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ORIGINAL ARTICLE

Autocrine amplification of immature myeloid cells by IL-6 in multiple myeloma-infiltrated bone marrow

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Multiple myeloma (MM) invariably develops in the bone marrow (BM), indicating the strong requirement of this tumor for the peculiar BM microenvironment, rich in cytokine and hematopoietic precursor cells. Interleukin-6 (IL-6) and a proliferation inducing ligand (APRIL) are key cytokines implicated in MM development. Here, we show that MM cells changed the hematopoietic microenvironment early upon BM infiltration by strongly downregulating hematopoietic precursor cells from all lineages except myeloid precursor cells. Myeloid precursor cells constituted a major source of APRIL in MM-infiltrated BM, and their proliferative response to IL-6 upregulation explained their relative resistance to MM infiltration. The osteolytic molecule receptor activator of NF-kB ligand (RANK-L) expressed by MM cells started this myeloid proliferation by inducing in a contact-dependent manner IL-6 production by myeloid precursor cells themselves. Taken together, our data demonstrate that MM cells do not simply displace hematopoietic cells upon BM infiltration, but rather selectively modulate the BM microenvironment to preserve a pool of high APRIL-producing myeloid precursor cells. Our data also identify a positive regulation of APRIL by IL-6 in myeloid precursor cells.

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INTRODUCTION

APRIL is a member of the tumor necrosis factor superfamily implicated in the differentiation and survival of plasma cells.[1] APRIL is also implicated in the development of multiple myeloma (MM), a tumor originating from plasma cells developing in bone marrow (BM).[2,3] We recently reported that most of the APRIL production in healthy BM originates from a specific hematopoietic lineage, the myeloid cells.[4] Myelopoiesis is an extremely active hematopoietic process, as a human adult produces more than 10^{11} mature myeloid cells per day.[5] This insures a constitutively high level of APRIL in BM that may benefit from the long-term survival of BM plasma cells. APRIL pattern of expression did not change upon BM infiltration by MM with myeloid precursor cells remaining the main source.[2] This latter observation is consistent, at least in part, with the strong dependency of MM cells on their microenvironment.[6] However, it also implies that MM cells may also limit their development by displacing APRIL-producing cells in BM.

Interleukin-6 (IL-6) has a key role among the cytokines increased in MM patients.[7,8] IL-6 acts directly via the JAK/STAT3 signaling pathway to induce expression of anti-apoptotic molecules such as myeloid cell leukemia 1.[9] IL-6 also confers drug resistance to MM.[10] Based on these findings, clinical trials on IL-6 antagonism have been launched in MM. Although early results were unsatisfactory,[11] trials are still ongoing to find the best condition to block this molecule.[12] Despite this advanced clinically translated research for IL-6 in MM, the cellular source of IL-6 has still not been clearly identified. Although early studies reported autocrine and paracrine origins,[7,13] more recent reports favor the paracrine origin.[6,14] In the present report, we use a new mouse model to confirm the paracrine nature of IL-6, and to precisely characterize for the first time its cellular source. We further show that IL-6 contributes to the maintenance of a high-APRIL production in MM-infiltrated BM.

MATERILAS AND METHODS

Animal experimentation

The veterinary office of Geneva and Grenoble approved animal experimentations. The syngeneic mouse MM model was established from IgA-producing plasmocytoma MOPC-315 cells.[15] Briefly, MOPC-315 cells were injected intravenously into syngeneic Balb/c mice, and BM resident cells were recovered from mouse tibiae/femora. This procedure was repeated nine times to obtain MOPC-315.BM variant cells. Selected cells were propagated in tissue culture with RPMI-1640 containing 10% fetal calf serum. For tumorigenesis experiments, 2×10^5 MOPC-315.BM cells were injected intravenously into female Balb/c mice aged 5–10 months. Mice were observed regularly, and BM cells were recovered from tibiae/femora at the first sign of mouse paraplegia, usually after 4–6 weeks. The MOPC-104E and J588 plasmocytomas originating from Balb/c were kindly provided by M. Wabl (UCSF, San Francisco, CA, USA) and obtained at the American tissue culture collection, respectively. The blocking rat IgG anti-mouse IL-6Rα (15A7), kindly provided by Dr Van Snick (Brussels, Belgium), was injected intraperitoneally (1 mg/mouse) 4 days and 1 day before BM analysis.

Flow cytometry

Fluorochrome-conjugated antibodies against Gr-1 (RB6-8C5), F4/80 (6F12), CD11c (HL3), CD11b (M1/70), B220 (RA3-682),

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TER-119 (TER-119), CD41 (MWReg30), CD115 (T38-320), Ly6-G (1A8), LY6-C (AL-21), Sca-1 (D7), CD34 (RAM34), c-kit (ACK 45), CD45 (30-F11), IgA (C10-3) and receptor activator of NF-kB ligand (RANK-L; IK22-5) were from BD Biosciences (San Jose, CA, USA), except the anti-IL-6Rβ (KGP130, eBiosciences, San Diego, CA, USA). The anti-IL-6Rα used was a biotinylated form of the 15A7 monoclonal antibody. A biotinylated anti-mouse IgA from goat was used (Southern Biotech, Birmingham, AL, USA). Cell suspensions from flushed BM were stained in the presence of CD16/CD32 blocking monoclonal antibody after red blood cell lysis. Propidium iodide (Sigma, Saint Louis, MO, USA) staining was used to exclude dead cells from the flow cytometry analysis. IL-6 intracellular staining was performed on cells pre-treated 10 h with 20 μM Brefeldin A (Sigma) at 37 °C, fixed with 1% formaldehyde and permeabilized with 1% saponin. Fluorescence analysis and cell sorting were performed on a FACS Calibur and FACS Aria, respectively (Becton Dickinson, San Jose, CA, USA). Cytospins were prepared from FACS-sorted cells for cytology analysis with Giemsa staining.

