On epigenetic regulation in atherosclerosis pathology

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A role for KMT1C in monocyte to dendritic cell differentiation

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Abstract

Monocytes play a key role in immune system function. Chromatin remodeling is crucial for various differentiation and gene regulation processes and is rather well studied in T cells. However, for monocytes not much is known regarding how the epigenetic machinery influences the differentiation into various effector cell types. In the work presented here, we explore the epigenetic underpinnings of monocyte differentiation. By transcriptional profiling we show that transcription of lysine methyltransferases (KMTs) and in particular KMT1C is markedly up regulated after differentiation of monocytes into immature dendritic cells (iDCs). Specifically inhibiting KMT1C function, using the small-molecule inhibitor BIX-01294, changes the transcription levels of the DC marker DC-SIGN, but does not affect surface protein expression. Blocking global KMT activity, using DZNep, does influence monocyte differentiation into iDCs, indicated by a loss of DC-SIGN surface expression. When BIX-01294 and DZNep treatment was combined DC-SIGN expression was almost lost completely. This work shows that the activities of KMTs are required for successful differentiation of monocyte-derived dendritic cells. Furthermore it shows the importance of KMT inhibitors in the field of epigenetic immune therapy, which is still much focused around HDAC inhibitors.

Introduction

Monocytes are key players in the functioning of our immune system. They are the precursor cells that upon differentiation form dendritic cells (DCs) and macrophages.[1,2] In diseases such as atherosclerosis and multiple sclerosis, monocyte dysfunction contributes to a great extend to disease initiation and progression.[3] In atherosclerosis particularly monocytes navigate to sites of endothelial damage, adhere to the vessel wall and transmigrate over the endothelial cell layer to infiltrate the underlying tissue.[4] When monocyte entry of the subendothelium is inhibited, by blocking chemokines or their receptors, atherogenesis is retarded or prevented in mouse models.[5]

Differentiation of monocytes into macrophages is a critical step in the pathogenesis of atherosclerosis. Upon transendothelial migration, monocytes differentiate into functionally defined subsets: the classically activated or M1 macrophages or the alternatively activated or M2 macrophages. Classically activated macrophages, a profile induced by Th1 cytokines, mainly display an inflammatory phenotype and produce pro-inflammatory cytokines such as IL-6, IL-12 and TNF-α. In contrast, alternatively activated macrophages are induced by Th2 cytokines and display a more atheroprotective phenotype, exemplified by the secretion of e.g. IL-10.[6] In addition, monocytes may also differentiate into DCs, depending on the stimulus.[7–9]

Dendritic cells, once activated, play an important role in the pathogenesis of atherosclerosis and are particularly harmful in destabilizing the atherosclerotic plaque.[6] Studies of DCs in patients indicate that aberrant DC activation or functions are associated with different autoimmune diseases including rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, psoriasis, and inflammatory bowel disease (e.g. Crohn's disease and ulcerative colitis).[10]

How epigenetic regulation potentially influences various pathological conditions has been subject of several studies in recent years.[11,12] Epigenetic regulation of gene expression is thought to regulate lineage-specific gene expression in a manner that is stable across cell divisions. In humans, the chromatin remodelling involved in T cell differentiation has been rather well studied.[13–16] The epigenetic processes underlying differentiation of monocytes into dendritic cells and into macrophages, however, have not yet been studied in much detail. The work published on epigenetic regulation involving monocytes focuses on the epigenetic regulation of single genes, including following the response to interferon stimulation.[17–19] In addition to epigenetic regulation at a single gene level, some studies have been published

on genome-wide distribution of epigenetic markers, in particular H3K4me3 and H3K27me3.[20,21] That epigenetic processes indeed play an important role also in monocyte differentiation is also illustrated by the observation that inhibition of HDAC activity by the HDAC inhibitor (HDACi) butyrate results in inhibition of functional differentiation of human monocyte-derived dendritic cells.[22] While all previous work has mainly focused on the activity of epigenetic regulatory enzymes, in this study we present for the first time the expression characteristics of the genes encoding epigenetic regulatory enzymes. The transcription characteristics of these genes were measured during monocyte differentiation into various effector cells. By using specific inhibitors, an important functional role for lysine methyltransferases (KMTs) and in particular KMT1C in monocyte differentiation into dendritic cells is revealed.

Materials and Methods

Cell isolation and culturing

For the initial screening of transcript levels of epigenetic modifiers, peripheral blood was obtained by venipuncture from three individuals undergoing phlebotomy at Sanquin (Amsterdam, the Netherlands). For the validation of *KMT1C* transcript levels peripheral blood of institutional volunteers was drawn by venipuncture and collected into sodium citrate tubes (Greiner). For both screening and validation experiments, peripheral mononuclear cells (PBMCs) were then isolated by density gradient centrifugation using Ficoll Isopaque plus (GE Healthcare). Written informed consent was obtained from all participants. The study protocol conforms to the Declaration of Helsinki and was approved by the ethics committees of the institutions. Five parts of blood were diluted with one part of buffer consisting of phosphate buffered saline (PBS; LUMC Pharmacy) + 10% v/v GPO (Sanquin) + 10% v/v Sodium Citrate (LUMC Pharmacy). PBMC fraction was recovered and washed three times in buffer. Monocytes were isolated from the PBMC fraction by magnetic separation with anti-CD14 magnetic beads (MACS; Milteny Biotech).

