec;208:252-66. PubMed PMID: 16313353.

40. Andrews SW, Kabrah S, May JE, Donaldson C, Morse HR. Multiple myeloma: the bone marrow microenvironment and its relation to treatment. BrJBiomedSci. 2013 2013;70(3):110-20.

41. Sallusto F, Lanzavecchia A. Heterogeneity of CD4+ memory T cells: functional modules for tailored immunity. European journal of immunology. 2009;39(8):2076-82.

42. Trentin L, Miorin M, Facco M, Baesso I, Carraro S, Cabrelle A, et al. Multiple myeloma plasma cells show different chemokine receptor profiles at sites of disease activity. BrJHaematol. 2007 9/2007;138(5):594-602.

43. Moller C, Stromberg T, Juremalm M, Nilsson K, Nilsson G. Expression and function of chemokine receptors in human multiple myeloma. Leukemia. 2003 1/2003;17(1):203-10.

44. Bishop GA, Moore CR, Xie P, Stunz LL, Kraus ZJ. TRAF proteins in CD40 signaling. AdvExpMedBiol. 2007 2007;597:131-51.

45. Teoh G, Tai YT, Urashima M, Shirahama S, Matsuzaki M, Chauhan D, et al. CD40 activation mediates p53-dependent cell cycle regulation in human multiple myeloma cell lines. Blood. 2000 2/1/2000;95(3):1039-46.

Figure Legends

Figure 1. Polyclonally activated Th cells stimulated the proliferation of MM cells. We tested the ability of anti-CD3/28 beads (abbreviated as anti-CD3/28 in figure) polyclonally activated Th cells to stimulate purified $CD138^+$ MM cells. (**a**) Proliferation (BrdU incorporation) and cell size (FSC scale) of purified $CD138^+$ MM cells in the presence of anti-CD3/28 beads and allogeneic Th (allo Th) cells or CD138-depeleted autologous BM cells. Left contour plot shows purified MM cells ($CD138^+CD56^+$) in a co-culture with allogeneic Th cells ($CD4^+CD138^-CD56^-$). Right panels show gated MM cells (MM104) as indicated in the presence of anti-CD3/28 beads, MM cells in the presence of anti-CD3/28 beads + allogeneic Th cells, MM cells in the presence of anti-CD3/28 beads + MM cell depleted BM cells. The mean cell sizes (downward arrows) are indicated vs. control (upward arrow) on the FSC axis. (**b**) Proliferation of MM cells in response to polyclonally activated autologous Th cells from patient BM (Th_{MYEL}), vs. MM cells alone, or MM cells with beads. BrdU *vs*. surface λ light (L) chain expression on gated $CD138^+CD56^+$ MM cells from the λ^+ patient MM106. The rightmost plots show the proliferation of gated $CD138^-CD56^-CD4^+$ BM Th cells in the presence of MM cells and anti-CD3/28 beads. Bottom: Percentage of proliferating MM cells in stimulated cultures vs. control as indicated, N=4. Data represent mean±s.e.m. Paired student T test, P<0.007. (**c** & **d**) Kinetic analysis of Th_{MYEL}-supported MM cell proliferation (details of materials and methods are in Supplementary Information). (**c**) CellTracker-labeled Th_{MYEL} cells (red) were mixed in a 1:1 ratio with autologous $CD138^+$ MM cells (non red-labeled) from patient MM159 in the presence of anti-CD3/28 beads and T cell cytokines (IL-2 + IL-15). Representative images of cells at 0 h and 60 h. Contour plot: Distribution of $CD138^+$ MM cells and $CD4^+$ Th_{MYEL} after 98 h. (**d**) Percent confluence of non-red MM cells in wells from 0 h to 60 h in co-culture with autologous Th_{MYEL} cytokines and anti-CD3/28 beads (left histogram), with cytokines and anti-CD3/28 beads or with cytokines and autologous Th_{MYEL} (right histogram). Data represent mean ± s.d. Patients (N=4) MM104, MM106, MM107 and MM159 were IgG^+ and previously untreated. See also Supplementary Figure S1.

Figure 2. Phenotype and cytokine profile of BM Th cells from healthy individuals and MM patients. (a & b) Analysis of transcription factors and cytokines from polyclonally short term activated BM Th cells from healthy individuals (Th_{NORM}) and those of MM patients (Th_{MYEL}) cell lines. In histogram, white bar: Th_{NORM}; black bar: Th_{MYEL}. (**a**) Intracellular staining of IFN-γ, IL-2 and T-bet, and surface CCR5, CCR6 and CXCR3 expression of short term activated Th_{NORM} and Th_{MYEL} cell lines, representative examples are shown. Right histogram: Ratio of IL-2, IFN-γ and TNF-α positive cells after normalization (Percentage of respective cytokine positive cells in Th_{MYEL} divided by that of Th_{NORM} is shown as Th_{MYEL}/Th_{NORM}). Grey bars indicate normalized ratios. Data represent mean±s.e.m. (**b**) Cytokine secretion in supernatant from these short term activated Th_{NORM} (*N*=4) and Th_{MYEL} cell lines (*N*=6). Top: cytokines secreted by stimulated BM Th cells Th_{MYEL} cell lines. Bottom: Ratio Th_{MYEL}/Th_{NORM}, i.e. the secretion of respective cytokines of Th_{MYEL} divided by that found with Th_{NORM}. Only ratios >3 and <1/3 are indicated as the numbers above or below respective bars. Data represent mean±s.e.m. Th_{MYEL} cells were from the previously untreated IgG^+ MM patients MM104, MM105, MM106, MM107, MM108, MM122, MM123, and MM133. Th_{NORM} were from 5 healthy individuals. See also Supplementary Fig. S3.