Cell culture and ELISA

Mouse BM cells were stimulated with recombinant murine IL-6 (10 ng/ml, R&D systems, Abingdon, UK), IL-1β (1 ng/ml, Peprotech EC Ltd, London, UK), vascular endothelial growth factor (10 ng/ml, Peprotech EC Ltd), basic fibroblast growth factor (10 ng/ml, Peprotech EC Ltd), stromal-derived factor-1β (10 ng/ml, Peprotech EC Ltd). In these experiments, we also used mouse cross-reactive recombinant human insulin-like growth factor-1 (10 ng/ml, Peprotech EC Ltd) and tumor necrosis factor (10 ng/ml, Adipogen, Epalinges, Switzerland). In some experiments, cells were labeled with carboxyfluorescein succinimidyl ester as previously described.[16] IL-6 concentration was measured with a mouse IL-6 ELISA kit from R&D systems. BM cells were seeded at 2.5×10^6 cells/ml for supernatant conditioning. Unless indicated, MOPC cells represented 1% of the total cellularity when mixed to BM cells. For contact-independent experiments, BM cells and MOPC-315.BM cells were added in the lower and upper compartments, respectively, of 0.4 μm transwells from 24-well plates. Heat-inactivated fetal calf serum was used in experiments using FACS-sorted cells.

Quantitative real time PCR

Total RNA was extracted from FACS-sorted cells using Trizol (Life technologies, Paisley, UK), and complementary DNA were generated using random primers and the SuperScript II reverse Transcriptase (Life technologies). mRNA expression was measured by real-time PCR using the iCycler iQ Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA) and a SYBRgreen-based kit (iQ Supermix, Bio-Rad). Primers have already been described.[4] Samples were assayed in triplicates and the experiment was performed twice. Results are shown as relative expression.

Statistical analysis

Means or means ± s.d. are shown. Statistical analyses were performed for sample size superior to five. Two-tailed unpaired *t*-tests were performed on samples of equal size using the Prism software (Graphpad Software Inc., San Diego, CA, USA). *P*-values superior to 0.05 were considered as non-significant.

RESULTS

High APRIL-producing myeloid precursor cells resist the most to MM infiltration

To analyze in detail the hematopoiesis modulation in MM, we used a new mouse model constituted by a variant of the MOPC-315 plasmocytoma cell line, called MOPC-315.BM. MOPC-315.BM efficiently colonized BM following intravenous injection.[15] At an end stage, mice developed bilateral hind-leg paraplegia due to high tumor burden and subsequent spinal cord compression. BM infiltration severely impaired hematopoiesis, as the amount of total BM cells decreased by 4.6 ± 2.7-fold ($n = 17$) in paraplegic mice compared with healthy mice. Erythroid (FSC^{high} $TER\text{-}119^+$ cells) and ($B220^{low}$) B-lymphocyte precursors decreased in frequency and number (Figure 1a). Notably, the percentage of $Gr\text{-}1^{low}$ myeloid precursor cells increased. Considering the important reduction in total BM cells, this increase did not avoid a loss of myeloid precursor cells, but the decrease was minimal. Megakaryocytes were also strongly reduced (Figure 1b). In total, fold decrease in the absolute number of hematopoietic precursors was 14, 6, 7 and 2 for the megakaryocyte, erythroid, B lymphoid and myeloid lineages, respectively, showing that $Gr\text{-}1^{low}$ myeloid cells were the most resistant cells to MOPC-315.BM infiltration. Decrease of precursors correlated with a strong reduction in the mature cell pool recovered from infiltrated BM, excluding an increased hematopoietic activity in our model (Figure 1c). The long half-life, in the month range, of mature erythrocytes did not allow to observe their decrease during the course of the experiment. To specifically study the myeloid compartment, we decided to monitor the ratio between mature and immature cells. This ratio ($Gr\text{-}1^{high}/Gr\text{-}1^{low}$) averaged 5.2 ± 1.4 in BM from healthy mice (Figure 1d). In BM from paraplegic mice, this ratio was shifted toward an increased proportion of $Gr\text{-}1^{low}$ cells. Notably, we detected a significant decrease of this ratio very early in MOPC-315.BM development with BM with no more than 1% of infiltration.

We next precisely characterized the resistant $Gr\text{-}1^{low}$ cell population in infiltrated BM. This cell population contained a minority of $CD11c^+$ dendritic cells, $CD115^+$ monocytes and $F4/80^+$ macrophages (Figure 2a). Compared with $Gr\text{-}1^{high}$ myeloid cells, this population expressed lower levels of the myeloid markers CD11b, Ly6-G and Ly6-C. These cells were well committed to the myeloid lineage, as they almost completely lost expression of the CD34, Sca-1 and C-kit early hematopoietic markers (Figure 2b). Their early features of nuclear segmentation was reminiscent of the promyelocyte/myelocyte stage previously defined in mouse,[17] and clearly distinguished them from the $Gr\text{-}1^{high}$ population that contained more mature metamyelocytes and band cells (Figure 2c, left panel). They also produced higher levels of APRIL mRNA than $Gr\text{-}1^{high}$ cells (Figure 2c, right panel). APRIL mRNA upregulation was more than twofold in immature cells compared with mature cells, very similar to the differences already reported in healthy mouse BM,[4] and comparable to human MM.[2] Taken together, these results indicate that $Gr\text{-}1^{low}$ cells resisting MM infiltration were *bona fide* myeloid precursor cells. These experiments demonstrate that MM cells strongly decrease all hematopoietic precursors upon BM infiltration with the exception of high APRIL-producing myeloid precursor cells.