Between 0.25 and 1.0×10^6 freshly isolated monocytes were seeded in 1 mL in a 24-wells plate. Cells were cultured in RPMI-1640 medium (Gibco, Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (FCS; PAA), 100 IU/mL streptomycin, 100 IU/mL penicillin (both Lonza) and 2 mM L-glutamine (Gibco). Cytokines were added to the medium to obtain DCs (1000 U/mL GM-CSF, 500 U/mL IL-4), M1 (50 U/mL GM-CSF) and M2 (50 ng/mL M-CSF) as previously described

by Verreck et al.[23] When indicated, cells were cultured in presence of lysine methyltransferase inhibitors (KMTi's): 5 μM 3-Deazaneplanocin A (DZNep, kind gift of V.E. Marquez), 5 μM BIX-01294 (Sigma Aldrich) or a combination of BIX-01294 and DZNep (both in final concentration of 5 μM). The used concentrations of inhibitors are based on the maximum tolerated concentration, allowing for at least 80% viable cells (assessed by eosin staining), when cells were harvested.

After 5 days of culture, fresh medium and cytokines (and inhibitors) were added to the cells and the cells were cultured for an additional 2 days. To obtain activated macrophages or maturated dendritic cells (mDCs), the fresh medium was supplemented with 100 ng/mL (final concentration) LPS at day 5 of culture and cells were cultured for an additional 2 days.

RNA isolation and cDNA synthesis

Either directly after isolation, after 24 hours or 7 days of culture, cells were scraped of the culture plate and centrifuged for 5 min at 500×g. Thereafter the pellet was lysed in RNAbee (Tel-Test) according to manufacturer's instructions. Lysates were stored at 80°C until further processing. Total RNA was extracted according to manufacturer's instructions.

From 1 μg total RNA, cDNA was synthesized using SuperScript III (Invitrogen), with random hexamers (Promega), at 50°C for 1 hour according to manufacturer's instructions.

qPCR

Quantitative PCR was performed on a Bio-Rad iCycler 5 with SYBR Green supermix (Bio-Rad) and the primers shown in supplementary table 3–S1. For each reaction, 125 ng of cDNA and 5 pmol of forward and reverse primer were used in a total volume of 25μL. The thermal profile for the PCR reactions was 3 min 95°C (enzyme activation) followed by [15s 95°C; 30s T_{ann}; 30s 72°C] for 50 cycles. All primers were designed for 60°C annealing temperature. For some primers however, optimal performance was achieved at a higher annealing temperature. The used annealing temperatures are shown in supplementary table 3–S1. Primers were designed to pick up all splice variants of a given gene, with the use of NCBI Primer design tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). All primers were tested beforehand to produce only a single amplicon and for a PCR efficiency between 90% and 110%. Amplicons were sequenced to confirm the identity of the

target sequence. Amplicon length varies between the primer-pairs from 114bp to 246bp. All PCR reactions were performed in duplo. For the screening experiment, transcription levels were normalized against RNA polymerase II (*RPII*) transcript levels. To minimize normalization-induced error, the transcription levels in the validation experiment and BIX-01294 treatment experiment where normalized against the geometric mean Cq-value of *RPII*, Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), ß-glucuronidase (*GUS*), and Hypoxanthine-guanine phosphoribosyltransferase (*HPRT*).

Western Blotting

Monocytes were isolated from peripheral blood and *in vitro* differentiated to immature Dendritic Cells (iDCs), with and without BIX-01294 present in the medium, as described on page 73 and harvested after 5 days of culture. From freshly isolated monocytes and iDCs, two independent histone lysates were prepared by acid extraction according to Shechter et al.[24] Lysates were separated by electrophoresis in a 15% Sodium Dodecyl Sulfate (SDS)-Polyacrylamide gel and transferred overnight to a PVDF membrane at 10V at 4°C. This membrane was blocked with 2% w/v Elk (dried skimmed milk; Campina) in PBST buffer consisting of PBS with 1% v/v Tween-20 (Sigma-Aldrich) and probed subsequently with anti-Me2K9H3 antibodies (1:5000, Abcam) and anti-mouse-HRP (1:1000, Dako). Antibodies where diluted in 0.2% w/v Elk-PBST. To correct for the concentration difference in the loading the same blot is stripped at 50°C using a buffer consisting of 2% w/v SDS (Sigma Aldrich), 62.5 mM Tris-HCl (Sigma Aldrich) pH6.8 and 0.8% v/v ß-mercaptoethanol (Sigma Aldrich). After stripping, the blot was rinsed with tap water for 30 min and washed extensively with PBST. Thereafter it was probed again with total-H3 antibody (1:10000, Abcam) followed by anti-mouse HRP (1:1000, Dako).

Separated proteins were visualized using ECL (GE Healthcare) and recorded using radiographic film (Fuji). The films where scanned and multiple exposures of each independent experiment were densitometrically analyzed using the ImageJ software.[25]

Flow Cytometry

Monocytes were isolated from peripheral blood and differentiated to iDCs *in vitro* in the presence of BIX-01294, DZNep, a combination of BIX-01294 and DZNep, or without inhibitor as described on page 73. After 7 days of culture the iDCs

were harvested by scraping of the dish and washed twice in FACS buffer: PBS, supplemented with 1% FCS (PAA) and 0.1% Sodium Azide (LUMC Pharmacy). The cells were kept on ice during the rest of the procedure. Cells were stained after dilution of the antibody in FACS buffer for 45 min. with DC-Sign (Rat Anti-Human CD209 APC; eBioscience; clone eB-h209; dilution 1:80) or the appropriate isotype control (Rat IgG2a, κ Isotype Control APC; eBioscience; clone eBR2a; dilution 1:80). Cells were washed twice in FACS buffer and taken up in 1% paraformaldehyde. Hereafter the cells were kept at 4°C until data acquisition. Flow cytometry data acquisition was performed on a FACSCalibur flow cytometer (Becton Dickinson) using Cell Quest software. Data was analyzed using the FlowJo software package. Live cells were selected based on forward and side scatter properties and analyzed for CD-209 expression.

