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The cytokine-mediated crosstalk between primary human acute myeloid cells and mesenchymal stem cells alters the local cytokine network and the global gene expression profile of the mesenchymal cells

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ABSTRACT

Interactions between acute myeloid leukemia (AML) blasts and neighboring stromal cells are important for disease development and chemosensitivity. However, the molecular mechanisms involved in the cytokine-mediated crosstalk between mesenchymal stem cells (MSCs) and AML cells are largely unknown. Leukemic cells derived from 18 unselected AML patients were cultured with bone marrow MSCs derived from healthy donors; the populations then being separated by a semipermeable membrane. Coculture had only minor effects on MSC proliferation. The unique cytokine network in cocultures was determined by high constitutive MSC release of certain cytokines (especially IL-6 and vascular endothelial growth factor) and constitutive release of a wide range of soluble mediators by primary AML cells. However, the AML cell release varied considerably between patients, and these differences between patients were also reflected in the coculture levels even though supra-additive effects were seen for many mediators. These effects on the local cytokine network were dependent on a functional crosstalk between the two cell subsets. The crosstalk altered the global gene expression profile of the MSCs, especially expression of genes encoding proteins involved in downstream signaling from Toll like receptors, NFκB signaling and CCL/CXCL chemokine release. Thus, primary AML cells alter the functional phenotype of normal MSCs.

1. Introduction

Acute myeloid leukemia (AML) is a heterogeneous malignancy characterized by bone marrow infiltration of immature leukemia blasts (Network, 2013; Welch et al., 2012; Reikvam et al., 2013a). The AML cell population has a hierarchical organization including a minority of leukemic stem cells (Bonnet & Dick, 1997) that often are included in the $CD34^+/CD38^-$ compartment (Bonnet & Dick, 1997). These cells show self-renewal and long-term in vitro proliferation and have the capacity to produce leukemic progenitors showing proliferative but not self-renewal capacity (Huntly & Gilliland, 2005). Both leukemic as well as normal hematopoietic stem cells (HSCs) depend on support from the bone marrow microenvironment (Lane et al., 2009). This support is at least partly mediated through the local cytokine network and the leukemic progenitors (i.e. the more mature leukemia cell subset) as well as the stromal cells contribute to this network through their constitutive cytokine release.

Mesenchymal stem cells (MSCs) can be isolated from various adult tissues (including bone marrow), they are multipotent and can differentiate into cells of the mesodermal (e.g. adipocytes, osteocytes, chondrocytes) as well as other embryonic lineages (Jiang et al., 2002). MSCs can also be cultured in vitro and retain their multilineage potential (Pittenger et al., 1999). It is not known whether AML cells can alter the biological functions of bone marrow MSCs. However, several observations suggest that cells with a stromal phenotype are important in leukemogenesis (Blau et al., 2011; Blau et al., 2007). MSCs may even mediate resistance against antileukemic chemotherapy (Kojima et al., 2011), possibly through their effects on the local cytokine network of the bone marrow (Bruserud et al., 2007; Uccelli et al., 2008). Finally, MSCs may also have immunomodulatory effects (Uccelli et al., 2008; Le Blanc et al., 2007; Le Blanc et al., 2008), but it is not known whether this is important for leukemogenesis or chemosensitivity. We have

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previously shown that the cytokine-mediated crosstalk between AML cells and differentiated stromal cells supports leukemia cell proliferation (Bruserud et al., 2004; Hatfield et al., 2006), and in the present study we investigated the cytokine-mediated crosstalk between primary human AML cells on immature MSCs.

2. Material and methods

2.1. AML patient population and preparation of primary AML cells

The study was approved by the local Ethics Committee (REK III, University of Bergen, Norway) and samples collected after written informed consent. Our department is responsible for the treatment of all AML patients in a defined geographic area, and the present study included 18 consecutive and thereby unselected patients (10 males and 8 females; median age 67 years with range 18–87 years) with high relative and/or absolute peripheral blood blast counts ($>7 \times 10^9$/L) (Supplementary Table 1). All patients were diagnosed according to the WHO criteria (Döhner et al., 2010), and most of them had *de novo* disease. All AML cell samples were obtained from peripheral blood, the leukemic cells were isolated by density gradient separation (Lymphoprep, Axis-Shield, Oslo, Norway) and gradient-separated cells contained at least 95% leukemic blasts. Cells were stored in liquid nitrogen until used in the experiments (Bruserud et al., 2003).

2.2. In vitro culture of mesenchymal stem cells and normal hematopoietic cells

2.2.1. Normal human MSC

Human MSCs derived from the bone marrow of healthy Caucasian donors (MSC24429, 45 years old male; MSC24539, 24 years old female; MSC25200, 38 years old male) were purchased from Lonza (Cambrex BioScience, Walkersville, MD, US). The cells showed no evidence for differentiation towards the adipogenic, chondrogenic and ostogenic lineages, the donors had no evidence for HIV-1, hepatitis B or hepatitis C infections, and all three cell preparations tested negative with regard to mycoplasma, bacterial and fungal infections (distributor's information). The MSCs were expanded in complete Mesenchymal Stem Cell Growth Medium (MSCGM™) (Lonza). Cells were obtained in passage two before being expanded, trypsinated and used for the experiments in passage 3–4. Viability determined by trypan blue exclusion was 50–80%..

2.2.2. Normal hematopoietic cells

Normal human hematopoietic progenitor cells (Lonza) were obtained by bone marrow aspiration from the posterior iliac crest of healthy donors. The cells were cultured for ten days in Poietics™ primary human hematopoietic progenitor growth medium that was supplemented with stem cell factor (SCF) 25 ng/mL, thrombopoietin (TPO) 50 ng/mL and FLT3-ligand (FLT3-L) 50 ng/mL. After culture 83.7% of the nucleated cells were $CD45^+/117^+/13^+/10^-/14^-$/HLA-$DR^+$ blast-like cells, 5.8% expressed lymphoid markers and 10.5% expressed neutrophil markers. The blast like population included 47% of cells showing low CD34 expression whereas the remaining 53% were highly positive for CD34.

2.2.3. Transwell cocultures

Coculture s of MSCs with normal or leukemic hematopoietic cells were prepared in six well plates (Costar 3401 transwell plates; Costar, Cambridge, MA, USA) where cells in the lower chamber are separated from the cells in the upper chamber by a semipermeable membrane (0.4 μm pore size). Cultures were prepared in complete MSCGM™ medium and MSCs were seeded in the lower chamber (6000 cells/cm^2). Pilot experiments showed that primary human AML cells cultured in this medium showed cytokine-dependent proliferation that was comparable to the response in the StemSpan SFEM medium (Stem Cells, Vancouver, Canada) (data not shown). After four days, 1×10^6 normal hematopoietic stem cells or primary human AML cells were added to the upper well. Cells were cocultured for three additional days before cells/supernatants were harvested or proliferation assayed by ^{3}H-thymidine incorporation as described in detail previously (Bruserud et al., 2004; Hatfield et al., 2006; Bruserud et al., 2003). The mesenchymal cells did not reach confluence during this culture.

2.2.4. Microtiter cultures of MSC

As described in detail previously (Bruserud et al., 2004; Hatfield et al., 2006) proliferation of MSCs was tested by ^{3}H-thymidine incorporation in microtiter cultures.

2.2.5. AML cell culture supernatants

In accordance with previous studies the AML cells (1×10^6/ml, 2 ml/well, 24 well culture plates) were cultured in Stem Span medium for 48 h before supernatants were harvested; at this time point the differences between patients with regard to ability of AML cells to show constitutive cytokine release are clearly seen (Bruserud et al., 2000).

2.3. Analyses of soluble mediator release

Supernatants were stored at −80 °C until analyzed. Cytokine levels determined by Luminex analyses (R&D Systems, Abingdon, UK) included (i) the growth factors tumor necrosis factor α (TNFα), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), heparin binding-epidermal growth factor (HB-EGF), granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF); (ii) the interleukins (IL) IL1-RA, IL-1β, IL-6, IL-10 and IL-33; (iii) the chemokines CCL2-5, CXCL1/5/8/10/11; and (iv) matrix metalloproteases 1 (MMP-1) and 2 (MMP-2). IL-8/CXCL8, MMP-9, tissue inhibitor of MMP1 (TIMP-1) and angiopoietin-1 (Ang-1) levels were analyzed by enzyme-linked immunosorbent assays (ELISAs) (R&D Systems). All analyses were performed strictly according to the manufacturer's instructions.

