Differential regulation of chemokine CCL5 expression in monocytes/macrophages and renal cells by Y-box protein-1

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The Y-box protein-1 (YB-1) belongs to the family of cold shock proteins that have pleiotropic functions such as gene transcription, RNA splicing, and mRNA translation. YB-1 has a critical role in atherogenesis due to its regulatory effects on chemokine CCL5 (RANTES) gene transcription in vascular smooth muscle cells. Since CCL5 is a key mediator of kidney transplant rejection, we determined whether YB-1 is involved in allograft rejection by manipulating its expression. In human kidney biopsies, YB-1 transcripts were amplified 17-fold in acute and 21-fold in chronic allograft rejection with a close correlation between CCL5 and YB-1 mRNA expression in both conditions. Among three possible YB-1 binding sites in the CCL5 promoter, a critical element was mapped at −28/−10 bps. This site allowed up-regulation of CCL5 transcription in monocytic THP-1 and HUT78 T-cells and in human primary monocytes; however, it repressed transcription in differentiated macrophages. Conversely, YB-1 knockdown led to decreased CCL5 transcription and secretion in monocytic cells. We show that YB-1 is a cell-type specific regulator of CCL5 expression in infiltrating T-cells and monocytes/macrophages and acts as an adaptive controller of inflammation during kidney allograft rejection.

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Immunosuppressive regimens for renal transplant patients have improved over the last decades. Nevertheless, the occurrences of one or more episodes of acute allograft rejection are highly predictive of a shortened transplant survival.[1,2] A key event for acute as well as chronic transplant rejection is in both cases the recruitment of inflammatory cells from the circulation into the renal allograft. Such alterations may be classified according to the Collaborative Clinical Trials in Transplantation (CCTT) criteria for diagnosis of renal allograft pathology.[3] These encompass, among others, alloantigen activated T and monocytic cells.[4,5] Experimental data revealed that the leukocyte recruitment into transplants is mediated by chemokines and chemokine receptors.[6,7] Blockade of chemokine receptors or neutralization of single chemokines reduces the degree and in some instances even prevents the occurrence of (acute) transplant rejection in experimental models.[8–11] Although there is substantial knowledge on underlying immunologic mechanisms that trigger and orchestrate organ loss in acute as well as chronic rejection, it is still far from complete. A pivotal role in mediating recruitment of immune cells has been attributed to the C-C chemokine 'regulated upon activation, normal T cell expressed and secreted' (RANTES/CCL5) that is shown to be upregulated in acute transplant rejection.[12] In a rat kidney transplant model, the application of Met-RANTES, a specific CCL5 antagonist,[13] ameliorated acute and chronic allograft rejection.[14] In rat tissue and human biopsies with transplant rejection CCL5 mRNA expression was localized to tubular epithelial, endothelial cells, and infiltrating mononuclear cells by *in situ* hybridization and real-time PCR.[15]

Bone marrow-borne monocytes travel through peripheral blood vessels along gradients of chemoattractants. Once they reach a target tissue, a local gradient of chemokines permit them to infiltrate and propagate a process denoted 'differentiation' into macrophages.[16,17] Cell recruitment as well as

the monocytic cell differentiation process are triggered and orchestrated by several factors, such as interferon-γ, CCL5, cell-surface receptor CCR2, and transcription factor Foxp1.[16,18]

The cold shock proteins are highly conserved in evolution and are involved in a wide variety of cellular functions, such as regulation of DNA transcription,[19,20] RNA splicing,[21] and translational control of protein synthesis.[22] The prototypic member, denoted Y-box (YB) protein-1, has been shown to associate with DNA elements encompassing inverted CAATT-box sequences (Y boxes). YB-1 may act as a cell-type-specific regulator of gene transcription,[23] with preferential binding to single-stranded DNA.[20] Recent evidence from our laboratory hints at a pivotal regulatory role for YB-1 in *CCL5* gene transcription and atherogenesis.[24] These results prompted us to hypothesize that YB-1 is also involved in kidney transplant rejection by triggering CCL5 expression.