IL-6 induces myeloid precursor cell proliferation in MM-infiltrated BM

The early occurrence of increased myeloid precursor cell proportion strongly suggests an active phenomenon involving a soluble mediator. MM development is associated with upregulation of several cytokines/growth factors. Some of these factors, like IL-6,[18] insulin-like growth factor-1,[19] fibroblast growth factor,[20] IL-1,[21] vascular endothelial growth factor,[22] tumor necrosis factor[23] and stromal-derived factor-1[24] act on myelopoiesis. Among them, only IL-6 decreased the $Gr\text{-}1^{high}/Gr\text{-}1^{low}$ ratio when added to BM cell cultures (Figure 3a). This decrease was due to a proliferation selectively induced in $Gr\text{-}1^{low}$ compared to $Gr\text{-}1^{high}$ cells (37 ± 8% vs 6 ± 5%, $P < 0.01$; Figure 3b). $Gr\text{-}1^{low}$ cells could initiate a proliferative response to IL-6, as they coexpressed the

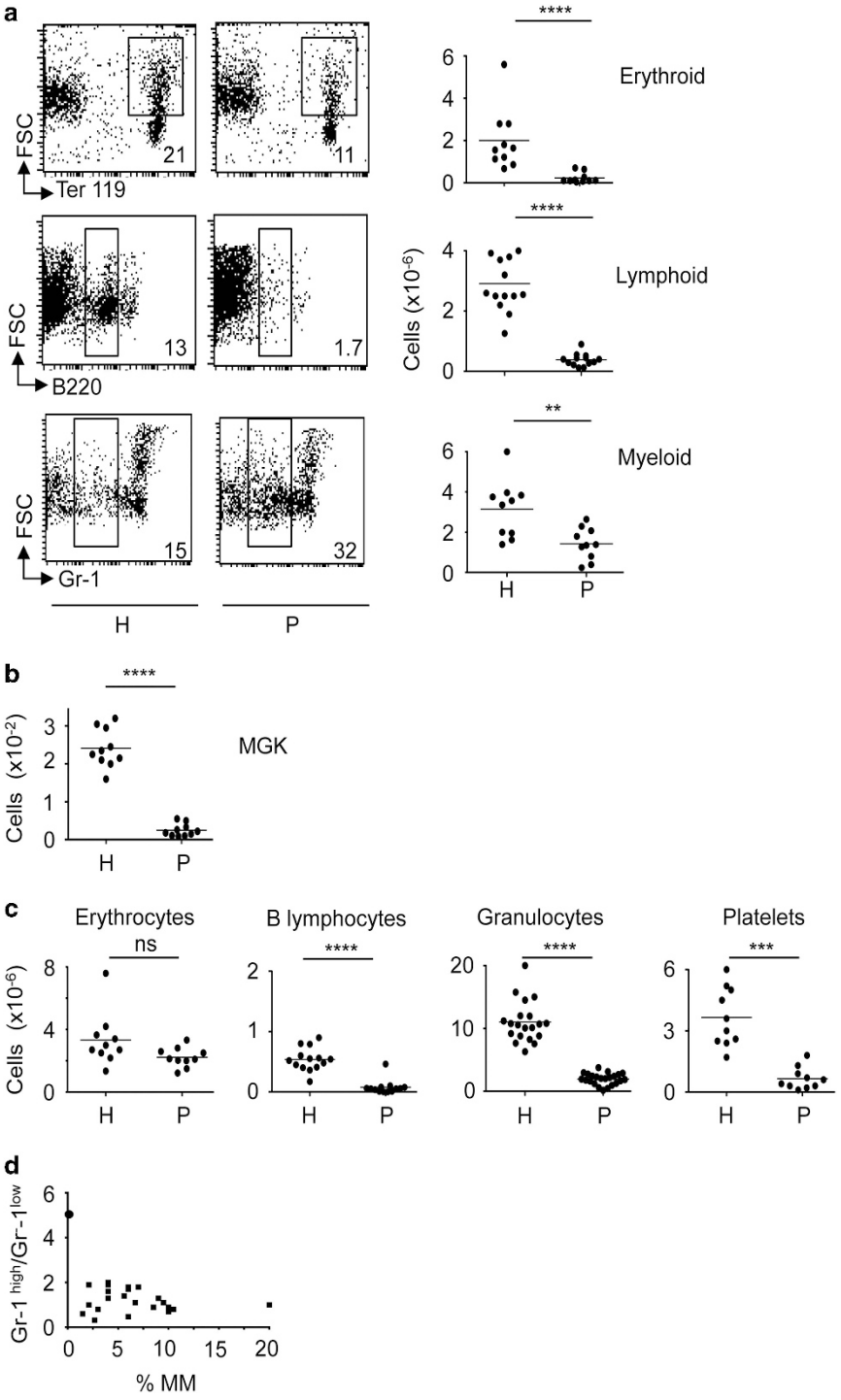

Figure 1. Gr-1^{low} cells are relatively resistant to BM infiltration in the MOPC-315.BM mouse model of MM. (**a**) Dot plots showing hematopoietic precursor cells identified by size and the indicated lineage markers in BM from healthy (H) and MOPC-315.BM-infiltrated paraplegic (P) mice. Populations corresponding to precursor cells with their percent among total BM cells are marked (left panel). Total numbers are also shown (right panel). (**b**) Megakaryocytes (MGK) numeration after morphological identification on BM sections. (**c**) Quantification of mature FSC^{low} Ter119^{+} erythrocytes, B220^{high} B lymphocytes, Gr-1^{high} mature granulocytes and CD41^{+} platelet cells in BM from H and P mice. (**d**) Correlation between the Gr-1^{high}/Gr-1^{low} ratio and level of MOPC-315.BM infiltration (%). Unless indicated, quantification was performed by flow cytometry. Absolute numbers are shown with mean values. $^{**}P < 0.001$; $^{***}P < 0.0001$ and $^{****}P < 0.00001$. NS, not significant.