2.4. Analysis of intracellular mediator levels

MSCs were trypsinized and the cell pellets (after centrifugation at 600 ×*g* for five minutes) were resuspended in Shieh lysis buffer. After 30 min of incubation on ice, the suspension was centrifuged at 13.000 ×*g* for 30 min. The clear lysate was harvested and stored at −80 °C until the levels of (1) p65 (RelA) that is the association partner of the mature subunit of NFκB, (2) the p65 inhibitor IκB, and (3) the p65 activator, IKKα/β were determined by Luminex analyses (Merck Millipore, Darmstadt, Germany).

2.5. RNA preparation, microarray analysis of global gene expression and PCR analyses

The MSCs from the lower wells were lysed in RLT plus buffer with added β-mercaptoethanol according to QIAgen's RNeasy protocol and thereafter homogenized by the aid of QIAshredder columns according to the manufacturer's recommendations. The lysates were stored frozen at −80 °C until thawing at 37 °C in a water bath and handled according to QIAgen's instructions in the RNeasy with DNase treatment protocol for the QIAcube. All microarray assays were performed using the Illumina iScan Reader and based on fluorescence detection of biotin-labeled cRNA. For each sample 300 ng total RNA was reversely transcribed, amplified and Biotin-16-UTP-labeled using the Illumina TotalPrep RNA Amplification Kit (Applied Biosystems/Ambion; Foster City, CA, USA). Amount and quality of the biotin-labeled cRNA were controlled both by the NanoDrop spectrophotometer and Agilent 2100 Bioanalyzer before 750 ng of biotin-labeled cRNA was hybridized to the HumanHT-12 v4 Expression BeadChip according to manufacturer's

instructions. This chip targets 47 231 probes derived primarily from genes in the NCBI RefSeq database (Release 38).

Real-time PCR was carried out in triplicate on an ABI Prism 7900HT sequence detector system (Applied Biosystems) using cDNA synthesized by means of a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) as template. Primers were designed using Primer (Rozen & Skaletsky, 2000). RT-PCR relative gene expression levels were determined using the comparative ΔCt method, using β-actin (Actb) and ribosomal protein, large, (Rplp0) as endogenous controls.

2.6. Statistical and bioinformatical analyses and presentation of the data

All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 21.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA), and p-values <0.05 were regarded as statistically significant. The data from the scanning of arrays (IlluminaiScan Reader) were examined in GenomeStudio and J-Express 2012 (MolMine AS, Bergen, Norway) for quality control (Stavrum et al., 2008). All arrays were quantile normalized before being compiled into an expression profile data matrix. Genes were collapsed using the max probe for merge statistics, before analysis. Gene ontology (GO) terms were identified using the Gene Ontology website (http://www.geneontology.org/GO.current.annotations.shtm). Other bioinformatical analyses were performed using the J-Express 2012 analysis suite (MolMine AS) (Stavrum et al., 2008).

3. Results

3.1. Coculture of MSCs with primary AML cells has no or only minor effects on MSC proliferation

We compared MSC proliferation (MSC24429, MSC24539, MSC25200) for transwell cocultures with primary AML cells (Supplementary Table 1) and AML-free control cultures; proliferation was then assayed as ^{3}H-thymidine incorporation. We seeded 20,000 MSCs in the lower chamber of each transwell, and the cells were harvested when the cells showed ≤70% confluence. No difference in MSC viability was observed by microscopic examination during culture. The three MSCs showed a similar and relatively weak proliferation in repeated experiments (range 2706–6072 cpm); ^{3}H-thymidine incorporation <1000 cpm was regarded as no detectable proliferation whereas cytokine-dependent AML cell proliferation (50,000 cells per well) often corresponds to 20, 000–50,000 cpm (Bruserud et al., 2003). When using this assay for other cell types we have defined a significant difference in the proliferation to correspond to 2000 cpm (Bruserud et al., 2003); a difference >2000 cpm for at least two of the MSCs was only seen for three patients (always increased proliferation). A twofold alteration was not observed for any patient for MSC24429 and MSC25200, whereas this was seen for seven patients when testing MSC24539 (Supplementary Fig. 1). Thus, when using a ^{3}H-thymidine incorporation assay all three MSCs showed relatively low proliferation and coculture with primary AML cells caused either no significant alteration or only a minor increase in MSC proliferation.

3.2. Different MSC populations show similar cytokine release profiles

MSC24429, MSC24539 and MSC25200 were cultured in medium alone and the MSCs then showed similar cytokine release profiles with (Tables 1 and 2 for MSC24429 and MSC24539): (i) undetectable (only CCL3) or relatively low absolute levels (<100 pg/mL) of CCL4–5, CXCL10, IL-1β/1RA/10, TNFα, bFGF, G-CSF and GM-CSF; (ii) 100–500 pg/mL of CXCL5 and HGF; (iii) 500–5000 pg/mL of CCL2, IL-6, VEGF and MMP-1; and (iv) >5000 pg/mL of MMP-2 and TIMP-1. A limited variation between MSCs corresponding to less than fivefold variations and/or an absolute difference less than 200 pg/mL was seen for CXCL1, CXCL8 and CXCL11. Only MMP-9 (range 4.8–329 pg/mL) showed a relatively wide variation between the three MSCs. This mediator release profile was reproduced in independent experiments and is consistent with previous studies (Kastrinaki et al., 2013; Kemp et al., 2005; Ranganath et al., 2012).

Table 1
The levels (pg/mL) of soluble mediators in supernatants derived from cultures containing primary AML cells alone (18 patients examined), MSC24539 alone and cocultures of MSC and AML cells.

Mediator	MSC alone	AML alone	MSC + AML coculture
Chemokines			
CCL2	2351	2362 (19.8–3902)	4713 (2371–6253)
CCL3[a]	nd	1067 (34–41518)	2092 (86–42167)
CCL4[a]	17.5	487 (4.9–9377)	1254 (42–17629)
CCL5[a]	26	226 (9–55553)	10578 (29.7–55553)
CXCL1[a]	20	1049 (nd–9830)	13705 (120–13044)
CXCL5[a]	263	376 (nd–55553)	10427 (297–55553)
CXCL8[a]	307	31105 (257–115815)	49342 (768–379791)
CXCL10[a]	1.1	42 (1.3–542)	3.6 (1.1–889)
The interleukin system			
IL-1β	0.1	9.6 (1.1–133)	68 (nd–1622)
IL-1RA[a]	0.8	9867 (17–>14441)	>14441 (20–>14441)
IL-6	1488	220 (0.5–>2775)	2775 (705–>2775)
IL-10	nd	0.7 (nd–60.0)	3.8 (nd–245)
Growth factors			
TNFα	1.3	9.4 (nd–1487)	10.6 (2.1–1489)
HGF	152	17.7 (3.1–266)	92 (27.6–641)
VEGF	1147	7.4 (0.9–108)	1163 (1147–1254)
bFGF[a]	5.4	nd (nd–4.3)	14.6 (8.5–24.5)
G-CSF[a]	nd	nd (nd–357)	263 (nd–4049)
GM-CSF[a]	1.2	2.8 (0.4–834)	118 (1.6–2207)
The MMP system			
MMP-1[a]	700	152 (nd–14263)	3987 (303–14263)
MMP-2	20367	6888 (854–12918)	23357 (18594–22826)
MMP-9[a]	329	13718 (235–215189)	23032 (279–227465)
TIMP-1	121752	5956 (nd–37990)	105858 (61019–145948)

The concentrations of the MSCs alone are presented as the mean of duplicate determinations, the variation between duplicates being generally <10% of the mean. The AML and coculture results are presented as the median and the variation range, respectively. Abbreviation: nd, not detected.

[a] Means at least 9 patients showing supra-additive levels.

3.3. Supra-additive cytokine levels can be seen in MSC-AML cell cocultures

MSC24429, MSC24539 and MSC25200 were cocultured in transwell cultures with primary human AML cells derived from all 18 patients and levels of soluble mediators determined in the supernatants. Control cultures were prepared with AML cells and MSCs alone. The overall results for MSC24539 are summarized in Table 1, and the results for MSC24429 are summarized in Fig. 1. MSCs cultured alone showed relatively high levels (i.e. levels comparable or higher than the corresponding median level for AML cells alone; for absolute levels see Table 1) of CCL2, IL-6, HGF, VEGF, MMP-1, MMP-2 and TIMP-1. The AML–MSC coculture levels of these mediators were also relatively high. In addition, bFGF showed a similar pattern with relatively high levels for MSCs compared with the AML cells (i.e. MSC release higher than the median AML release); although both cell types showed relatively low absolute levels compared with several other cytokines. For all these mediators the variations between patients were relatively small both for CCL2 (<4.0 fold variation), VEGF (3.4 fold), bFGF, (4.3 fold) MMP-2 (1.2 fold) and TIMP-1 (2.4 fold). Even the variation for HGF (23.2 fold) is much lower than for many of the mediators constitutively released only by AML cells. Similar results were observed for the two other MSCs cocultures (data not shown). Finally, the clustering shown in Fig. 1B does not show any association with differentiation (FAB classification, $CD34^{+}$ expression) or genetic abnormalities.