RESULTS

Under circumstances of renal allograft rejection YB-1 transcript numbers are upregulated in human kidneys

Given our hypothesis that YB-1 is relevant for CCL5 expression in kidney transplant rejection, we first quantified YB-1 transcript numbers in biopsy samples obtained from patients classified into five distinct groups by immunohistochemistry. In kidney tissue obtained from human living donors without abnormalities ($n=8$), cadaveric donors ($n=8$) and patients with minimal change glomerulonephritis ($n=8$), average transcript numbers were equal and set as 1 (Figure 1a). Compared to these groups transcript numbers were markedly upregulated in kidney tissue with CCTT I ($n=7$; 17.4 ± 20.8) and CCTT II transplant rejection ($n=8$; 20.7 ± 23.8), as depicted in Figure 1a. The intrarenal expression levels of CCL5 in biopsy samples are shown in Figure 1b.

YB-1 and CCL5 transcript numbers correlate in acute and chronic transplant rejection

In the same human kidney biopsies quantification of CCL5 transcript numbers was performed and the correlation coefficients were calculated. A highly significant correlation between the expression of both transcripts was present in acute ($R^2=0.875$) and chronic rejection ($R^2=0.847$; Figure 1c). When outliers were removed from statistical analyses, the correlation coefficient in acute transplant rejection even slightly increased ($R^2=0.895$), whereas the coefficient in chronic rejection was lower ($R^2=0.626$) however still statistically significant.

Cell-type-specific effects of YB-1 on the CCL5 promoter activity

As YB-1 and CCL5 transcript numbers correlate under conditions of renal allograft rejection and previous studies demonstrated a cell-type-specific regulation of target genes