Statistics

The relative expression of transcripts is represented as mean ± SEM with propagated errors. Initial screening experiments where analysed with a linear model (ANOVA). First, we applied a variance-stabilizing (cube-root) transformation to the expression values. Next, we tested for expression differences between cell types among specific groups of genes (F-test). To correct for multiple testing, we used the inheritance procedure.[26] Briefly, this method is applicable to situations where the hypotheses that are to be tested can be structured in a tree. Starting from the root of the tree (all genes), we proceed to test ever-smaller subsets of genes, until we arrive at the leaves of the tree (single genes). The inheritance procedure, guarantees that the family-wise error rate across all tested gene sets does not exceed 5%. The analysis was performed in the statistical software R (R, http://www.r-project.org). Sequential tests were performed following the tree-model depicted in figure 3–2B. Statistical testing of the validation study was performed using the Graphpad Prism software by means of a Mann–Whitney U test. Differences were considered to be significant if $p < 0.05$ after multiple testing correction.

Results

Transcription levels of epigenetic modifiers

To assess the role of epigenetic processes in monocyte differentiation, we studied the expression profiles of the genes encoding epigenetic regulatory enzymes during monocyte differentiation. Monocytes were isolated from peripheral blood of

three volunteers and were cultured for 24-hours in medium alone or for a total of 7 days in the presence of stimuli to induce differentiation into type 1 macrophages (M1) and type 2 macrophages (M2) or immature dendritic cells (iDCs). To monitor the effect of activation by LPS, after 5 days part of the cell cultures were activated for an additional 2 days by addition of LPS. Quantitative PCR was performed to measure the transcript levels of 51 genes encoding epigenetic regulatory enzymes after extraction of total RNA and cDNA synthesis. These genes represent the different classes of epigenetic proteins i.e. DNA methyltransferases (DNMTs), lysine acetyltransferases (KATs), histone deacetylases (HDACs) and sirtuins, lysine methyltransferases (KMTs) and lysine demethylases (KDMs). Additionally, we studied the expression profile of members of the two Polycomb Repressive Complexes: PRC2 (involved in initiation of gene repression) and PRC1 (involved in maintenance of repression). The relative transcription levels were normalized against RNA polymerase (*RPII*) transcription. Relative transcription levels are shown in figure 3–1 and supplementary figure 3–S1.

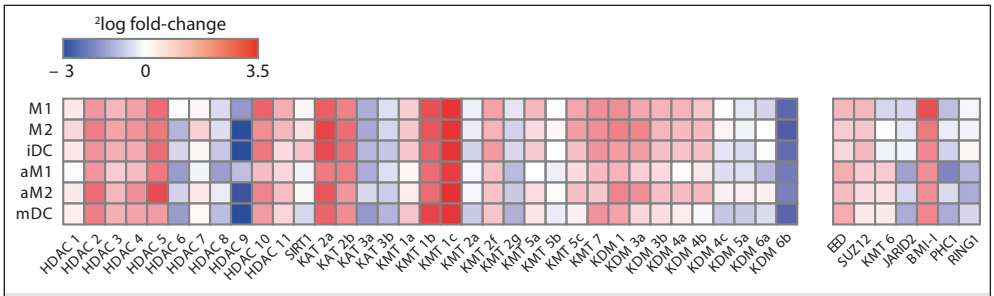

Figure 3–1. Heatmap of transcription levels of epigenetic modifying enzymes in M1, M2 iDC and activated M1, M2, mDC. Monocytes were isolated from PBMCs of three independent donors and differentiated to the depicted cell types in-vitro. All transcription levels were normalized to *RPII* transcription levels. Data is shown as ^{2}log fold-change relative to monocytes cultured for 24h. Polycomb components are shown separately, as these components were also analysedd separately. Data was visualized using GENE-E (http://www.broadinstitute.org/cancer/software/GENE-E/).

Differential deployment of epigenetic modifiers

To aid in the analysis of transcript levels, the analyzed genes were divided into several (sub-)groups based on their biological properties. In a first division, genes were classified according to global substrate specificity (i.e. histone acetylation, histone methylation or DNA methylation). Within the acetylation and methylation groups, genes were then further subdivided into writing or erasing post-translational histone modifications (i.e. HDACs vs. KATs and KMTs vs. KDMs). Finally, the HDACs were further subdivided into the common HDAC classes (i.e. HDAC class I,

II and IV; based on structural properties), and sirtuins of which only *SIRT1* was investigated.[27] A schematic representation of these divisions is shown in figure 3–2A. Genes who are a member of one of the PRCs were analysed separately. The lysine methyltransferase KMT6 is the catalytic subunit of the PRC2 complex (see Figure 3–2A) and is therefore not analyzed together with other KMTs.

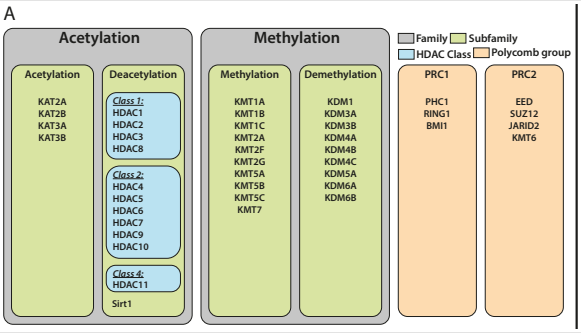

Figure 3–2. (A) Classification of epigenetic modifying enzymes into their respective classes used for statistical testing. (B) All classes showing significant difference in transcription levels ($p<0.05$ after multiple-testing correction) after differentiation are shown in black. The data were tested for expression differences between cell types among specific groups of genes (F-test). Correction for multiple comparisons was done using the inheritance procedure for tree-structured hypotheses.[26]