Supra-additive levels were often detected in the cocultures (i.e. coculture levels higher than the summarized levels for MSCs and AML cells cultured alone): (i) at least nine out of the 18 patients showed

Table 2
Supernatant level (pg/mL) of various mediators after *in vitro* culture of MSC24539 alone, HSCs alone and transwell cocultures of MSCs + HSCs. Supra-additive levels in the cocultures are given in bold.

	MSC alone	HSC alone	MSC + HSC
Chemokines			
CCL2	**822.2**	**224.2**	**1662**
CCL3	23.8	39.8	29.0
CCL4	66.9	57.7	101
CCL5	0.98	37.2	27.2
CXCL1	210	207	376
CXCL5	**141**	**99.2**	**416**
CXCL8	647	312	1352
CXCL10	2.4	2.3	2.7
CXCL11	172	131	196
Interleukins			
IL-1β	**6.8**	**5.1**	**13.4**
IL-1RA	**7.8**	**86.4**	**906**
IL-6	630	45.0	468
IL-10	26.8	24.4	14.7
Growth factors			
TNFα	6.6	3.0	9.1
HGF	**124**	**16.4**	**325**
bFGF	81.0	64.9	74.3
Ang-1	767	710	1225
G-CSF	50.4	34.8	42.5
GM-CSF	17.7	19.5	18.4
MMP/TIMP			
MMP-1	**3502**	**87.7**	**6716**
MMP-2	17,691	3736	19,076
MMP-9	**4.8**	**5.9**	**13.8**
TIMP-1	58,600	5800	56,800

All concentrations are given in pg/mL. All analyses were performed in duplicates and the difference between the two measurements generally corresponded to <10% of the mean. The MSC alone results are the mean level for 4 independent cultures, the HSC and coculture results represent the mean of two independent cultures each.

supra-additive levels of CXCL1/5/8, IL-1RA and MMP-9 when AML cells were cocultured with all three MSCs; and (ii) supra-additive levels for at least half of the patients with two out of the three MSCs were observed for CCL3/4/5, CXCL10, IL-6, IL-10, bFGF, G-CSF, GM-CSF and MMP-1 (data not shown). Thus, supra-additive levels in cocultures were uncommon only for eight of the 24 mediators tested; these exceptional mediators included IL-1β, TNFα and CXCL11 that showed low release for most cultures and the five mediators that showed relatively high release by MSCs cultured alone (CCL2, HGF, VEGF, MMP-2 and TIMP-1; see above) (data not shown).

To further illustrate the observation of supra-additive effects we did a cluster analysis (Fig. 1). For each mediator and patient we estimated the relative increase, i.e. (the level in cocultures minus the corresponding level for MSCs cultured alone): (the corresponding level for AML cells cultured alone). Hence, a ratio >1 indicates a supra-additive level. These values were log(2) transformed before used in a hierarchical clustering analysis, a value >0 then indicates a supra-additive effect. The mediators then formed two main clusters: one subset consisted of mediators showing minor alteration in cocultures and included several chemokines (CCL3-5, CXCL10/11 together with TNF-α, MMP-9, bFGF, HB-EGF, IL-1RA/β/10 and GM-CSF. The other subset consisted of the mediators showing strong supra-additive effects in cocultures (ratio being >1.0) and included CCL2, CXCL1/5/8, TIMP-1, IL-6, G-SCF, HGF, and Ang-1. The detection of supra-additive levels is summarized in the Supplementary Fig. 2; the figure shows the levels for those 15 cytokines that showed supra-additive levels for at least 9 patients and for at least 2 MSCs. Supra-additive levels were common and were detected for most patients and with all three MSCs, they were also seen for the four exceptional patients identified as described in section 3.7 (patients 4, 6, 12, 14) and detection of supra-additive levels showed no significant association with AML cell differentiation or genetic abnormalities (see Supplementary Table 1).

3.4. Individual differences between patients in constitutive AML cell release of soluble mediators are also reflected in the coculture levels

There was a wide variation among patients in the release of several mediators, and we therefore investigated whether there was any correlation between coculture levels and the corresponding levels for AML cells cultured alone. The results for CCL3, IL-1RA and MMP-9 are presented in Fig. 2; these results illustrate those significant correlations between AML culture and coculture levels could be detected for different mediators even when they showed supra-additive effects in cocultures. Significant correlations ($p \le 0.004$ unless otherwise stated) between AML and coculture levels were also observed for CCL4, CCL5 (only MSC24429), CXCL1 ($p = 0.023$ for MSC25200), CXCL5, CXCL8, CXCL10 ($p = 0.008$ for MSC24539), IL-1β, TNFα, HGF ($p = 0.0023$ for MSC24429), bFGF ($p = 0.012$ for MSC24539), GM-CSF, G-CSF (only for MSC24429) and MMP-9 (data not shown). Thus, a majority of the 17 soluble mediators showing undetectable or relatively low constitutive release by normal MSCs compared with the AML cell (i.e. the MSC release being lower than the median AML cell release) shows a wide variation between patients with regard to constitutive primary AML cell release, and this patient-dependent variation is maintained in AML–MSC cocultures. Despite this heterogeneity with regard to clinical and biological characteristics (Supplementary Table 1) among our consecutive patients (including differences in the constitutive cytokine release), a majority of our patients showed a similar qualitative (i.e. supra-additive) but not quantitative modulation of the local cytokine network during coculture.

3.5. The altered cytokine network in AML–MSC cocultures depends on a crosstalk between the cells

We compared the local cytokine network for (i) AML–MSC24539 cocultures; and (ii) MSCs alone cultured with 50% of the medium being replaced with 50% AML cell supernatant derived from five patients showing high constitutive cytokine release. The mediator levels were higher in the corresponding cocultures for all five patients for (i) the chemokines CCL2/4, CXCL5/10; (ii) IL-1β/6/10; (iii) the growth factors G-CSF, GM-CSF; and (iv) MMP-1/9. Furthermore, we cultured MSC24429 and MSC25200 in the presence of exogenous single cytokines (CCL2-4, CXCL1, CXCL8, IL-6), dual combinations (IL-6 + CXCL1, IL-6 + CXCL8, CXCL8 plus each of CCL2-4) or triple combinations (CXCL8 + IL-6 plus each of CCL2-4) (data not shown). The levels of 24 soluble mediators were determined in the supernatants; even though the effects differed between mediators/combinations, they were always similar for both MSC donors: (i) a general decrease for at least 12 of the 14 cultures was seen for CCL2, CXCL1, IL-6, HGF, MMP-1; and (ii) TNFα levels increased 5–10 fold for cultures containing CCL2, and CXCL5 levels increased approximately 2–3 fold for cultures containing CXCL8 (data not shown). Thus, single cytokines, cytokine combinations and AML supernatants could alter the constitutive MSC cytokine release, but such effects could not explain the more extensive or different effects seen in the cocultures. The more extensive effects can only be explained by an active crosstalk between AML cells and MSCs.