ligand-binding α- and the signaling β-chains of the IL-6 receptor in healthy BM, and this pattern of expression did not change significantly in MOPC-315.BM-infiltrated BMs (Figure 3c and d). In healthy mice, IL-6 blockade did not modulate the myeloid compartment in BM (Figure 3e, left panel). However, this treatment increased and decreased the number of Gr-1^{high} and Gr-1^{low} cells, respectively, in BM from paraplegic mice. Overall, the Gr-1^{high}/Gr-1^{low} ratio in paraplegic mice was partially

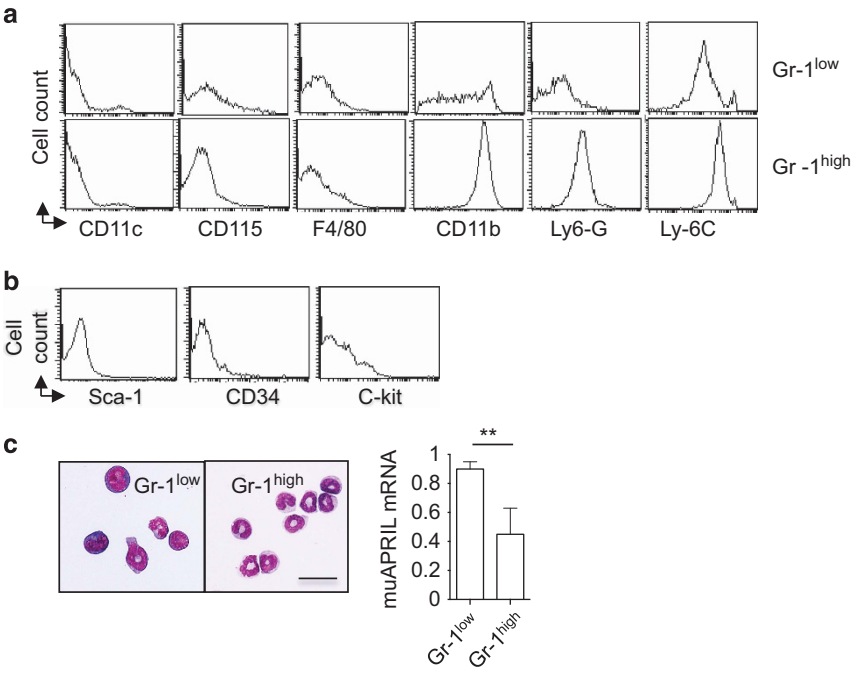

Figure 2. Resistant Gr-1^{low} cells are high APRIL-producing myeloid precursor cells. (**a**) Histogram plots showing myeloid marker expressions on gated Gr-1^{low} and Gr-1^{high} cells. (**b**) Similar analysis on gated Gr-1^{low} cells for early hematopoietic marker expressions. (**c**) FACS-sorted Gr-1^{low} and Gr-1^{high} cell population analysis for morphology (scale bar, 20 μm; left panel) and APRIL mRNA expression (mean ± s.d.) by qRT-PCR (right panel). A value of 1 was given arbitrarily to immature myeloid cells from healthy BMs. Data are representative of four P mice. $^{**}P < 0.001$.

restored to the normal value (Figure 3e, right panel). Altogether, these show a role for IL-6 in the myeloid deviation observed in MM-infiltrated BM.

IL-6 is autocrine to myeloid precursor cells in MM-infiltrated BM

In vivo, we detected a slight but not significant IL-6 increase in the serum of paralytic mice compared with healthy mice (Figure 4a). IL-6 upregulation has also been reported in MM patients' serum, but high IL-6 concentrations were best detected in supernatant from cultures of MM-infiltrated BM cells.[13] In our mouse MM model, we observed a similar high-IL-6 upregulation in cultures of BM cells from paraplegic compared with healthy mice (Figure 4b). We reproduced this IL-6 upregulation in mixed cultures of BM cells from healthy mice and MOPC-315.BM cells (Figure 4c). In this experiment, IL-6 production was rapid, detectable 24 h after culture initiation and dependent on a minimal number of tumor cells (as low as 0.01% of the total cellularity). IL-6 production required contacts between tumor cells and their environment (Figure 4d), consistent with a previous report made in the human system.[25] Notably, separation of CD138$^-$ healthy cells and CD138$^+$ MOPC-315.BM cells from BM of paraplegic mice stopped IL-6 production, indicating that constant contacts are required in this process (Figure 4e). Such rapid induction of IL-6 was not limited to variant MOPC-315.BM cells, as we also detected IL-6 in supernatants from BM cells incubated with parental MOPC-315 wild-type cells, and the two other plasmocytomas, MOPC-104E and J588 (Supplementary Figure sup1). Plasmocytomas themselves did not produce detectable level of IL-6 in these experiments.