Following differentiation, the transcription levels of the genes encoding the enzymes involved in histone methylation as well as histone acetylation were significantly altered in M1, M2 and iDC when compared to the expression levels of undifferentiated monocytes that were cultured in medium alone. Transcription levels of PcG genes however were not altered by the differentiation stimulus (Figure 3–2B). Within the group of (de)methylating enzymes both the expression of KMTs and KDMs was significantly altered ($p = 1.3\times10^{-6}$ for KMTs and $p = 1.7\times10^{-5}$ for KDMs). Within (de)acetylating enzymes, the expression of HDACs was significantly altered ($p = 0.015$), but notably not the transcription levels of KATs. (Figure 3–2B) The alteration of transcription of KATs and HDACs could not be pointed to a specific enzyme as none of the individual transcription levels was significantly altered. Although *HDAC9* transcription was decreased dramatically, this decrease did not reach statistical significance (Figure 3–3; $p = 0.08$ by ANOVA, after correction for multiple testing). Within the group of KMTs, *KMT1C* was significantly higher expressed in differentiated monocytes then in monocytes cultured for 24h without cytokines (Figure 3–2B & Figure 3–3; $p = 0.002$ by ANOVA, after correction for multiple testing). Maturation or activation of the differentiated cells by LPS did not have an impact on transcription levels of all the genes tested.

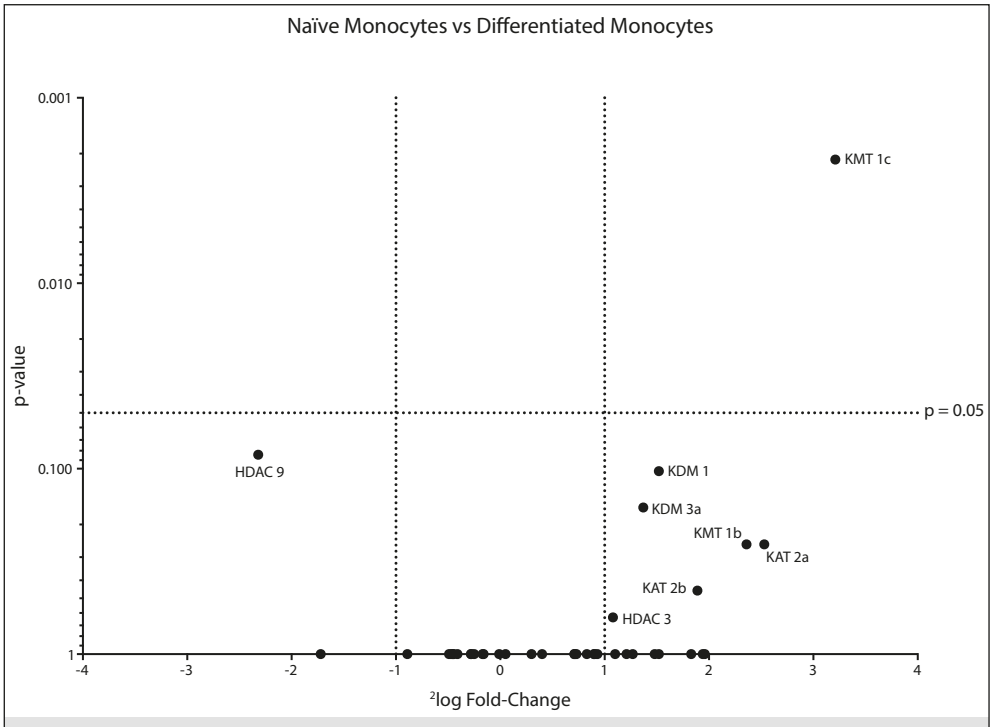

Figure 3–3. Volcano-plot showing transcript level fold-change (as shown in figure 3–1) and their respective p-values. Transcript levels of naïve monocytes were compared to monocytes after differentiation. Data was analysed using a linear model (ANOVA) and correction for multiple comparisons was done using the inheritance procedure for tree-structured hypotheses.[26]

Validation of KMT1C transcription levels

Given the important role of dendritic cells in various diseases,[10] this cell type was used for further validation of the findings above. An additional, unrelated, five healthy individuals were used in a validation experiment. Furthermore, as 24-hours of culture may already induce differentiation of monocytes, we compared immature DCs (iDCs) after 7 days of differentiation to freshly isolated monocytes instead of monocytes that had been cultured for 24 hours.

The transcription levels of *KMT1C* in this validation experiment are shown in figure 3–4. A significant increase of *KMT1C* transcription ($p = 0.0159$; Mann-Whitney U test; M0 median = 24.52, range: 14.42–45.27; iDC median = 49.77, range: 41.24–73.43) was observed in iDCs compared to freshly isolated monocytes. This increase in transcription in iDC is comparable to the increase observed earlier when the transcription profiles were compared to those of 24h cultured monocytes.

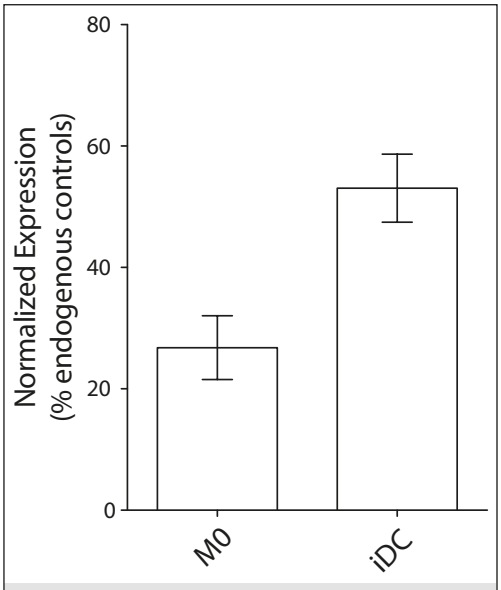

Figure 3–4. Transcription levels of KMT1C in freshly isolated monocytes and iDCs after 7 days of differentiation. There results presented here were obtained in a validation cohort (n=5) to verify the data depicted in figure 3–1 and supplementary figure 3–S1. KMT1C transcription levels are significantly increased in the differentiation process (p=0.0159; Mann-Whitney U test; M0 median=24.52, range: 14.42–45.27; iDC median=49.77, range: 41.24–73.43). Expression levels where normalized to the geometric mean Cq-value of 4 endogenous controls (GAPDH, RPII, GUS and HPRT); error bars indicate SEM with propagated error.