Figure 3. MM cell stimulation is contact and CD40L dependent

Analysis of cell-cell contact dependency and CD40 ligation. (**a-c**) Gated cells are shown as indicated in top rows, bottom rows show L chain expression of $CD138^+$ cells. (**a**) BrdU vs. FSC and BrdU vs. κ light (L) chain expression of $CD138^+CD56^+$ MM cells from Transwells either alone (left), from inserts with ongoing Th cell/MM cell interactions in the bottom well (middle), or from MM cells stimulated by anti-CD3/28 beads (abbreviated as anti-CD3/28 in figure) activated autologous Th cells (right). (**b**) Analysis of the effect of CD40L on MM cell proliferation, BrdU vs. FSC/λ L $chain^+$ is shown. Gated MM cells ($CD138^+$, λ L $chain^+$) in the presence of control L cells (left); gated MM cells stimulated by $CD40L^+$ L cells (right). (**c**) Th_{MYEL} cells were co-cultured with

autologous MM cells in the presence of anti-CD3/CD28 beads as in Figure 1c. Cells were cultured with anti-CD40L mAb or isotype control mAb from day 3-5. Left: Isotype control, right: Anti-CD40L mAb. BrdU vs. FSC (top) and BrdU vs κ L chain (bottom) are shown of gated $CD56^+IgG\kappa$ L $chain^+$ MM cells. MM cells were from untreated patients with IgG^+ MM cells: MM104 in (**a**), MM159 in (**c**) and a patient IgA^+ (MM111) that had relapsed after high-dose chemotherapy and autologous stem cell transplantation in (**b**).

Figure 4. CD40-activated MM cells secrete cytokines/chemokines that attract Th cells. Purified MM cells from patients (N=6) were activated by agonistic anti-CD40 mAb, supernatants (SN) were analyzed for cytokines and chemokines and compared to unstimulated control. (**a**) Change from background (Δ) is shown for significantly increased cytokines (P<0.05) in all depicted cytokines. Data represent mean ± s.e.m. (N=6). (**b**) Migration of purified patient Th cells through transwell chambers towards supernatant from unstimulated or anti-CD40 stimulated MM cells. Spontaneous migration towards fresh medium (negative control) has been subtracted; change of migratory response (Δ migration) is shown. Mann Whitney test, (N=6), $^{**}P$<0.01. MM cells were from the previously untreated IgG^+ MM patients MM104, MM106, MM107, MM122, MM123 and MM133.

Figure 5. MM cells can activate alloreactive Th cells and elicit help for proliferation. Analysis of HLA-DR expression and mixed leukocyte reactions (MLR). **(a)** Percentage of HLA-DR positive MM cells from patients with IgG and IgA MM (N=25, N=7 respectively). Data represent mean ± s.e.m. (**b**) MM cells (MM107) in the absence or presence of alloreactive Th cells. BrdU vs. surface κ light (L) chain is shown on gated $CD138^+CD56^+$ MM cells. Right panel: Response of $CD138^-$ $CD56^-CD4^+$ Th cells. (**c**) Percentage of proliferating MM cells from patients in the absence or presence of alloreactive Th cells (N=7). Data represent mean ± s.e.m. Two tailed Mann-Whitney P=0.002. MM cells were from MM107 (IgG^+ patient, untreated) in (**b**); 2 relapsed IgA^+ patients (MM101, MM103) and 5 IgG^+ untreated (MM104, MM105, MM107, MM112, MM117) in (**c**).

Figure 6. Autologous Th cells support MM cell proliferation *in vivo.* Analysis of autologous BM Th cell support of MM cells in NSG mice. (**a, b**) $CD4^+$ Th cells and $CD138^+$ MM cells were purified; Th cells were activated and co-injected *i.v.* with MM cells into conditioned NSG mice. MM cells alone were injected *i.v.* in control NSG mice. Details are described in the Supplementary Information. (**a**) Serum samples were drawn to measure M-component; results from 4 patients expressing IgGκ are shown: mice injected with MM cells alone (MM), mice injected with both MM cells and autologous Th cells (MM+Th) (N=4). Mice were killed on day 50. Mean and s.d. is shown , two tailed Mann-Whitney P<0.02. (**b**) *In vivo* proliferation of MM cells in the absence or presence of injected Th cells in the bone marrow of recipient mice. Left panels: representative BrdU staining for proliferative MM cells. Right panels: Percentage of proliferating MM cells from the mice injected with MM vs. MM+Th. Box & whiskers, min-max is shown, two tailed Mann-Whitney P=0.02. MM cells were from untreated IgG^+ MM patients MM122, MM123, MM126 and MM133. **c)** Bone marrow cells were depleted of T cells (without (wo) Th and $CD8^+$ cells, left) or $CD8^+$ T cells and were cultured in autologous serum and IL-2. After 14 days, MM cells were identified by intracellular κ L chain stain (Igκ L chain vs. Igλ L chain). Proliferation (BrdU) vs. size (FSC) of MM cells (gated Igκ L $chain^+$) in cultures without T cells (left) and without $CD8^+$ cells (right), N=4. **d)** Transfer of bulk cultures as in c) after 14 days in culture into NSG mice for 42-51 days. Ungated MM cells (Igκ L $chain^+$) in spleens from mice that received cultures depleted of T cells, or cultures depleted of $CD8^+$T cells (Igλ vs. Igκ L chain) and proliferation (BrdU) vs. size (FSC) is shown. Right: numbers of proliferating ($BrdU^+$) MM cells in the BM of NSG mice (numbers per mouse femur). Mean + s.d. is shown, two tailed Mann-Whitney: P=0.0061, P=4 patients, cells from relapsed IgG^+ MM patients: MM.C100 MM182, MM185, and from relapsed IgA^+ MM patient MM183. See also Supplementary Figure S6.