We next characterized the cellular source of IL-6 in our mixed cultures of BM and MOPC-315.BM cells by intracellular IL-6 staining. IL-6-producing cells were CD45$^+$ hematopoietic cells, but neither IgA$^+$ MOPC-315.BM tumor cells, nor B220$^+$ cells (Figure 5a). We could also exclude NK1.1$^+$ CD49b$^+$ NK cells, CD3$^+$ T cells, CD31$^+$ platelets and TER-119$^+$ erythrocytes due to their absence in our 1-day culture (data not shown). In fact, these cells were myeloid precursor cells expressing low levels of CD11b and Gr-1. In this experiment, we constantly observed a minority but reproducible fraction of cells (11.3 ± 5.2% of the total IL-6-producing cell pool, $n = 10$) devoid of Gr-1 expression. BM Gr-1$^-$ cells encompassed two populations differing in cell size, and IL-6-producing cells were present in the FSC^{high} population (Figure 5b, upper panel). We further observed that the Gr-1$^-$ FSC^{high} cells could be further subdivided according to B220 and CD11b expression, and IL-6-producing cells were present in the CD11b$^+$ cells (Figure 5b, middle panel). CD45 and FcγR expression demonstrated the myeloid origin of these Gr-1$^-$ IL-6-producing cells. This latter cell population neither exhibited dendritic cell (CD11c) nor monocyte/macrophage markers (F4/80 and CD115; Figure 5b, lower left panel). Histologic analysis of this cell population showed a large size and the absence of nuclear segmentation, revealing a more immature stage than the resistant Gr-1^{low} cells described above. (Figure 5b, lower right panel). Taken together, this cell population was consistent with the myeloblast stage. IL-6-producing cells were absent from lymph nodes and peripheral blood, confirming the immature nature of these cells (Figure 5c). Taken together, our data identify myeloid precursor cells from the myeloblast to the myelocyte stages as the cellular source of IL-6 in MM-infiltrated BM.

MM-expressed RANK-L induces IL-6 in myeloid precursor cells

RANK-L is a molecule expressed by MM cells primarily involved in osteolytic lesions associated to tumor development.[26] In addition, RANK-L also induces cytokine production including IL-6 in myeloid cells.[27] MOPC-315.BM cells expressed surface RANK-L (Figure 6a). Blockade of RANK-L with a soluble form of its receptor inhibited by 55% ± 14% intracellular IL-6 in myeloid precursor cells (Figure 6b). This show that the role of RANK-L is not limited to bone destruction, but is also a mean for MM cells to deviate hematopoiesis toward maintenance of an APRIL rich environment. The full process of IL-6 production and amplification of the