3.6. The cytokine network during coculture of MSC and normal hematopoietic cells

We compared the cytokine release for MSC24429 cultured alone, normal hematopoietic cells cultured alone (one donor, two independent experiments) and cocultures of MSC24429 with normal hematopoietic cells (Table 2). Supra-additive effects were seen for several mediators. Thus, there is also a crosstalk via the soluble mediator network between MSCs and normal hematopoietic cells, but the final effect

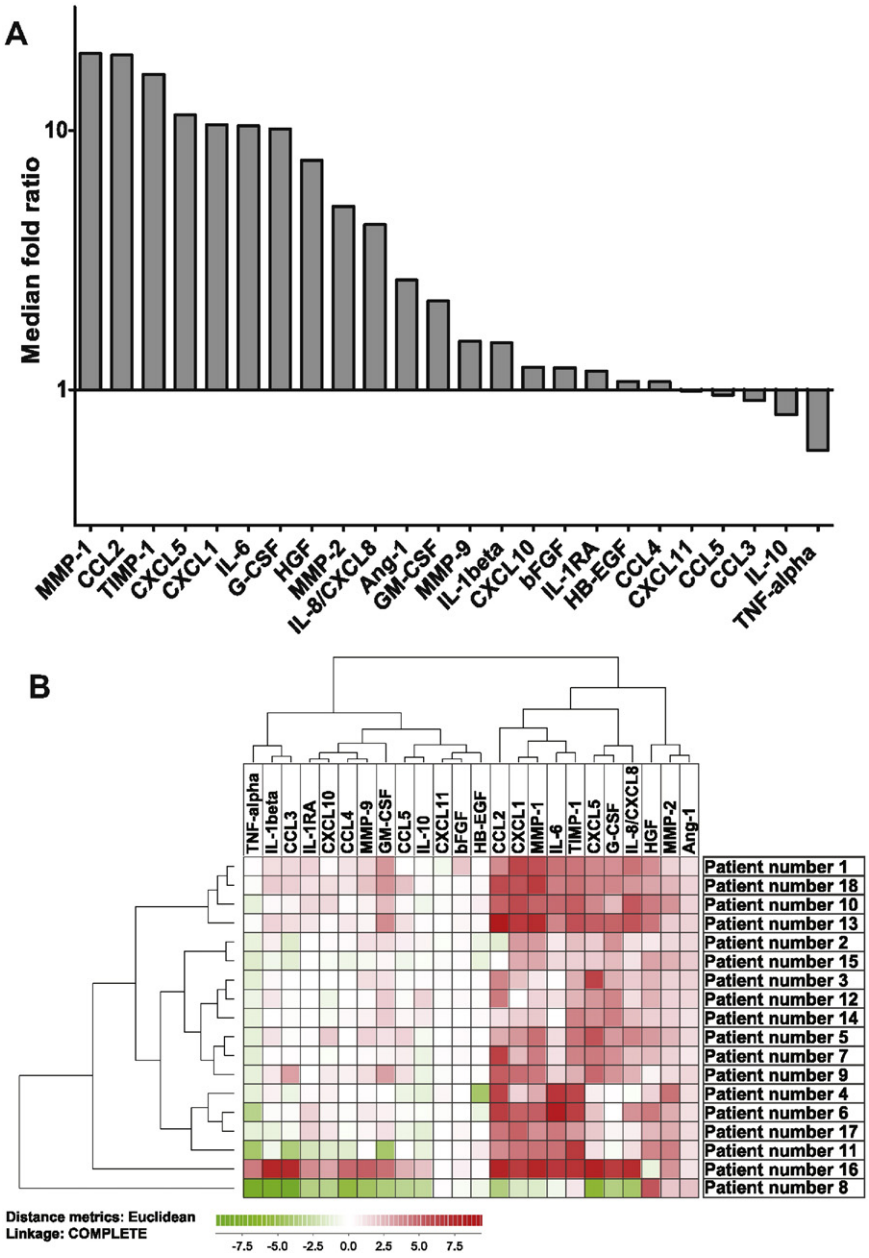


Fig. 1. The effect of the cytokine-mediated crosstalk between primary human AML cells and MSCs on the local cytokine network. MSC24429 derived from a healthy donor were cocultured in transwell cultures together with primary AML cells derived from 18 patients. We determined the cytokine levels in supernatants harvested from AML–MSC cocultures and corresponding control cultures only containing AML cells or MSCs. (A) The fold change was defined as the level in coculture relative to the corresponding level for AML cells cultured alone. The figure presents the median fold change for all 18 patients and for each soluble mediator. (B) We also performed a hierarchical clustering analysis based on the ratio of cytokine levels in AML–MSC cocultures and AML cell cultures. Each horizontal row in the figure represents the observation for one patient, and a vertical column represents the observations for one of the soluble mediators. Red indicates increased fold change, white no difference and green decreased fold change. Two major cytokine subsets were identified; the left main cluster included mediators usually showing no or only minor alterations in AML–MSC cocultures, whereas the right main cluster included mediators that are usually showing increased fold change in cocultures.

(i.e. which cytokines showing supra-additive effects) differs for normal and leukemic cells. For the normal cells supra-additive levels were seen for fewer (7 versus 15) and partly other mediators (CCL2, IL-1β, HGF) than for the AML cells, the common mediators with supra-additive effects being CXCL5, IL-1RA and MMP-1/9).

3.7. AML cells alter the global gene expression profile of MSCs whereas normal hematopoietic stem cells have only minor effects

We examined the global gene expression profile for MSC24429 cultured alone (four parallel cultures), together with normal hematopoietic cells (two parallel cultures) and together with primary human AML cells derived from all 18 patients (Supplementary Table 1). First we investigated the co-variance between samples and performed a correspondence analysis that allowed the display of low-dimensional projection of the data and identification of samples with similar behavior (Fig. 3) (Fellenberg et al., 2001). The X-component variance distinguished the majority of patient samples both from the four cultures containing MSC alone and the two cultures containing MSC with normal hematopoietic cells, whereas the patients were heterogeneous and AML cells derived from four exceptional patients localized relatively close to the control samples compared with the other 14 patients. Despite the Y-component variation between the 4 MSC controls, it can be seen that the majority of the 14 AML patients localized away