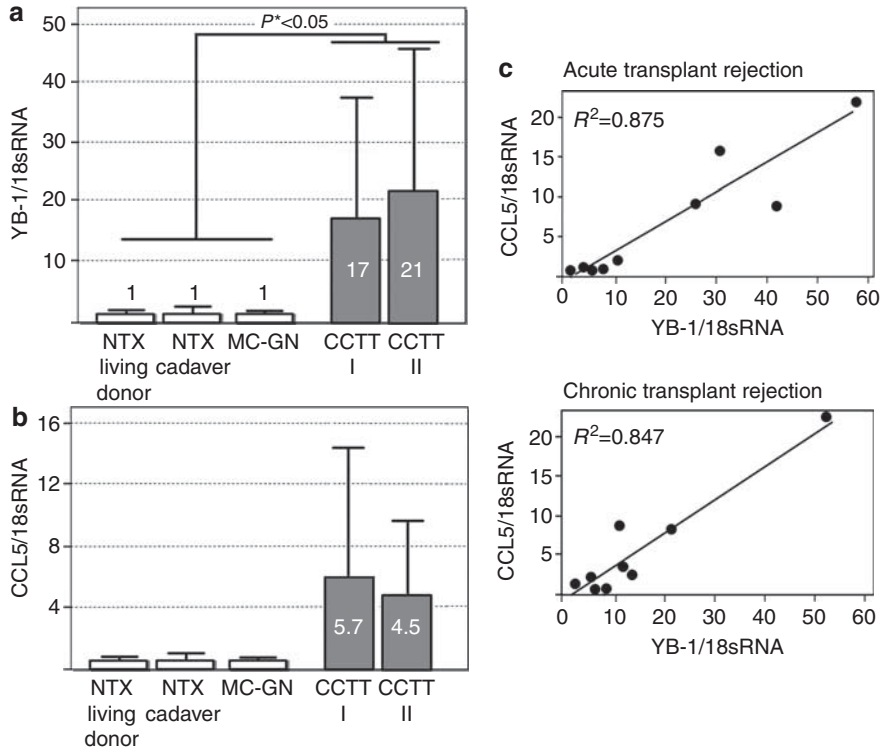

Figure 1 | YB-1 and CCL5 expression correlate in renal allograft rejection. Quantification of YB-1 (**a**) and CCL5 (**b**) transcript numbers was performed in biopsy samples from human kidney transplants (NTX living donor, cadaver, CCTTs I and II) and patients with minimal changes glomerulonephritis (MC-GN) collected in the renal cDNA bank in Munich. Biopsies were scored according to the CCTT criteria. YB-1 and CCL5 mRNA abundance was normalized to 18S rRNA content. Data are expressed as mean values ± s.d. (**c**) Correlation of YB-1 and CCL5 expression in human kidney biopsies with acute and chronic transplant rejection.

by YB-1,[23,25] we characterized the *trans*-regulatory effect that YB-1 has on the *CCL5* promoter in the context of different cell lines. The YB-1 content of rat mesangial cells (MCs) was manipulated by ectopic overexpression and knockdown using a Tet-off system and small-interfering RNA.[20] The corresponding transcript numbers of YB-1 and CCL5 revealed a direct, linear correlation over a broad range of transcript numbers (Figure 2a). This result indicated a direct influence of YB-1 protein levels on *CCL5* gene transcription in MCs. In the human HUT78 T-cell line, ectopic expression of YB-1 elicited an up to fourfold increase of promoter activity (Figure 2b, left panel) that was paralleled by upregulation of CCL5 transcript numbers (middle panel) and a fivefold increase in secreted protein (right panel). Given that the CCL5 mRNA levels were only 40% higher the data furthermore suggest that posttranslational effects on CCL5 synthesis take place. Notably, depletion of endogenous YB-1 content by introducing short-hairpin RNA led to lower CCL5 transcript numbers (middle panel, white columns).

A similar response on *CCL5* gene transcription and protein synthesis was observed in monocytic THP-1 cells (Figure 3a). Knockdown of endogenous YB-1 resulted in a significantly lowered CCL5 secretion rate (Figure 3a, right panel, white column).

Contrary to these findings, overexpression of YB-1 in a mouse macrophage cell line, RAW264.7, resulted in suppression of *CCL5* promoter activity (Figure 3b, left panel). This negative effect of YB-1 on CCL5 expression was also seen at the protein level with a reduction of secreted CCL5 protein