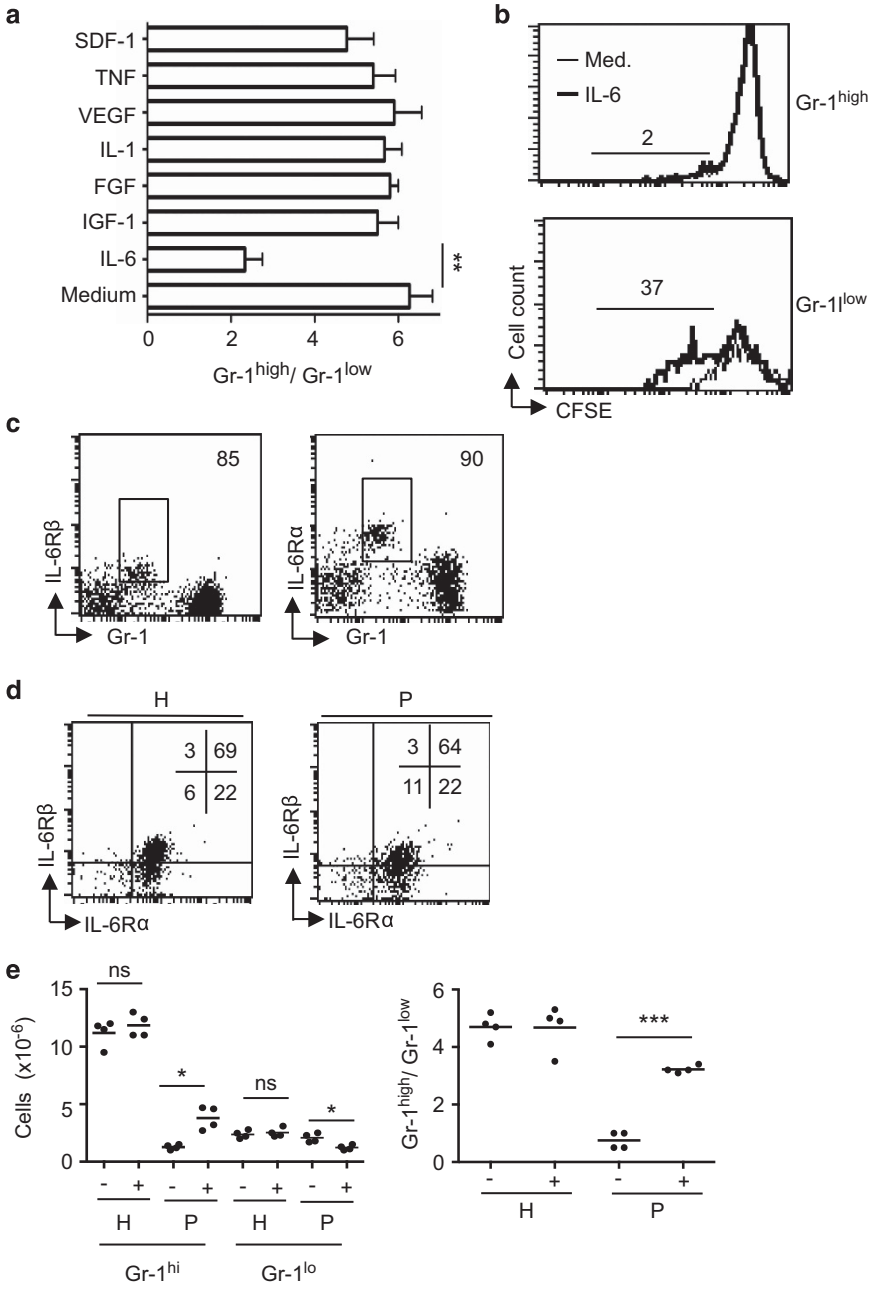

Figure 3. IL-6 induces proliferation of myeloid precursor cells in MM-infiltrated BM. (**a**) Gr-1^{high}/Gr-1^{low} cell ratio (mean ± s.d.) obtained after a 4-day *in vitro* treatment of total BM cells from healthy mice with the indicated growth factors. Data are representative of two independent experiments performed with triplicate cultures. (**b**) Histogram plots showing proliferation of CFSE-labeled total BM cells from healthy mice in the presence or absence of IL-6. The percent of dividing cells with the gating performed is indicated. Data are representative of three independent experiments. (**c**) Dot plots showing IL-6 Rα, and β-expression on Gr-1^{+} cells from mouse healthy BM. The percent of Gr-1^{low} cells expressing IL-6 Rs is marked. (**d**) Dot plots showing coexpression of IL-6 Rα, and β on gated Gr-1^{low} BM cells from healthy and paraplegic mice. Percent of cells is indicated. Data are representative of four healthy and two paraplegic mice. (**e**) Numeration of Gr-1^{high} and Gr-1^{low} cells after control Ig (−) and αIL-6R (+) blockade in healthy and paraplegic mice. Cell numbers with mean values obtained 1 day after the last antibody injection are shown (left panel). Deduced Gr-1^{high}/Gr-1^{low} ratios are also shown (right panel). Cell quantification was performed by flow cytometry. *$P < 0.05$; ***$P < 0.0001$.

APRIL-producing cell pool during MM infiltration of BM is illustrated in Figure 7.

DISCUSSION

During BM infiltration, MM obviously impairs hematopoiesis, at least when the tumor load is high enough. Our new mouse model of MM shows that hematopoiesis impairment is an early phenomenon in MM development, well before a high infiltration level. In addition, it is a selective process depending on the lineage analyzed. We observed that red blood cells, platelets and lymphocytes are strongly inhibited with the disappearance of precursor cells. The myeloid lineage behaves distinctly with a much less pronounced decline in precursor cells. In fact, myeloid precursor cells are quite resistant to MM infiltration compared with other precursors, and this is due to proliferation induced by

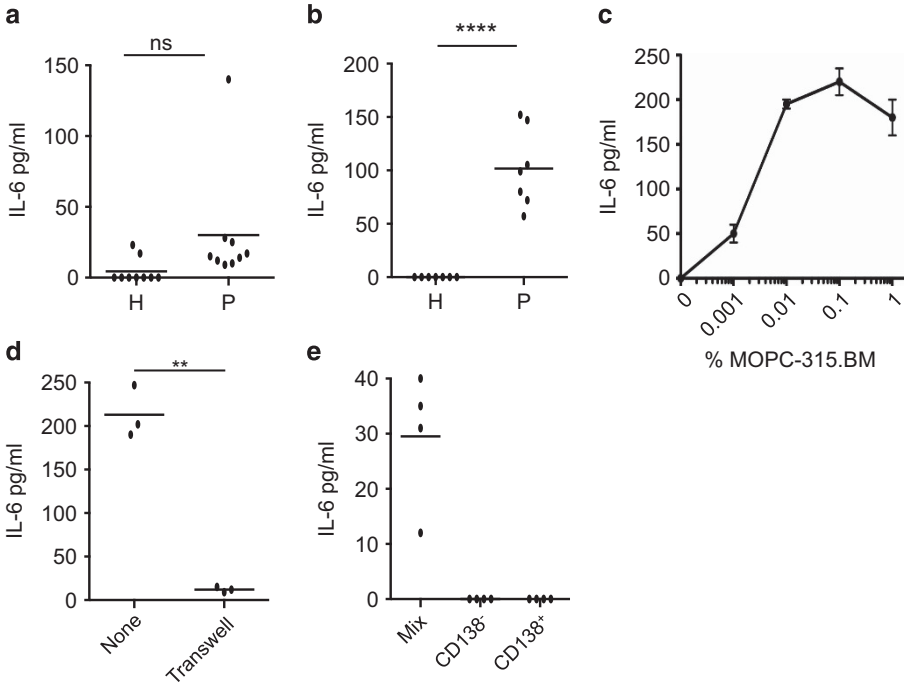

Figure 4. IL-6 upregulation in the MOPC-315.BM model. (**a**) IL-6 concentration in sera of healthy and paraplegic mice. (**b–e**) IL-6 concentration in supernatants from a 1-day culture of total BM cells recovered from healthy and paraplegic mice (**b**), total BM cells from healthy mice mixed with the indicated number of MOPC-315.BM cells (**c**), total BM cells from healthy mice mixed with MOPC-315.BM cells in the presence and absence of a transwell (**d**), total and FACS-sorted CD138$^+$ tumor and CD138$^-$ non-tumor BM cells from paraplegic mice (**e**). Data are representative of at least three mice. $^{**}P < 0.001$; $^{****}P < 0.00001$.