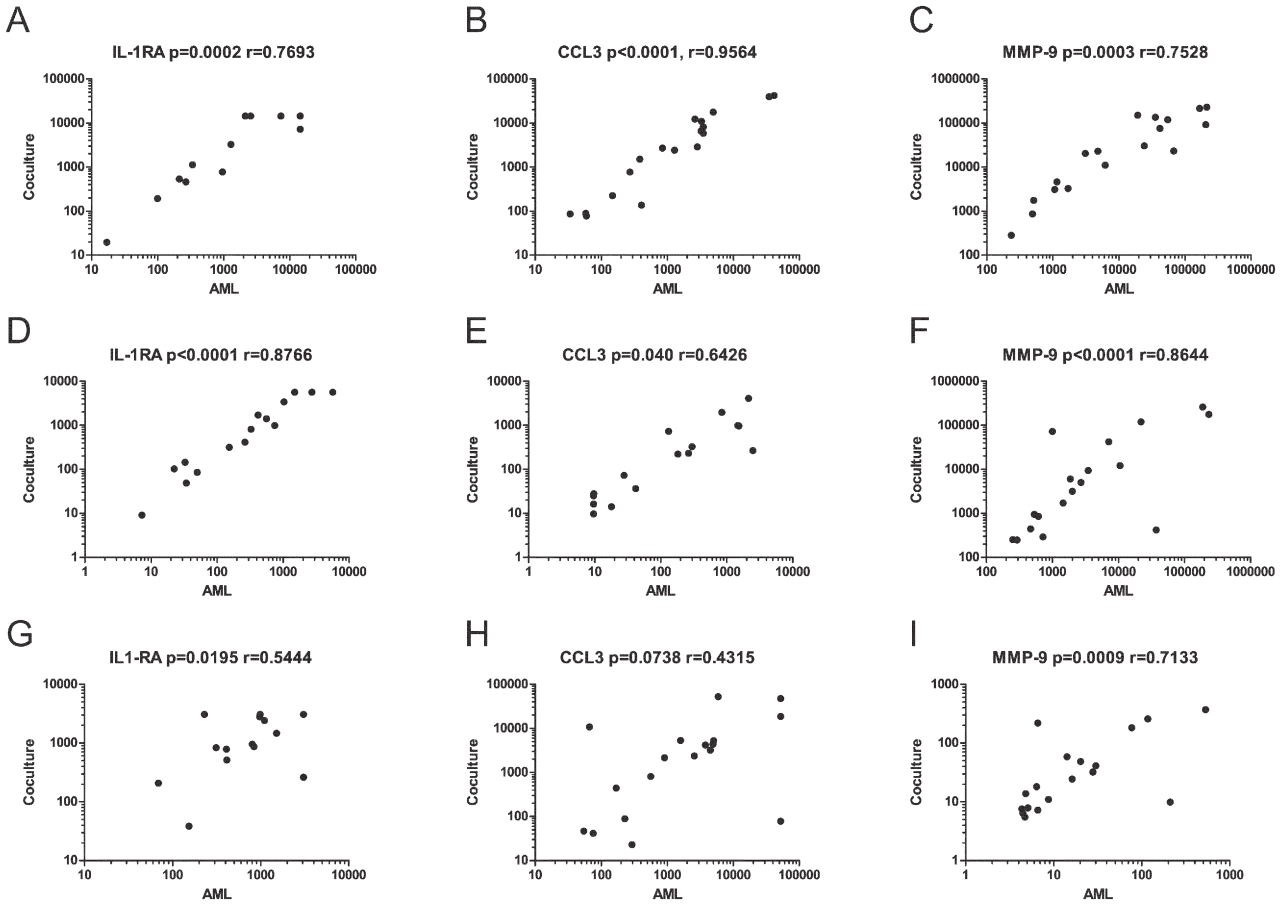


Fig. 2. Supernatant concentrations of IL-1RA (A, D, G), CCL3 (B, E, H) and MMP-9 (C, F, I) in cocultures of MSCs and primary human AML cells. MSC24539 (A, B, C) and MSC25200 (D, E, F) and MSC24429 (G, H, I) were cultured in transwell cultures together with primary human AML cells derived from 18 unselected patients. For a large majority of patients IL-1RA, CCL3 and MMP-9 showed supra-additive levels in cocultures compared with control cultures including MSCs or AML cells alone. We compared the mediator concentrations for cultures containing AML cells alone and the corresponding AML–MSC cocultures, and for each figure we show the results from this correlation analysis (p-value, r-value).

from the MSC controls, the normal hematopoietic cells and 4 exceptional patients.

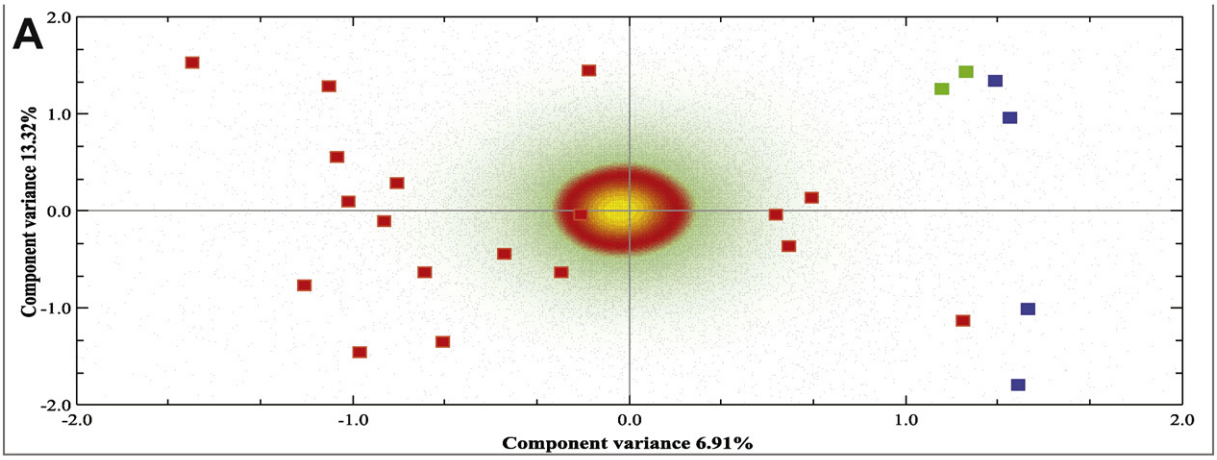
We used the significance analysis of microarrays (SAM) algorithm to circumvent the problems of multiple testing (Tusher et al., 2001) and compared the global MSC gene expression profile for cells cultured alone and together with AML cells. An algorithm of 400 permutations was used. When comparing the most differently expressed genes with a false discovery rate (FDR) <1.0, we identified 89 genes that showed a highly different expression in MSCs cultured with AML cells (45 up-regulated and 44 down-regulated). These 89 genes were then used for hierarchical clustering analysis (Pearson's correlation with complete linkage) (Fig. 3); one cluster then included MSCs cocultured with 14 of the AML samples whereas the other main cluster included the MSCs cultured in medium alone, together with normal hematopoietic cells and together with the four exceptional AML samples. Furthermore, within this last main cluster the four MSC samples, the two normal hematopoietic cell samples and the four exceptional patients formed separate subclusters.

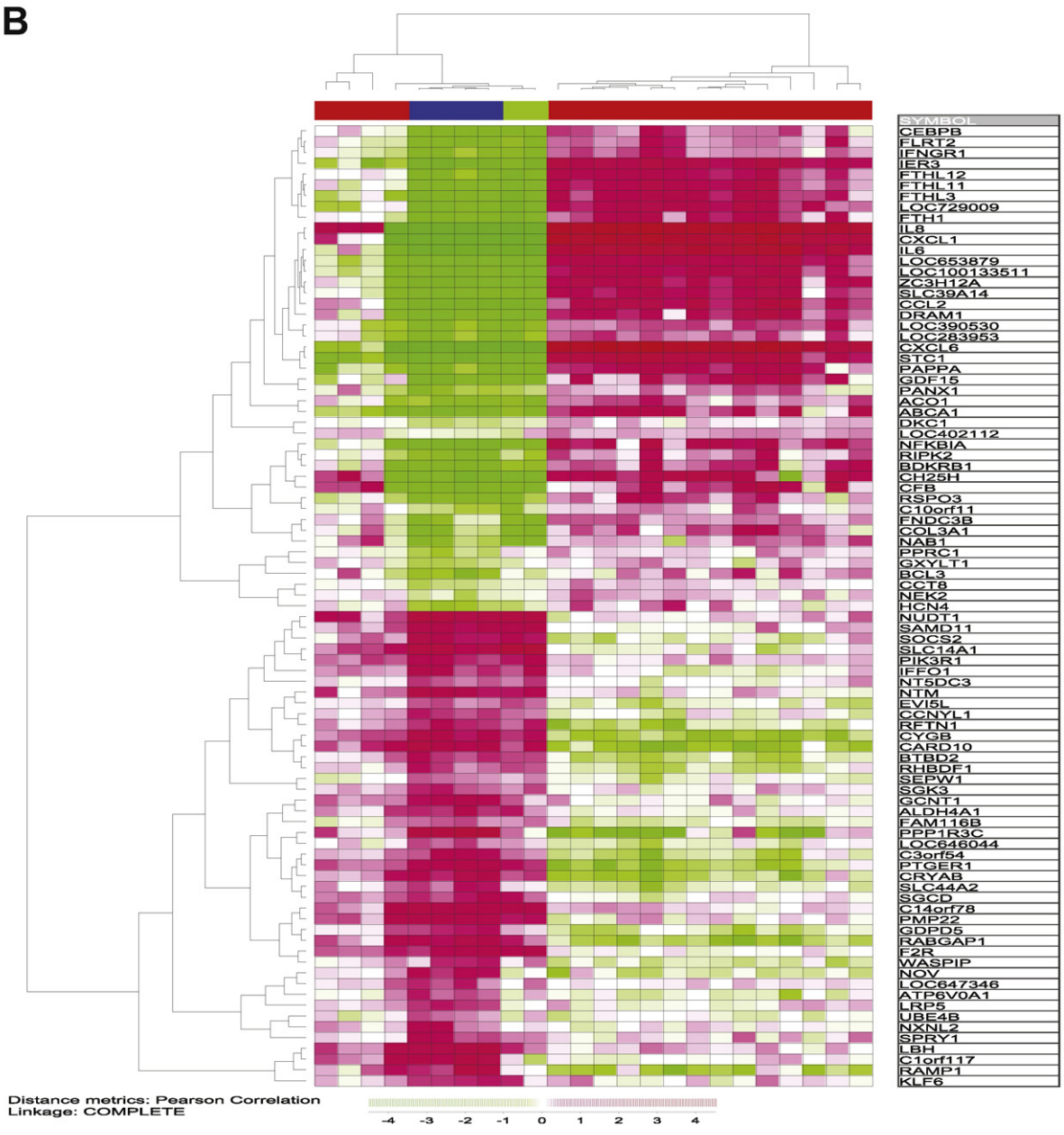
The AML cells derived from the four exceptional patients (see Figs. 3 and 4) were heterogeneous with regard to predisposition (2 secondary AML and 2 *de novo*), morphological signs of differentiation (FAB classification), CD34 expression and genetic abnormalities (Supplementary Table 1). We also performed a hierarchical clustering of all AML patients based on the constitutive cytokine release by AML cells cultured alone. The 18 patients could be divided into two main clusters and several subclusters based on their cytokine release profiles, but the four exceptional patients then clustered separately, i.e. they did not form a common cluster due to a similar and specific constitutive cytokine release profile (Supplementary Fig. 3). We finally identified those genes (i) included in the clustering analysis presented in Fig. 3 and (ii) showing a different expression for the majority of 14 patients compared with the 4 exceptional patients, i.e. all patients in one group showing increased and all patients in the other group showing decreased expression. The 13 identified genes are presented in Supplementary Table 2: six of the 11 genes with a known function are known to be important for NFκB signaling/function and two additional genes possibly have a role in transcriptional regulation. Our data have to be interpreted with care because the exceptional patients are few, but taken together our observations suggest that these exceptional AML patients differ from the majority of patients with regard to the regulation and/or role of NFκB in the MSCs whereas their common effect on MSC gene expression is not induced by a common and specific cytokine release profile.