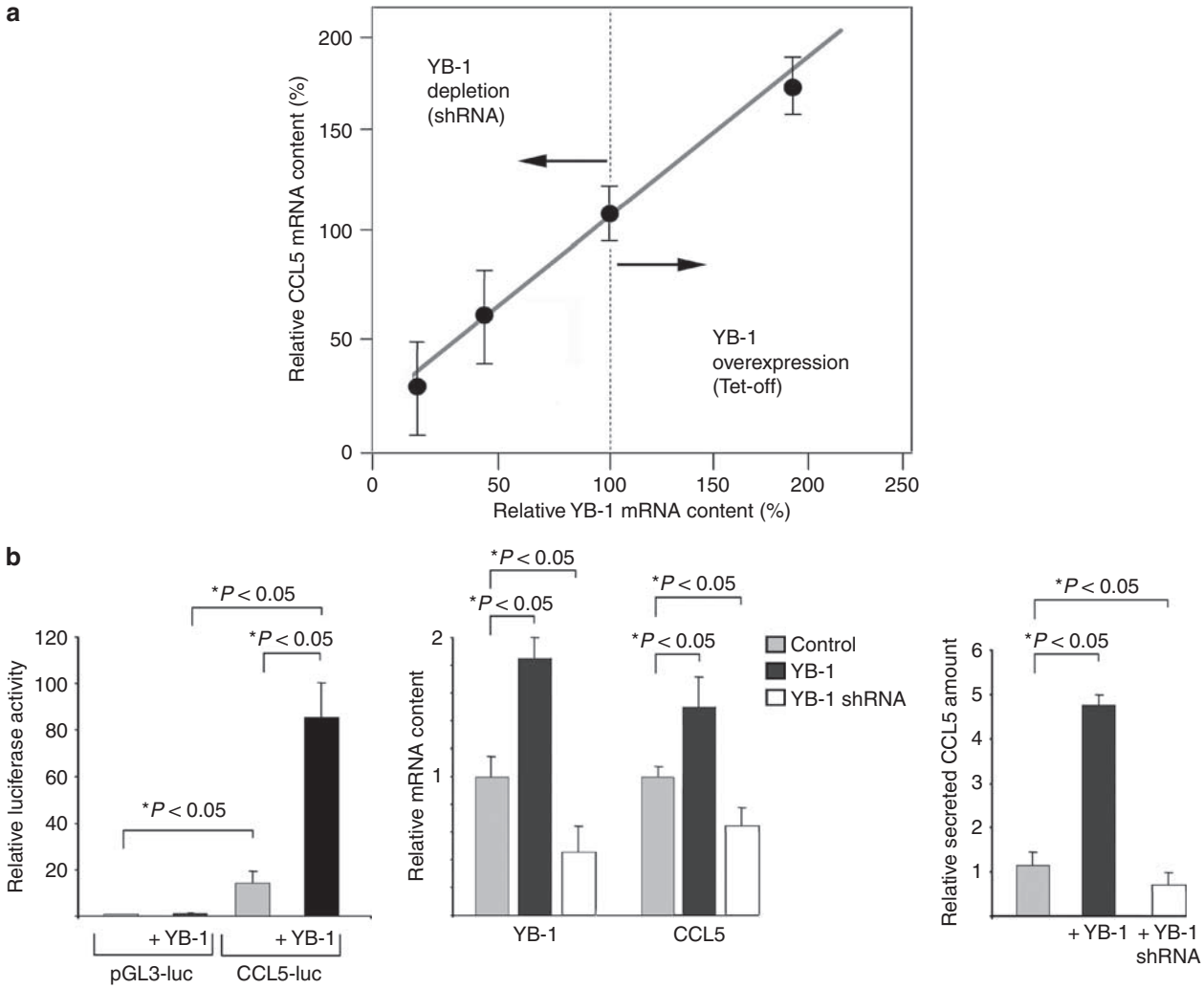

Figure 2 | *Trans*-regulation of the CCL5 promoter by YB-1 in rat mesangial cells and and T cells. (**a**) The effects of modified YB-1 content on CCL5 expression were determined at transcript level (RT-qPCR) by gradual depletion (shRNA) or overexpression (Tet-off system) of YB-1 in rat mesangial cells. All experiments were done at least in triplicate and repeated three times. Data are expressed as mean values ± s.d. (**b**) CCL5 promoter activity (left panel), mRNA (middle panel) and protein synthesis (right panel) were determined in human T lymphocytes (HUT78). A plasmid harboring the proximal 1014 bp of the 5′ regulatory sequence in context of a luciferase reporter gene was introduced to assess for YB-1 *trans*-regulation. Normalized luciferase activities were determined in three independent experiments, each performed in triplicate. Data are expressed as mean values ± s.d. Secreted CCL5 protein in the cell supernatant was quantified using ELISA technology and normalized to control transfection. The cellular YB-1 content was manipulated by introducing pSG5-YB-1 expression plasmid or achieving a knockdown with shYB-1.