Inhibition of KMT1C impairs monocyte differentiation

KMT1C is described to dimethylate lysine 9 in histone H3 (Me2K9H3).[28] Therefore, to establish whether the increase in *KMT1C* transcription levels also results in altered Me2K9H3 levels, global Me2K9H3 levels were determined by western blot analysis. For this purpose, monocytes were differentiated *in vitro* into iDCs for 5 days. Acid lysates were prepared from freshly isolated monocytes and iDCs as described on page 75. The results of the densitrometric analyses of two independent lysates are shown in figure 3–5A. A clear increase of Me2K9H3 levels can be seen in the iDCs compared to the levels of Me2K9H3 in monocytes.

To determine the functional consequences of this increase in Me2K9H3, monocytes were *in vitro* differentiated into iDCs in presence of the KMT1C-specific inhibitor BIX-01294. BIX-01294 was originally identified as a KMT1C inhibitor during a chemical library screen of small molecules and has previously been used in the generation of induced pluripotent stem cells.[29–31] Although BIX-01294 is generally considered as a KMT1C (G9a) specific inhibitor, it can also inhibit the G9a-like protein (KMT1d).[30] The used concentration of inhibitors allowed for at least 80% of viable cells after 5 days of culturing. After 5 days of culture, acid lysates where prepared to check for Me2K9H3 reduction by western blotting. Addition of BIX-01294 to the culture medium abrogated the increase in Me2K9H3 levels in iDC after monocyte differentiation and resulted in similar Me2K9H3 levels as in freshly isolated monocytes (Figure 3–5).

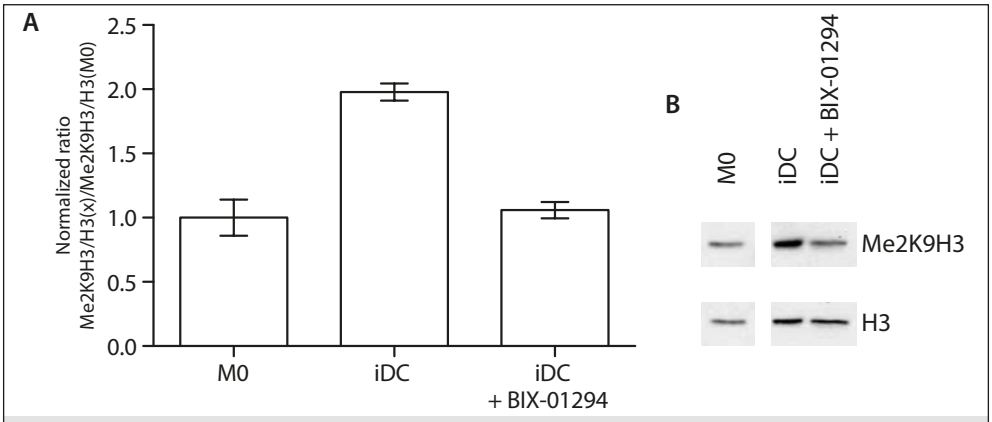

Figure 3–5. (A) Reduction of Me2K9H3 levels measured by densitometric analysis of western blots. Me2K9H3 was normalized to total H3 to correct for any loading differences. The Me2K9H3 ratio was then normalized to the ratio of freshly isolated monocytes. Shown are the results of two independent experiments. Error bars indicate SEM with propagated errors. (B) Representative western blot showing levels of Me2K9H3 and H3.

The transcription levels of the typical DC-marker *DC-SIGN* (*CD209*) were subsequently determined by qPCR analysis. RNA was extracted from freshly isolated monocytes, and iDCs, which were differentiated *in vitro* for 5 days with and without the presence of BIX-01294. Differentiating monocytes into iDCs in the presence of BIX-01294 resulted in a marked reduction of DC-SIGN transcript levels (Figure 3–6). Finally, the surface expression of DC-SIGN of iDCs and iDCs differentiated in presence of BIX-01294 was determined by flow cytometry (Figure 3–7). Although addition of BIX-01294 resulted in a reduction of DC-SIGN transcripts, surface expression of DC-SIGN was not altered by addition of this KMT1C inhibitor.

We also evaluated the impact of inhibition of multiple KMTs considering the fact that transcription of the entire group of KMTs was significantly altered during monocyte differentiation. For the purpose of these investigations we applied the lysine methyltransferase inhibitor (KMTi) 3-Deazaneplanocin A (DZNep) during the differentiation of monocytes into iDC, which is regarded as a general KMTi.[32–34] Monocytes where therefore differentiated into iDCs in presence of DZNep and a combination of DZNep and BIX-01294 and evaluated for cell surface expression of DC-SIGN.

Shown in figure 3–7, DC-SIGN surface expression levels were markedly decreased in the presence of DZNep. By adding both DZNep and BIX-01294 to the cell cultures DC-SIGN expression was almost completely abrogated. Together, this

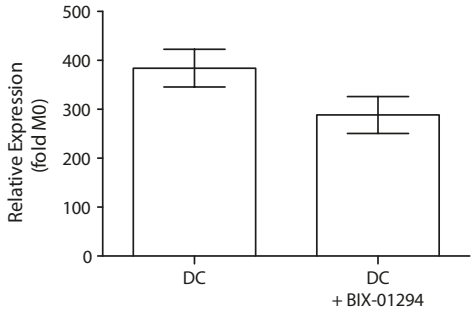

Figure 3–6. Transcription levels of the DC-marker *DC-SIGN*, determined by qPCR. The data is normalized to the geometric mean of 4 endogenous controls (*GAPDH*, *RPII*, *GUS* and *HPRT*) and represent as fold-change relative to monocyte transcription levels. Inhibiting KMT1C activity by addition of BIX-01294 results in lowered *DC-SIGN* transcription levels.

indicates that KMT1C has an important role in monocyte to DC differentiation and seems to act synergistically with the other KMTs in the differentiation of monocyte-derived dendritic cells based on expression of DC-SIGN.