IL-6 in myeloid precursor cells. It is interesting to note that this proliferation is mediated in an autocrine manner, as myeloid precursor cells produce IL-6 themselves. This highlights the existence of a positive feedback loop of IL-6 on its own production, confirming earlier data observed in anti-IL-6 treated MM patients.[28]

The paracrine origin of IL-6 has still not been well defined in MM. One group identified osteoblasts as IL-6-producing cells upon contact with MM cells.[29] Other studies identified long-term *in vitro*-propagated BM stromal cells as a cellular source of IL-6.[25,29,30] We have been able to identify IL-6-producing cells in a short-term (one day) cell culture assay. We confirm the previous detection of IL-6 mRNA in total myeloid cells from BM of MM patients,[31] and report a precise characterization of the BM myeloid-cell population involved. Upregulation of IL-6 upon contact with MM cells occurs in the early myeloblast to the intermediate myelocyte maturation stages. This population may also contain eosinophils, recently described as key cells in the medullary development of MOPC-315.BM cells.[32] In our hands, further differentiated cells are no more able to produce IL-6, and IL-6 production by myeloid precursor cells is not constitutive but under the influence of RANK-L, another member of the tumor necrosis factor superfamily. RANK-L has been extensively studied in MM, as it is a key mediator of associated bone lesions.[6] Here, we show that RANK-L expressed by MM cells mediates IL-6 induction in myeloid precursor cells. Despite the fact that myeloid precursor cells respond to RANK-L signaling,[33] surface expression of the RANK receptor has to date only been significantly detected on *in vitro* cultured primary cells.[34] We have also not been able to detect significant surface RANK *ex vivo* on the surface of myeloid cells from mouse BM (data not shown). Although it is likely that RANK mediates IL-6 induction, we cannot formally exclude the involvement of another receptor in the process.

Although we identified a common cellular source for the two MM-promoting factors IL-6 and APRIL, we noticed key differences in their regulation of expression. APRIL is constitutively produced. This is the case in healthy BM as previously reported.[4] This expression did not change upon BM infiltration as shown here. Notably, APRIL production peaks in immature cells from both healthy and MM-infiltrated BM. By contrast, IL-6 production is not constitutive. It is induced upon MM infiltration and requires constant contacts with MM cells. Differences also exist in the myeloid maturation stages involved. Although precursor cells produce both, mature cells produce APRIL but not IL-6. Finally, it is interesting to note that IL-6 positively controls the pool of APRIL-producing cells in MM-infiltrated BM. This implies that IL-6 upregulation upon BM infiltration not only directly insures MM development, but also indirectly by increasing the production of a second promoting factor, APRIL.

Our demonstration that MM-promoting factors originate from hematopoietic precursor cells may provide some answers to MM treatment failures. Indeed, intensive treatment in the form of combined high-dose chemotherapy still does not lead to disease cure.[35] According to our results, chemotherapy highly benefits to patients by eliminating proliferating myeloid precursor cells, producing IL-6 and APRIL, in addition to destroy most of the MM tumor. However, hematopoietic reconstitution will rapidly recreate a favorable IL-6/APRIL environment. Such process may contribute to persisting minimal disease and subsequent MM relapses. A prolonged targeting of survival factors provided by renewing hematopoietic cells may help to improve MM control.