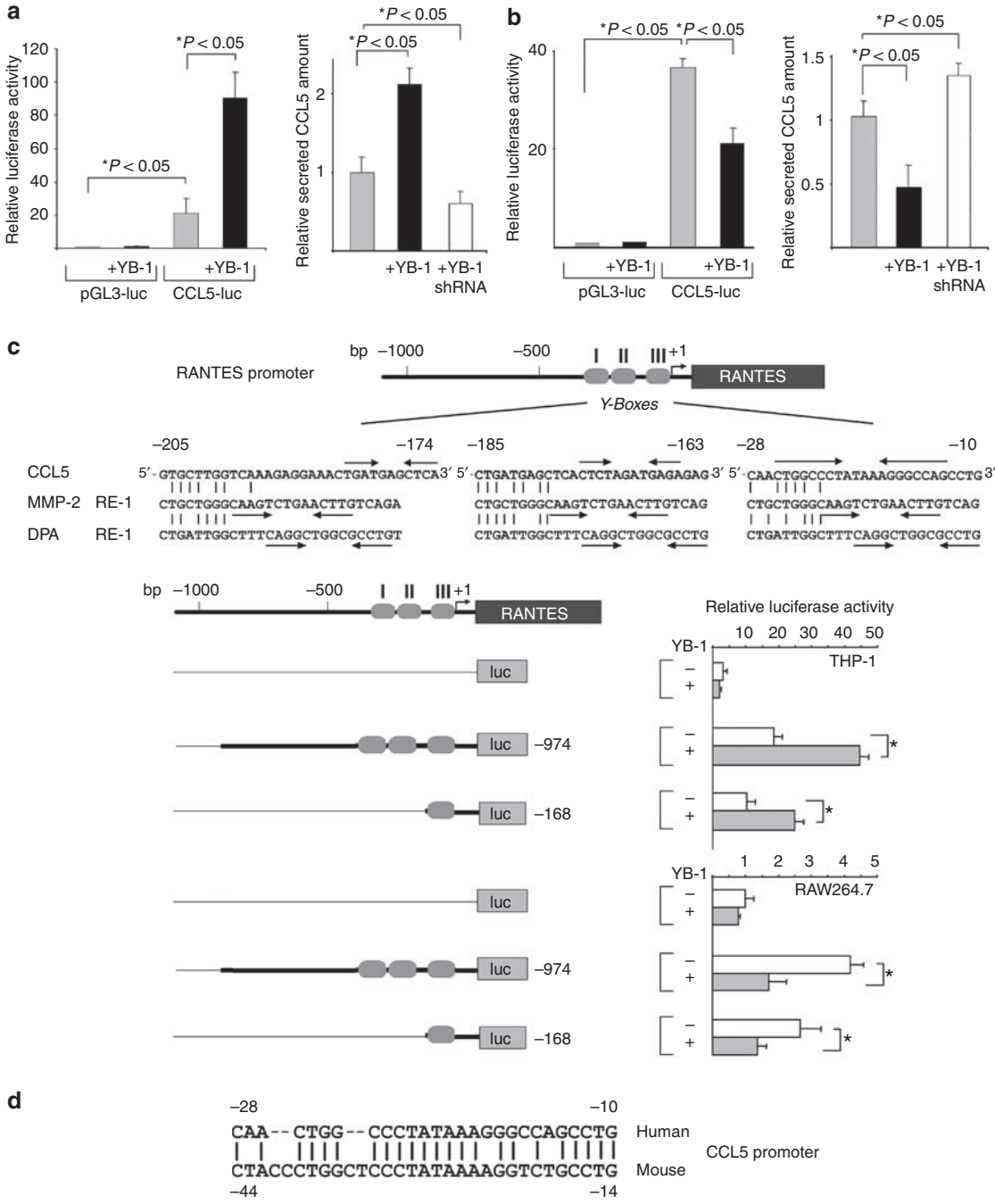

Figure 3 | *Trans*-regulation of the CCL5 promoter by YB-1 in monocytes/macrophages. *CCL5* promoter activity (left panel) and protein synthesis (right panel) were determined in (**a**) monocytes (THP-1) and (**b**) macrophages (RAW 264.7). Normalized luciferase activities were determined in three independent experiments, each performed in triplicate. Data are expressed as mean values ± s.d. (**c**) Inspection of the proximal human *CCL5* promoter and comparison with known YB-1-binding motifs of the *matrix metalloproteinase-2* (MMP-2) and *DNA polymerase-α* (DPA) genes revealed three putative YB-1-binding sites harboring homologies and an inverted repeat motif (indicated by arrows). Reporter construct analysis was performed using two plasmids harboring the proximal 1014 or 168 bp of the 5′ regulatory sequence of the *CCL5* promoter. The solid lines on the left represent the DNA content for each of the CCL5 reporter constructs as indicated by number. The putative YB-1-binding sites present in each construct are represented by small boxes. Relative promoter activity for each vector in THP-1 and RAW264.7 cells is indicated graphically as an average on the right side of the diagram. All experiments were done in triplicate. Data are expressed as mean values ± s.d. (**d**). Sequence homology between the human and murine *CCL5* promoter are shown for the proximal YB-1-binding site III (−28/−10 bp).

by 50%. A YB-1 knockdown resulted in marginally elevated CCL5 protein levels in the supernatant (right panel, white columns).

Our analyses for potential YB-1-binding sites within the proximal human *CCL5* promoter revealed three sequence elements that exhibit significant similarities with conserved

YB-1-binding motifs of the *metalloproteinase-2* and *DNA polymerase-α* genes. These are known to be directly *trans*-regulated by YB-1 binding (Figure 3c). A truncated *CCL5* promoter construct (−168/+1 bp), carrying solely the proximal YB-1-binding site III, was sufficient for YB-1 *trans*-regulation and a cell-type-specific effect of YB-1 in monocytes and macrophages was apparent (Figure 3c). The proximal putative YB-1-binding site is highly homologous between the human and rat *CCL5* genes, as depicted in Figure 3d.