Discussion

The current study shows that transcription of the genes encoding epigenetic modifying enzymes is modulated during monocyte differentiation into macrophages and into dendritic cells. We show that, with the notable exception of KATs and PcG genes, all major classes of the genes encoding epigenetic modifying enzymes are differentially transcribed when comparing monocytes to either macrophages (type 1 and type 2) or to immature dendritic cells. Further stimulation with LPS did not alter these expression patterns. Although the KDM, KMT and HDAC classes as a whole are differentially transcribed during differentiation, on a single gene level, only *KMT1C* was found to have significantly altered transcription levels during differentiation of monocytes into various lineages. Blocking KMT1C activity with BIX-01294 resulted in a reduction of *DC-SIGN* transcripts, while at the same time cell surface expression of DC-SIGN was hardly affected. However, surface expression of DC-SIGN was reduced when applying the general KMTi DZNep. Notably, DC-SIGN surface expression was almost completely lost when both DZNep and BIX-01294 were present in the culture medium. Together, these data indicate that KMT1C plays a role in monocyte to iDC differentiation. Furthermore, the additional studies with DZNep and both DNZep and BIX-01294 show that other KMTs are also involved in monocyte differentiation into iDC and that they might act synergistically with KMT1C in these differentiation processes.

Unfortunately we were unable to correlate the significantly increased levels of *KMT1C* transcripts with protein levels. Of the multitude of commercially available antibodies tested, none showed to be specific for KMT1C. Thus whether the rise in

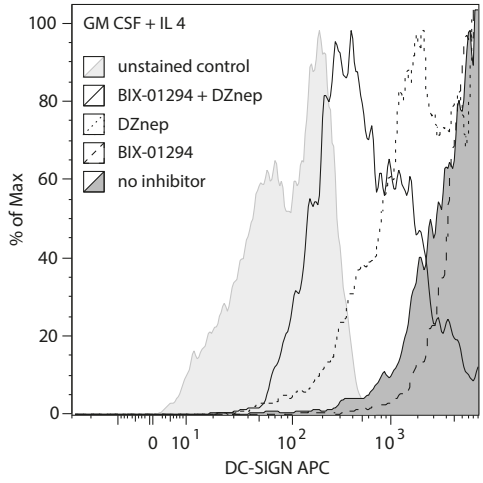

Figure 3–7. Determination of DC-SIGN surface expression on iDCs by flow cytometry. Live cells were selected based on forward and side scatter properties and analyzed for DC-SIGN surface expression. Shown histograms are normalized to peak-cell count (% of Max). Addition of BIX-01294 has little effect on the surface expression of DC-SIGN. However addition of DZNep clearly reduces the surface expression. When DZNep was combined with BIX-01294 surface expression of DC-SIGN was almost completely abrogated.

transcript levels is also associated with a rise in protein levels remains to be established.

Our data, revealing an effect of KMT inhibition on DC-SIGN expression, are in line with previous work, which has established a role for histone methylation at the DC-SIGN promoter.[19] In that study however, only H3K4, H3K9 and H3K20 triple methylation was studied and notably not dimethylation of H3K9.

Using a western blot and densitometry of X-ray film global Me2K9H3 levels were determined after BIX-01294 treatment. X-ray film has limited linearity[35] and the represented data should therefore be regarded as qualitative instead of quantitative. Although global Me2K9H3 levels where lowered after BIX-01294 treatment concomitant with a reduction in DC-SIGN transcript levels, this reduction apparently did not affect levels of DC-SIGN cell surface expression. The reduction in DC-SIGN transcripts also reveals that KMT1C contributes to DC-SIGN gene activation during monocyte differentiation. This co-activator function of KMT1C has been documented previously.[36–38] In addition, it could very well be that in the case of monocyte differentiation, methylation of non-histone proteins involved in DC-SIGN homeostasis are likely to compensate the lowered levels of DC-SIGN transcripts.[39,40] Protein surface expression can therefore be influenced both transcriptionally and post-transcriptionally by KMTs. Furthermore the observed difference in *DC-SIGN* transcription could be due to complex interactions of other regulatory factors involved in *DC-SIGN* transcription instead of directly influencing the *DC-SIGN* promoter. Thus the precise molecular interactions during differentiation remain however to be established.

The results presented here indicate that the transcription of KDM, KMT and HDAC genes are altered during monocyte differentiation. The work performed by Wang et al. is interesting in that respect, as they showed that butyrate functionally inhibited the differentiation of monocytes into DCs.[22] Butyrate is an HDACi with a specificity for class I and IIa (i.e. HDAC-4, -5, -7, -9) HDACs.[41] Both HDAC and KMT transcription levels rise during differentiation and blocking of HDAC or KMT activity seems to impair differentiation of monocytes into DCs. It would therefore also be interesting to see whether KDMi's will influence monocyte differentiation.

After the successful usage of HDACi as anti-epileptics and in the treatment of various cancers,[42–44] it has recently become apparent that epigenetic interference can be useful as well in the field of immunology and for treatment of autoimmune disorders.[45] Recently Immunology and Cell Biology ran a special issue on this emerging field. From the editorial it was evident that research in this field is mainly focused on HDACs and HDACi.[46] The work presented here not only underlines the potential of epigenetic therapy, but also illustrates that we should look beyond the HDAC-horizon.