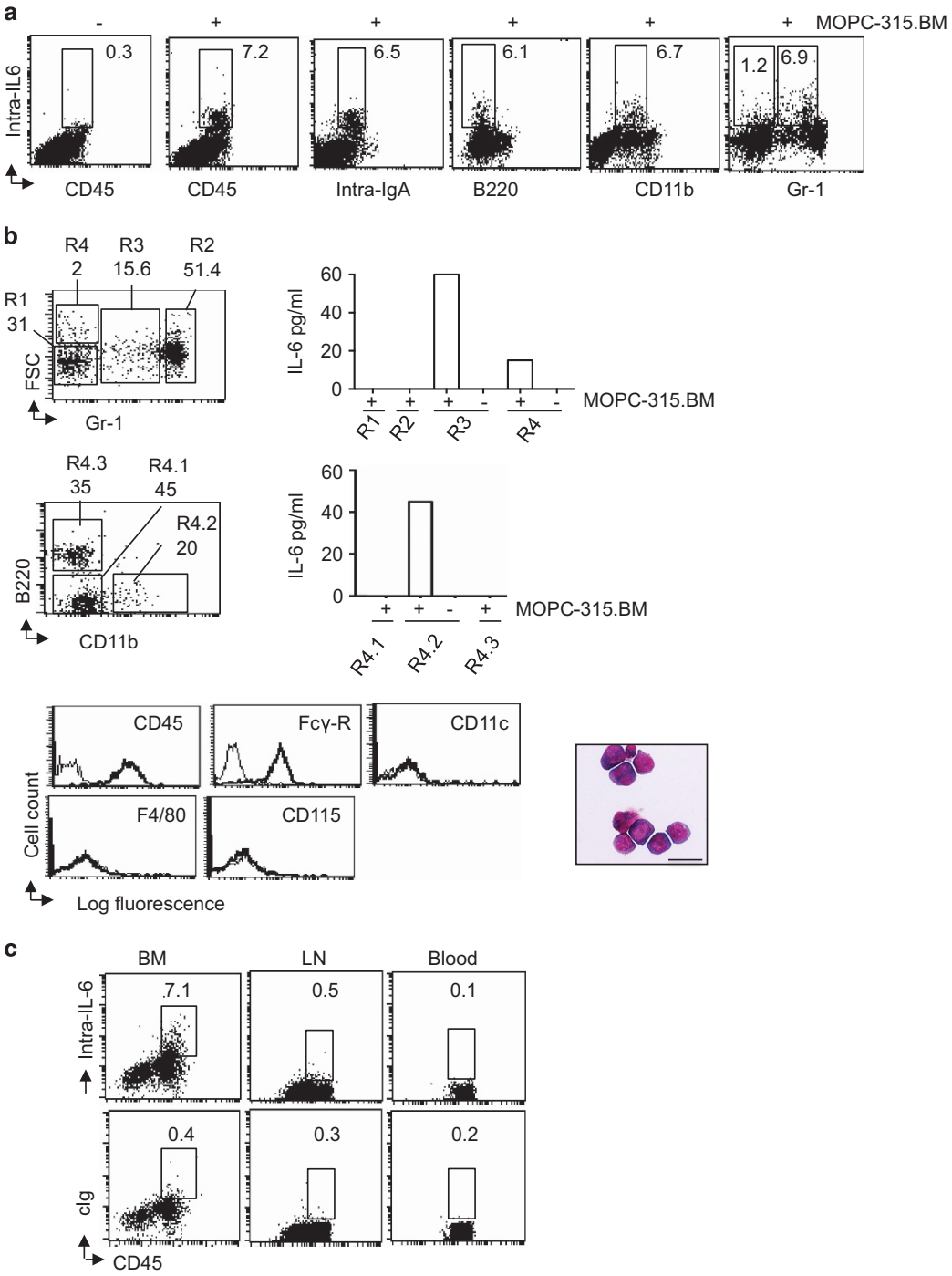

Figure 5. Myeloid precursor cells produce IL-6. (**a**) Dot plots showing intracellular IL-6 staining in BM cells from healthy mice in the absence or presence of MOPC-315.BM. IL-6-producing cells with their % among total cells from the culture are marked. Data are representative of a minimum of four experiments. (**b**) Dot plots showing cell populations from healthy BM according to size and Gr-1 expression (upper left panel). Cells were FACS-sorted and tested for IL-6 production in the presence or absence of MOPC-315.BM (upper right panel). Gr-1^{-} FSC^{high} cells (gate R4) were further subdivided according to B220 and CD11b expression. Population percentages are indicated (middle left panel). The different populations were FACS-sorted, and retested for IL-6 production (middle right panel). The R4.2 population (FSC^{high}, Gr-1^{-} and CD11b^{+}) was further analyzed for myeloid surface marker expression by flow cytometry (lower left panel) and morphology (scale bar, 20 μm; lower right panel). Data are representative of at least three experiments. (**c**) Dot plots showing intracellular IL-6 staining in CD45^{+} cells from BM, LN and blood incubated with MOPC-315.BM as in **a** (upper panel). CIg intracellular staining is also shown (bottom panel). The results are representative of six independent experiments.

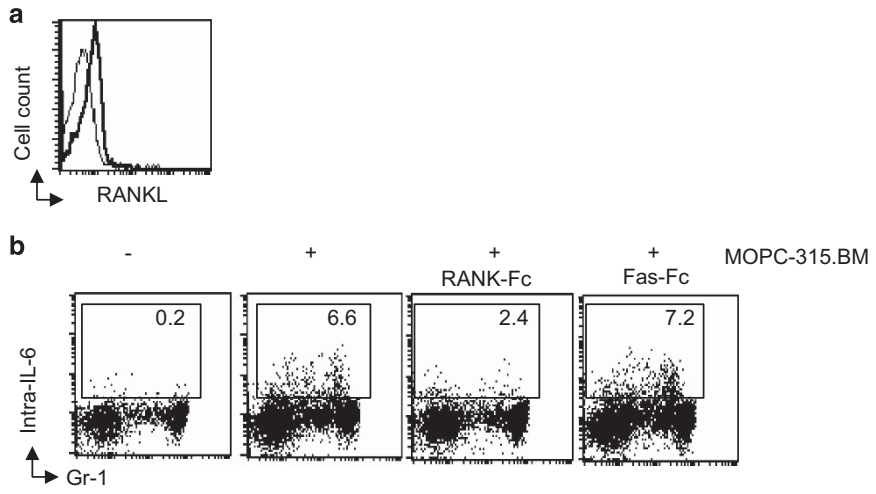

Figure 6. MM-expressed RANK-L induces IL-6 production in myeloid precursor cells. (**a**) Overlaid histogram plots showing RANK-L expression on MOPC-315.BM cells. Data are representative of three independent experiments. (**b**) Dot plots showing intracellular IL-6 staining from healthy mice as in Figure 5A. In some conditions blocking RANK-Fc or control Fas-Fc was added. Data are representative of four experiments.

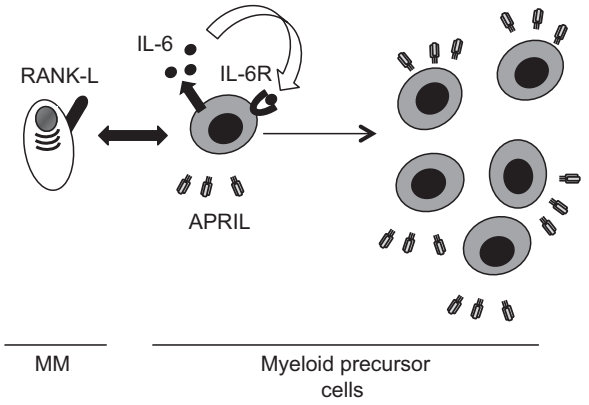

Figure 7. The pathway conducting to a stable expression of APRIL in MM-infiltrated BM. Myeloid precursor cells produce constitutively APRIL. BM infiltration by MM induces hematopoietic precursor cell downregulation. Upon interaction with RANK-L$^+$ MM cells, myeloid precursor cells expressing IL-6R produce IL-6 that triggers an autocrine proliferative loop, thus amplifying the pool of high APRIL-producing cells. This creates an environment rich in the two MM-promoting factors IL-6 and APRIL.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

ID-S, AZ, BM and BH performed experiments. TM and BB provided reagents. TM and BH analyzed data and wrote the manuscript. BH designed the study.

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