The expression of selected genes showing altered expression in MSC cocultured with primary human AML cells (*CCL2*, *CXCL6*, *IL8*, *IRAK2*, *NFKB1A*) were validated by PCR analyses, and for all these genes a strong correlation was observed between the microarray and the PCR data (Supplementary Fig. 4; $r \geq 0.068$ and $p \leq 0.003$ for five genes).

3.8. Gene set enrichment analysis detected upregulation of distinct MSC functions after coculture

Gene set enrichment analysis (GSEA) was used to identify gene ontology (GO) terms associated with coculture-induced differences in the gene expression profile of MSC24429 (all 18 patients included in the analysis) (Subramanian et al., 2005). Several GO-terms were highly enriched in MSCs cocultured with AML cells from the 18 patients, and


A
Component variance 13.32%
Component variance 6.91%
2.0
1.0
0.0
-1.0
-2.0
-2.0
-1.0
0.0
1.0
2.0


B
SYMBOL
CEBPB
FLRT2
IFNGR1
IER3
FTHL12
FTHL11
FTHL3
LOC729009
FTH1
IL8
CXCL1
IL6
LOC653879
LOC100133511
ZC3H12A
SLC39A14
CCL2
DRAM1
LOC390530
LOC283953
CXCL6
STC1
PAPPA
GDF15
PANX1
ACO1
ABCA1
DKC1
LOC402112
NFKBIA
RIPK2
BDKRB1
CH25H
CFB
RSPO3
C10orf11
FNDC3B
COL3A1
NAB1
PPRC1
GXYLT1
BCL3
CCT8
NEK2
HCN4
NUDT1
SAMD11
SOCS2
SLC14A1
PIK3R1
IFFO1
NT5DC3
NTM
EVI5L
CCNYL1
RFTN1
CYGB
CARD10
BTBD2
RHBDF1
SEPW1
SGK3
GCNT1
ALDH4A1
FAM116B
PPP1R3C
LOC646044
C3orf54
PTGER1
CRYAB
SLC44A2
SGCD
C14orf78
PMP22
GDPD5
RABGAP1
F2R
WASPIP
NOV
LOC647346
ATP6V0A1
LRP5
UBE4B
NXNL2
SPRY1
LBH
C1orf117
RAMP1
KLF6
Distance metrics: Pearson Correlation
Linkage: COMPLETE
-4
-3
-2
-1
0
1
2
3
4

41 of these terms had a false discovery rate (FDR) <1.0. These 41 GO-terms could then be further classified (AmiGo) into 12 main groups based on their biological function (Fig. 4 and Supplementary Table 3). These GO-terms included terms important for gene transcription (nucleotide binding, nucleic acid metabolism, NFκB), protein synthesis and stabilization (ribosome activity, heat shock protein/unfolded protein response), regulation of leukocyte functions/metabolism (leukocyte activation, interleukin activation, metabolism, chemokine activity, response to lipopolysacharide/bacteria, VEGF activity) and intracellular signaling (Toll-like receptor signaling pathway, NFκB). Thus, several of these terms describe interacting steps in the same signaling pathway: initial response to lipopolysaccharide/bacteria through ligation of Toll like receptors followed by downstream signaling involving NFκB that can translocate to the nucleus initiate expression of chemokines/interleukin through nucleotide binding.

When using the leading edge genes derived from these 41 GO-terms we identified 275 genes that belonged to at least one GO-term and were upregulated in cocultured MSCs. These 275 genes were used to perform a hierarchical clustering analysis and we then identified the same main clusters as with the SAM analysis (Fig. 4); one main cluster included the same 14 patients and within the other main cluster the four MSCs alone, the two normal hematopoietic and the same four exceptional patient samples formed three separate subclusters.

3.9. AML cells increased the MSC expression of genes involved in TLR initiated and NFκB mediated intracellular signaling together with increased CCL and CXCL chemokine expression

Using a Feature Subset Selection we were able to identify individual genes with highly increased expression in MSCs after coculture with primary AML cells. The 15 genes with the highest fold change then included genes encoding extracellular mediators (Bruserud et al., 2007; Reikvam et al., 2013b), i.e. seven chemokines (CCL2, CCL20, CXCL1/2/5/6/8) together with IL-1β, IL-6 and MMP-3. The expression of several of these mediators is regulated by TLR initiated and/or NFκB mediated intracellular signaling (Bruserud et al., 2007; Zeytun et al., 2010). Thus, both this last analysis as well as the identification of significantly altered GO-terms showed that the AML–MSC cytokine crosstalk modulates the TLR/NFκB/chemokine axis in normal MSCs. We therefore analyzed more in detail the three GO-terms TLR, NFκB and chemokine activity (Fig. 5). Several highly upregulated genes were included both in the TLR and NFκB terms; among the highest upregulated common genes were *NFKBIA*, *IRAK2*, *IRAK3*, *NKB1* and *IKBKE*. Furthermore, the increased mRNA expression of *CCL2*, *CCL20*, *CXCL1*, *CXCL2*, *CXCL6*, *IL8*, *IL1B* and *IL6* all corresponded to a more than 3.8 fold change, being highest for *IL8* (65.5), *CXCL1* (28.6), *CXCL6* (27.0) and *IL6* (18.6) (Fig. 5).

MSCs are able to differentiate in various directions including adipocytes and osteoblasts. The GO term Adipocyte differentiation includes 19 genes (*AACS*, *ADRB1*, *ADRB2*, *ALDH6A*, *ARL4A*, *CBY1*, *CCND1*, *CTBP1*, *DNAJA4*, *FABP4*, *FGF10*, *PPARG*, *PSMB8*, *RARRES2*, *RGS2*, *SDF4*, *SIRT1*, *TBLY1*, *TRIM32*) and the term Osteoblastic differentiation includes 13 genes (*DHX9*, *DNAJC13*, *EPHA2*, *ERH*, *GLI2*, *IGFBP5*, *PTH1R*, *PTHLH*, *RUNX2*, *SATB2*, *SMAD3*, *SPP1*, *TWIST2*). None of these 32 genes were among the most deregulated genes identified in any of the analyses described above. We then compared their expression for MSCs cultured alone and MSCs cocultured with the 18 primary AML cells. All these genes showed generally low expression with no significant difference between MSCs cultured alone and together with leukemic cells, the only exception being the osteoblast-associated IGFB1 that showed decreased expression in the presence of leukemic cells. IGFBP5 is secreted by preosteoblasts but decreases during their differentiation (Mukherjee & Rotwein, 2007). However, the IGF system is a growth regulator for many differentiated cells, and altered expression of this marker alone cannot be regarded as a sign of differentiation. The expression of these 32 differentiation markers did not differ between the majority of 14 patients and the 4 exceptional patients identified in Figs. 3 and 4 either.

3.10. MSCs cocultured with primary AML cells show altered NFκB signaling

MSC25200 cells were cocultured in transwell cultures with primary AML cells derived from the 18 patients before MSC lysates were prepared. The levels of the two stimulatory mediators p65/RelA and IKK and the inhibitory mediator IKBα/β were determined and the stimulator versus inhibitor ratios p65/IKB and IKK/IKBαβ estimated (Fig. 5). An increase of both ratios (i.e. increased stimulatory activity) was detected for 11 patients, additional 4 patients had one increased ratio and the last 3 patients showed no increase for any ratio. These 3 last patients included one of the exceptional patients from the global gene expression studies (Supplementary Table 1, patient 12) and two patients (patients 13 and 16) included in the major subset identified in Fig. 3. Thus, our results from these protein studies in cocultured MSCs also suggest that NFκB signaling is altered in the stromal cells after coculture with primary human AML cells.