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Table 3–S1: Primers used for the transcriptional profiling of genes encoding for epigenetic regulatory enzymes and the annealing temperatures used for the primers in qPCR.

Gene	Sequence (5'-3')	T_{ann}	Gene	Sequence (5'-3')	T_{ann}	Gene	Sequence (5'-3')	T_{ann}
HDAC1	F: GGACCAGATTTCAAGCTCCA R: CAGGCAATTCGTTTGTCAGA	61	KDM4A	F: GCTGGGAAGGACAACACAGT R: GGTCTCCTTCCTCTCCATCC	60	KMT5C	F: CGGTGAGAATGACTTCAGCA R: CTCACAGGTGTGGCATTCAC	60
HDAC2	F: CATGGCGTACAGTCAAGGAG R: TTCTTCGGCAGTGGCTTTAT	60	KDM4B	F: GCCCATCATCCTGAAGAAGT R: GCCACTTTGCCGTAGTCAAT	60	KMT6	F: AACACCGGTTGTGGGCTGCAC R: AGCCAGGTAGCACGGGCACT	64
HDAC3	F: TGGCTTCTGCTATGTCAACG R: TCTCTGCCCCGACTTCATAC	60	KDM4C	F: CACCGAAGACATGGACCTCT R: ATCTTGTGGCGAAGAAATGC	66	KMT7	F: TGTCCCCAGCCGCCATGGAT R: TACCCCTCCAGGGTGCTGCC	60
HDAC4	F: TTTCCGTGGAAATTTTGAGC R: AGGCAGTGAGAACTGGTGGT	60	KDM5A	F: CCTCCATTTGCCTGTGAAGT R: CCTTTGCTGGCAACAATCTT	61	EED	F: GCCTGCGGCCAAGAAGCAGA R: TCCTTCCAGGTGCATTTGGCGTG	64
HDAC5	F: CCCAACGAGTCGGCAGATGGGA R: CCCCGTAGCTCCACAGGGCT	60	KDM6A	F: ACCCCAGCTCAGCAGAAGTT R: GGAGGAAAGAAAGCATCACG	60	SUZ12	F: CGTCCAGGCTGACCACGAGC R: GCTGCAAATGAGCTGACAAGCTATGA	60
HDAC6	F: CCGTGTCCTAGCAGACACCT R: GTGATGTCCAGGAGGCCTAA	60	KDM6B	F: CGCCTCATCCCCCTCCTCGT R: TGGGCGCCCCTGGAGCATAA	61	JARID2	F: AACCAGTGCCAAAAAGATGC R: GAACTGGGCTTGTGGTGATT	60
HDAC7	F: AGCGGAGGTGATTCTGAAAA R: TCAGGTTGGGCTCAGAGACT	60	KMT1A	F: AAGAAGATCCGCGAACAGGA R: GGAACTGCTTGAGGATACGC	60	BMI1	F: TTGCCCATTGACAGCGGCGT R: ACAAAGCACACACATCAGGTGGGG	64
HDAC8	F: CGACGGAAATTTGAGCGTAT R: CCAACATCAGACACGTCACC	61	KMT1B	F: CGCTTTGCATCTTTCGAACT R: ACGTGATTCCCTTGTTGTCA	60	PHC1	F: AGCTCAGGGGGCAGCTCTCG R: ATTGTGGCCTGCTGGACGGC	60
HDAC9	F: CAGCAACGAAAGACACTCCA R: CAGAGGCAGTTTTTCGAAGG	60	KMT1C	F: AACATCGATCGCAACATCAC R: TAGAGCTGTAGCCGCACCTT	60	SIRT1	F: ACGCTGGAACAGGTTGCGGG R: AGCGGTTCATCAGCTGGGCA	60
HDAC10	F: ACAGAGGTGTCTGCGGTTGT R: GTGAAAGGTACTCGGGTGGA	60	KMT1D	F: GAGCGCTCTCATCGCCCTCGA R: GGGTCAATTCCGTCCACCAGCAT	60	DNMT1	F: GCTAAGCCTGAACCTTCACC R: TCCTTGATGGACTCATCCGA	65
HDAC11	F: CACCACTGCTCCAGCGACCG R: GCCTGATGGCCTGCTTGGCA	62	KMT2A	F: GAGGAAGACCTCCCACCTTC R: TTTGTACCCCCTTCCTTCCT	60	DNMT3A	F: TGATACGGCTGAGAAGAAAGC R: CTCCTCCACCTTCTGAGACT	61
KAT2A	F: AAGGGGAAGGAGCTGAAGGA R: GCTCAGTCATGGTCTTCAGGT	60	KMT2B	F: TCATCCCCTGAGAGCATCTT R: GGTATCTGGGAAGACTGGGA	60	DNMT3B	F: AAGCCCACTGGGATCGAG R: TCGAGTCTTGTTCTCGTATTTCC	60
KAT2B	F: GGAGCCACTTTAATGGGATG R: CTTTTCCACTCGGTTTCCAG	60	KMT2C	F: AGAGCCAGAAAGAAACCTCG R: TCCACTTCTGCTTCAGCATC	60	RING1	F: CTGAGCCGCCTGCACAACCA R: CCTTCTCCCTCCCCGGGCTC	63
KAT3A	F: CCTATGCTGCTCTCGGACTC R: CGTTCATCAGTGGGTTTGTG	61	KMT2D	F: TTGTGAACCCCGATGGTTTT R: AATGCGGATGGAACCAATGA	60	GAPDH	F: GGTCGGAGTCAACGGATTTG R: ATGAGCCCCAGCCTTCTCCAT	60
KAT3B	F: GGACAAAAAGGCAGTTCCTG R: TTGTGAGCATGCAAAAGGAG	60	KMT2E	F: AGACATCTCATGGGTTTGTGG R: GACGTTCGCCTCTGGTAAAT	60	RPII	F: CAGGAGTGGATCCTGGAGAC R: GGAGCCATCAAAGGAGATGA	60–66
KDM1	F: ATCTGCAGTCCAAAGGATGG R: GCCAACAATCACATCGTCAC	60	KMT2F	F: GGACACAAGCTTCTCCAGCA R: GCTGAAGATGCAGAGAAGTGG	60	GUS	F: GAAAATATGTGGTTGGAGAGCTCATT R: CCGAGTGAAGATCCCCTTTTTA	60
KDM2A	F: TCTTCCGCTACCTCAGCCGCA R: GGTCTTTCAGTCCTGGCAGCCT	61	KMT2G	F: ATCGACGCCACCAAGTGCGG R: GAGTCTGGTGCCGGGGCCTA	60	HPRT	F: GACCAGTCAACAGGGGACAT R: CTTGCGACCTTGACCATCTT	60
KDM3A	F: TGGTGTGTTGCGGGTAGAAGGC R: TCCAAGCAACCTGTCTGTGTGGC	60	KMT5A	F: CGCACCGACGGGGAGAACG R: CATGGCGCTCCGTACTGCGT	61	DC-SIGN	F: TCGAGGATACAAGAGCTTAGCA R: AAGGAGCCCAGCCAAGAG	60
KDM3B	F: CCGGCCACGCAGTGAGACAG R: GCCCCGGGCAGCATGTACCA	60	KMT5B	F: GCACACGGGGAAGGACACCC R: GCTCCTCGAAGGAAAGGCGCTAGT	60			