Taken together, these results indicate a context-dependent effect of YB-1 on the *CCL5* promoter in T cells and monocytic cells versus differentiated macrophages. Next we wanted to assess whether the opposing effects were due to the differentiation process from monocytes to macrophages. To mimick the situation of infiltrating monocytes with ensuing differentiation to macrophages, we added phorbol 13-myristate 12-acetate (PMA) and followed over time the cell differentiation process.[26]

Influence of YB-1 on CCL5 expression during PMA-induced monocytic cell differentiation

Phorbol 13-myristate 12-acetate-dependent effects on monocytes include changes in cell shape (Figure 4a), endocytosis rates, and shedding of membrane proteins, all of which resemble a more macrophage-like state.

Incubation with PMA led to a more than threefold increase of *CCL5* promoter activity within 6 h, which tapered off in the subsequent observation period until 48 h (Figure 4b). There was a markedly increased CCL5 synthesis rate in PMA-treated cells, with CCL5 protein content in the conditioned cell culture medium reaching more than 10-fold elevated levels after 48 h compared to control cells (Figure 4c).

Overexpression of YB-1 in nonstimulated THP-1 cells led to significantly increased *CCL5* promoter activity, as reported above (compare Figure 3a). Subsequent to PMA addition, within 6 h, the *trans*-stimulatory effect of ectopic YB-1 expression was no longer present for the ensuing time points (Figure 4d), nor did the CCL5 secretion change significantly under these conditions (Figure 4f). The little impact of YB-1 on CCL5 expression during PMA stimulation might be explained by the degradation of the protein (as can be seen in Figure 5). Depletion of endogenous YB-1 levels by introducing short-hairpin RNA had the expected inhibitory effect on the *CCL5* promoter in cells that were not treated with PMA (Figure 4e). However, after PMA addition, the cells showed a significant induction of promoter activity by YB-1 knock-