4. Discussion

AML is a heterogeneous malignancy with regard to the biology of the leukemic cells, and bone marrow stromal cells support the growth of primary human AML cells (Bruserud et al., 2004; Hatfield et al., 2006; Reikvam et al., 2013b; Reikvam et al., 2012). In the present study we focused on the cytokine-mediated crosstalk between AML cells and MSCs and how this communication alters the biological characteristics of normal MSCs.

Our MSCs were derived from three healthy individuals and showed several common characteristics; only one of them was therefore included in the global gene expression studies. Firstly, the MSCs expressed cell surface molecules consistent with a normal MSC phenotype (Kemp et al., 2005; Lv et al., 2014). Secondly, our MSCs showed only minor differences in their constitutive cytokine release profiles and several of these cytokines are constitutively released by normal MSCs during *in vitro* culture (Kastrinaki et al., 2013; Kemp et al., 2005; Ranganath et al., 2012). Thirdly, the presence of primary AML cells had no or only weak effects on MSC proliferation. Finally, all three MSC populations increased the *in vitro* proliferation and viability of primary human AML cells during coculture (Ito et al., 2014). Thus, our MSCs usually showed similar functional characteristics in various experimental models with only minor differences; these minor differences between our MSC populations are thus not caused by age-dependent differences (Liedtke et al., 2015; Lepperdinger, 2011). Taken together our observations suggest that the microarray data for the MSC24429 cells should be regarded as representative for our three normal human MSC populations. However, our results should be interpreted with care because of the limited

Fig. 3. Modulation of the MSC global gene expression profile caused by the cytokine-mediated crosstalk between primary human AML cells and MSCs in transwell cocultures. (A) Correspondence analysis; mesenchymal stem cells (MSC24429) derived from a healthy donor were either cultured alone in four independent cultures (blue squares in the figure), together with normal hematopoietic cells in two cultures (green squares) and together with primary human AML cells derived from 18 unselected patients (red squares). The global gene expression profile of the MSCs was determined for all these cultures. The correspondence analysis of the 24 profiles showed that the X-component could be used to distinguish between MSC populations with differences in their global gene expression profile, i.e. MSCs cultured alone, MSCs cultured together with normal hematopoietic cells and MSCs derived from a majority of the MSC–AML cocultures (14 out of the 18 patients). (B) We also performed a significance analysis of microarrays (SAM) of the MSC global gene expression profiles. We then identified 89 differentially expressed genes with a false discovery rate (FDR) <1.0. The results are presented as a heat map together with clustering denograms; 45 genes were upregulated in cocultured MSCs and 44 were downregulated. The 89 genes could be used for a hierarchical clustering analysis (Pearson correlation with complete linkage), and this analysis distinguished between MSCs cultured alone (blue bars), MSCs coculture with normal hematopoietic cells (green bars) and MSCs cocultured with AML cells (red bars). The same 4 exceptional AML patients could be identified in this analysis as in the correspondence analysis (A).

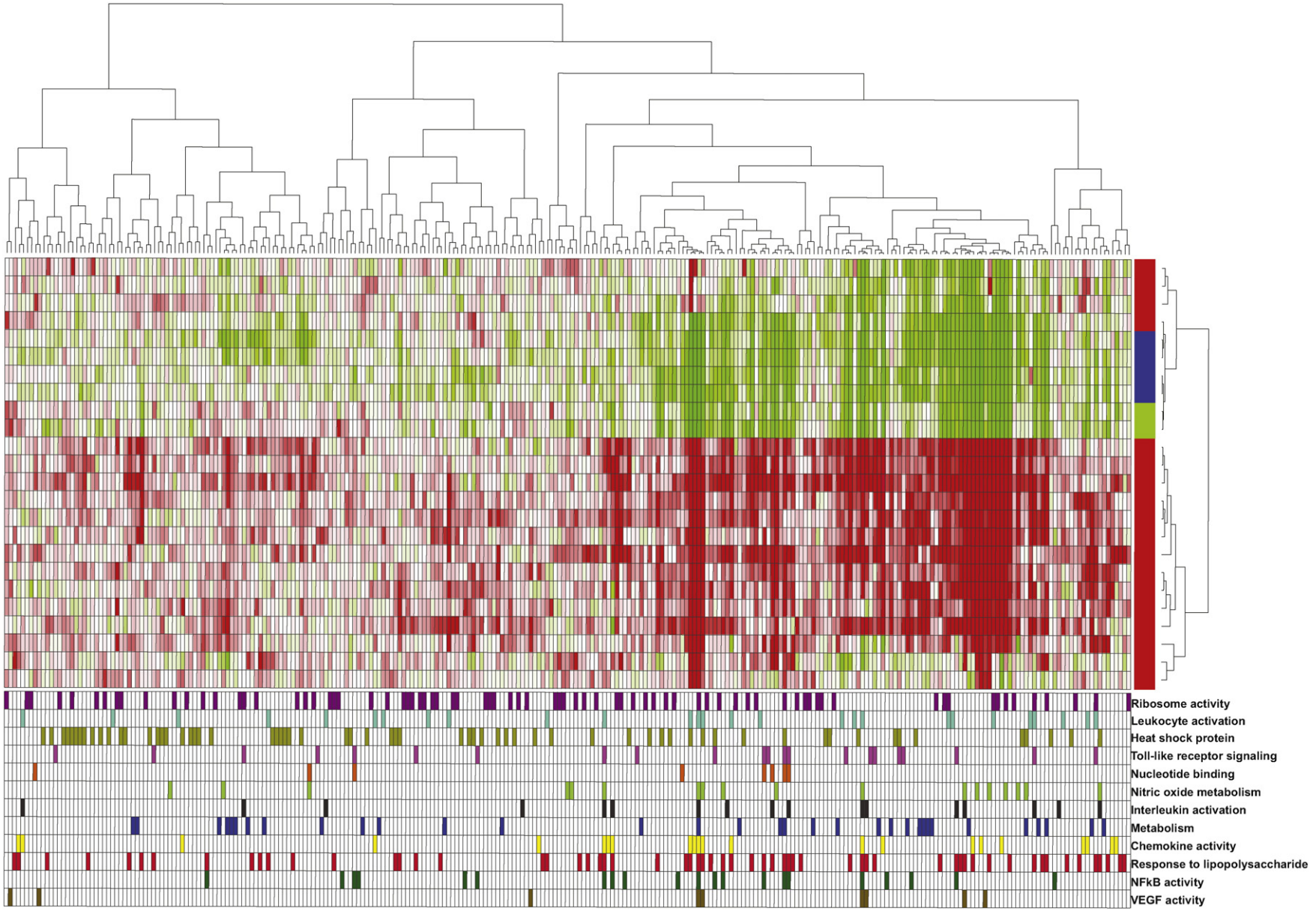


Fig. 4. Cytokine-mediated modulation of the MSC global gene expression profile during coculture (transwell cultures) with primary human AML cells — a gene set enrichment analysis (GSEA). The analysis was performed by comparing the profiles for MSC24429 cultured alone, cocultured with normal hematopoietic cells and cocultured with primary human AML cells (18 patients included). By using GO-terms with false discovery rates <0.01 we identified 41 GO-terms that could be classified into 12 main groups (Supplementary Table 2), and we then identified 275 genes belonging to the leading edge of at least one of these 41 GO-term. Finally these 275 genes were used to perform a hierarchical clustering analysis (Pearson correlation, complete linkage), and based on this analysis we could distinguish between MSCs cultured alone (right margin, blue color), MSCs cocultured with normal hematopoietic cells (green color) and MSCs cocultured with AML cells (red color). The same four exceptional patients were identified as in the previous analyses (Fig. 3). In the lower part of the figures the leading edge genes for each of the 12 major subgroups are identified (lower right part).

number of MSC donors examined, e.g. we do not know whether age-induced differences in MSCs will influence the effects of the cocultures.

Our *in vitro* model was based on the use of transwell cultures (Bruserud et al., 2004; Hatfield et al., 2006; Glenjen et al., 2002; Hatfield et al., 2009), and we could thereby investigate the cytokine-mediated crosstalk between MSCs and primary AML cells without the influence of direct cell–cell contact. We used a growth-factor supplemented culture medium that has been developed for MSC culture, and this medium could be used for AML cell culture. Our experimental model thereby represents a highly standardized methodological approach.