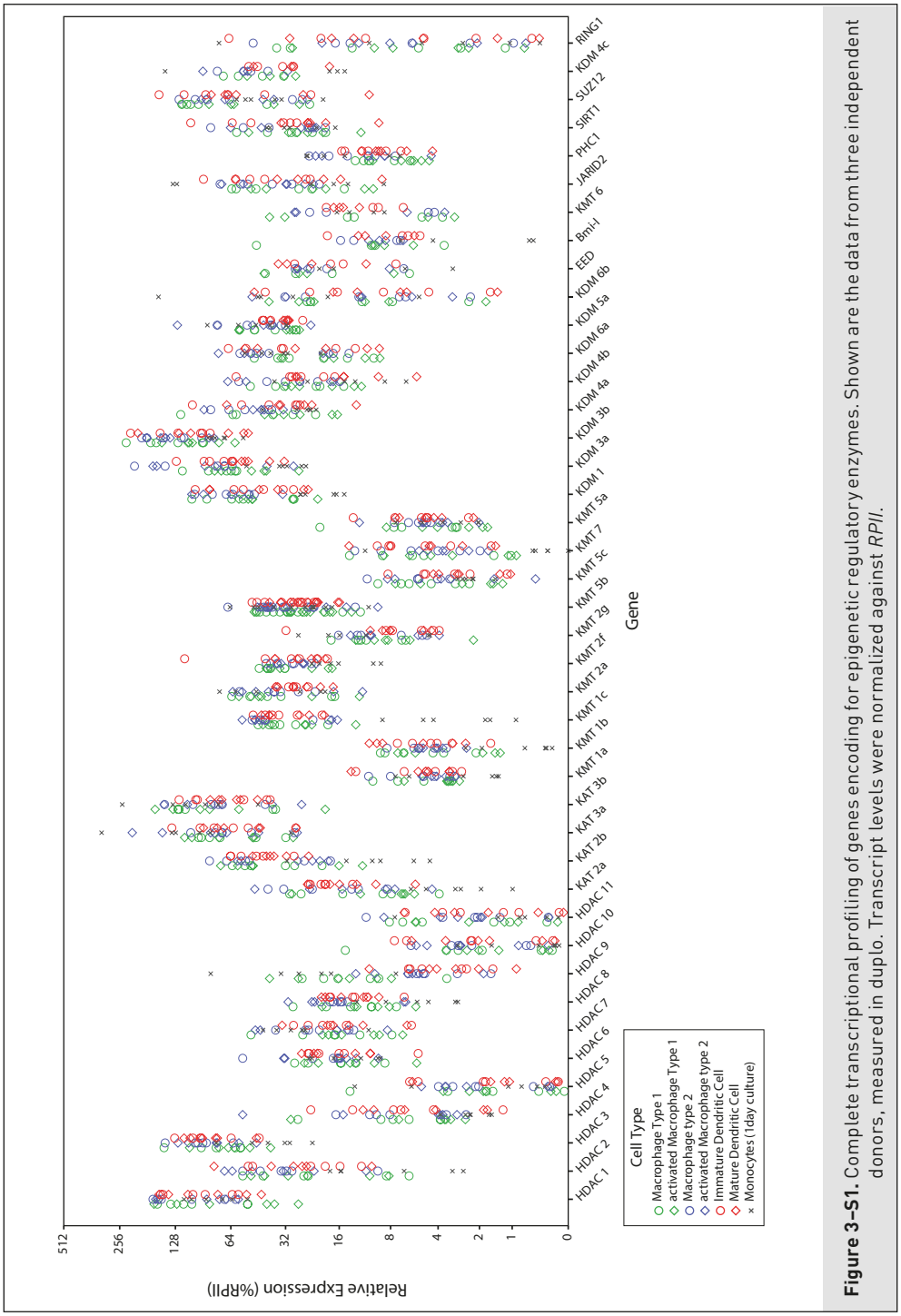

Figure 3–S1. Complete transcriptional profiling of genes encoding for epigenetic regulatory enzymes. Shown are the data from three independent donors, measured in duplo. Transcript levels were normalized against *RPII*.