We analyzed our results from the microarray studies by using different bioinformatical strategies. However, independent of the method used for data analysis we observed that patients were heterogeneous with regard to the effects of AML cells on MSCs and usually differed from normal hematopoietic cells, but a common effect of the AML cells was a modulation of the TLR–NFκB-chemokine axis in the normal MSCs. NFκB is also an important regulator of cytokine release by primary human AML cells (Bruserud et al., 2007). NFκB inhibitors are now available and this strategy thus represents a dual targeting of the cytokine release both in AML cells and MSCs (Reikvam et al., 2009).

Our coculture experiments demonstrated that all three MSCs showed a similar low *in vitro* proliferation; for most patients coculture of all three MSCs with primary human AML cells did not alter MSC proliferation but a minor increase in the proliferation was seen for a minority of patients. No or only minor effects on MSC proliferation were also seen when they were cultured with exogenous cytokines or AML supernatants. These observations suggest that the growth-enhancing effect of primary AML cells on MSCs depends on the overall intercellular crosstalk. It should in addition be emphasized that the MSC growth enhancement was observed for most patients despite the wide variation in cytokine release between patients both in AML cultures and AML–MSC cocultures.

We compared the cytokine network for MSC cocultures with both leukemic and normal hematopoietic cells. Because the normal hematopoietic cell population of the bone marrow is heterogeneous we then used a heterogeneous normal population including both immature cells, more differentiated myeloid cells and a minority of lymphoid cells. The constitutive mediator release by these normal cells differed from the AML cells, and their effects on the normal cytokine network as well as their effects on the MSC gene expression profiles differed from the majority of AML cells. Thus, for most AML patients the cytokine-mediated crosstalk between MSCs and AML cells results in a more extensive alteration of MSC gene expression than the normal bone marrow cells.

The cocultures showed a unique local cytokine network that reflected (i) the high constitutive release of certain mediators by the MSCs and thereby a relatively small variation between patients of these cytokines; and (ii) AML-associated differences between patients that were maintained during coculture even for mediators showing supra-additive levels. Despite the individual differences the effects of the cytokine-mediated crosstalk on MSC gene expression signatures were similar for most patients.

We compared the levels of 24 soluble mediators in culture supernatants when MSCs were cultured in AML cocultures and when only AML culture supernatants were added to MSC cultures. A large number of soluble mediators then showed higher levels in the cocultures than in the supernatant cultures. This observation strongly suggests that the effects of coculture on the local cytokine network are not caused simply by the presence of AML derived soluble mediators, but that a functional crosstalk between the cells is required.

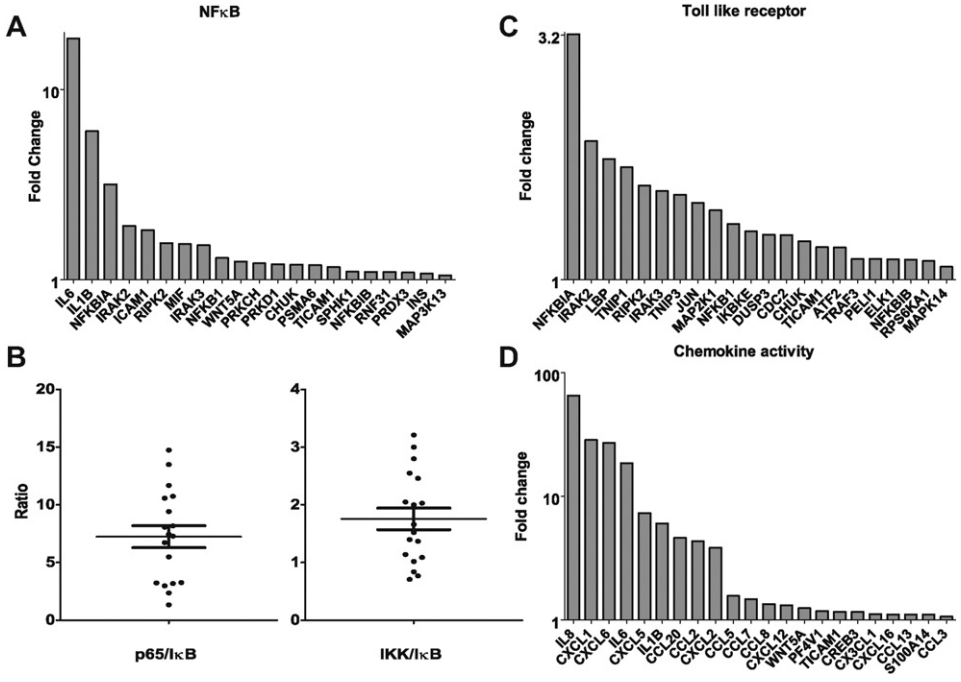


Fig. 5. Modulation of the global MSC gene expression profile after coculture with primary human AML cells derived from 18 patients; an overview of leading edge genes identified by the GSEA analysis and the balance between inhibitory and stimulatory NFκB signaling. MSCs were cultured in transwell cocultures either alone or in the presence of primary AML cells derived from 18 unselected patients. (A, B) The figure shows the altered expression of genes belonging to the GO-term NFκB signaling (A) and the balance between stimulatory versus inhibitory NFκB signaling (B). The mRNA levels are presented as the fold change, i.e. levels for MSCs cocultured with AML relative to the corresponding levels for MSCs cultured alone, and the figure shows the median fold change. NFκB signaling is presented as the ratio between stimulatory and inhibitory mediators; the levels of the two stimulatory mediators p65/RelA and IKK and the inhibitory mediator IKBα/β were determined in cell lysates and the results are presented as the ratios p65/IκB and IKK/IκB. The figure also shows the ratios for MSCs cultured in medium alone (horizontal line) together with the results for MSCs cocultured with leukemic cells derived from the 18 patients. The levels of the three intracellular mediators were determined in cell lysates by duplicate analyses. (B) The figure shows the altered expression of genes belonging to the GO-terms Toll like receptors (C) and chemokine activity (D). The mRNA levels are presented as the fold change, i.e. levels for MSCs cocultured with AML relative to the corresponding levels for MSCs cocultured with AML cells, these levels are presented as the median fold change.

Our microarray studies demonstrated that the cytokine-mediated crosstalk between MSCs and AML cells altered the global gene expression profile of the MSCs, and 14 of the 18 patients then showed similar alterations. These four exceptional patients (i) did not differ from the other patients with regard to clinical characteristics; (ii) did not have a common cytokine release profile; (iii) their effects on MSC proliferation were comparable to the other patients; and (iv) they showed supra-additive effects on cytokine release similar to the other patients. Thus, this crosstalk-induced difference in MSC gene expression profile is not secondary to any of the other clinical/biological differences described above.

Coculture of MSCs with primary AML cells especially altered the MSC expression of genes involved in TLR-initiated signaling (i.e. genes downstream to the receptors), regulation of NFκB and chemokine/interleukin expression (Fig. 4 and Supplementary Table 3). These three components form an interacting system at different levels of the cells (Bruserud et al., 2007). TLR receptors show transactivation with G-protein coupled receptors (e.g. chemokine receptors) (Abdulkhalek et al., 2012), NFκB is an important downstream target of TLR-initiated signaling and NFκB is in addition an important regulator of chemokine expression/release in various human cells, including primary AML cells. Therapeutic targeting of the NFκB system thus seems to represent an opportunity to modulate the local AML-supporting cytokine network in the bone marrow through inhibition of cytokine release both by the leukemic cells and normal stromal cells.

Our Feature Subset Selection Analysis demonstrated that several of the upregulated genes encoded cytokines that often showed supra-additive levels in cocultures of MSCs and AML cells, but this was not true for CCL2 and that were upregulated only at the mRNA but not at the protein level. One possible explanation is that additional posttranscriptional mechanisms are important for these two mediators, an alternative explanation is that these proteins are absorbed/consumed or degraded during the culture.

Ito et al. (2014) demonstrated that MSCs could support the growth of AML cells in cocultures, and our present study shows that there is a bidirectional crosstalk between AML cells and MSCs as the MSC characteristics were altered in our transwell cocultures. However, it is not known whether the cytokine network alone mediates an AML-supporting bidirectional crosstalk between mesenchymal and leukemic cells because the study by Ito et al. questioned the importance of the cytokine network and emphasized the importance of direct cell–cell contact for the MSC-associated growth enhancement of the AML cells (Ito et al., 2014).

To conclude, the cytokine-mediated crosstalk between primary AML cells and MSCs alters the functional characteristics of the MSCs, and these effects may thereby contribute to the AML supporting effects of normal MSCs.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2015.09.008.

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