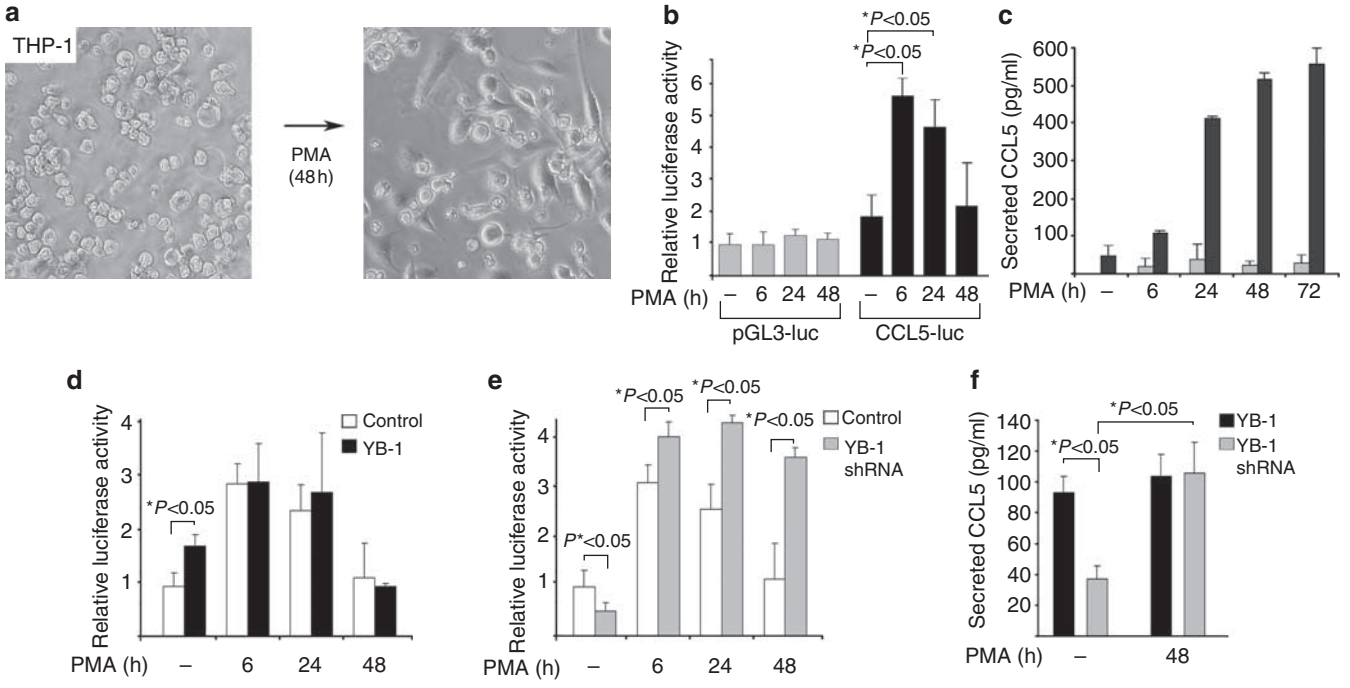

Figure 4 | Phorbol ester-dependent THP-1 cell differentiation and changes of *CCL5* promoter activity. (**a**) The cell morphology of THP-1 cells, untreated or exposed to 100 nM PMA for 48 h, changes from nonadherent globular morphology to adherent cells with cytoplasmic extensions. (**b**) The activity of the *CCL5* promoter (proximal 1014 bp) was determined following PMA stimulation in THP-1 monocytes. (**c**) Cell culture supernatants of THP-1 cells were collected after 72 h of incubation with PMA addition for the indicated time periods (black bars). CCL5 protein synthesis and secretion was assessed by ELISA. In the absence of PMA only minor amounts of CCL5 are accumulated during the time course (gray bars). (**d**, **e**) The *CCL5* promoter activity was determined in THP-1 cells with increased YB-1 content (**d**) or depletion of YB-1 (**e**) over time following incubation with phorbol ester (PMA). (**f**) Influence of YB-1 overexpression (black bars) or knockdown (gray bars) on PMA-stimulated CCL5 secretion in THP-1 cells. Representative results of three independent experiments performed as triplicates are shown. Data are expressed as mean values ± s.d.

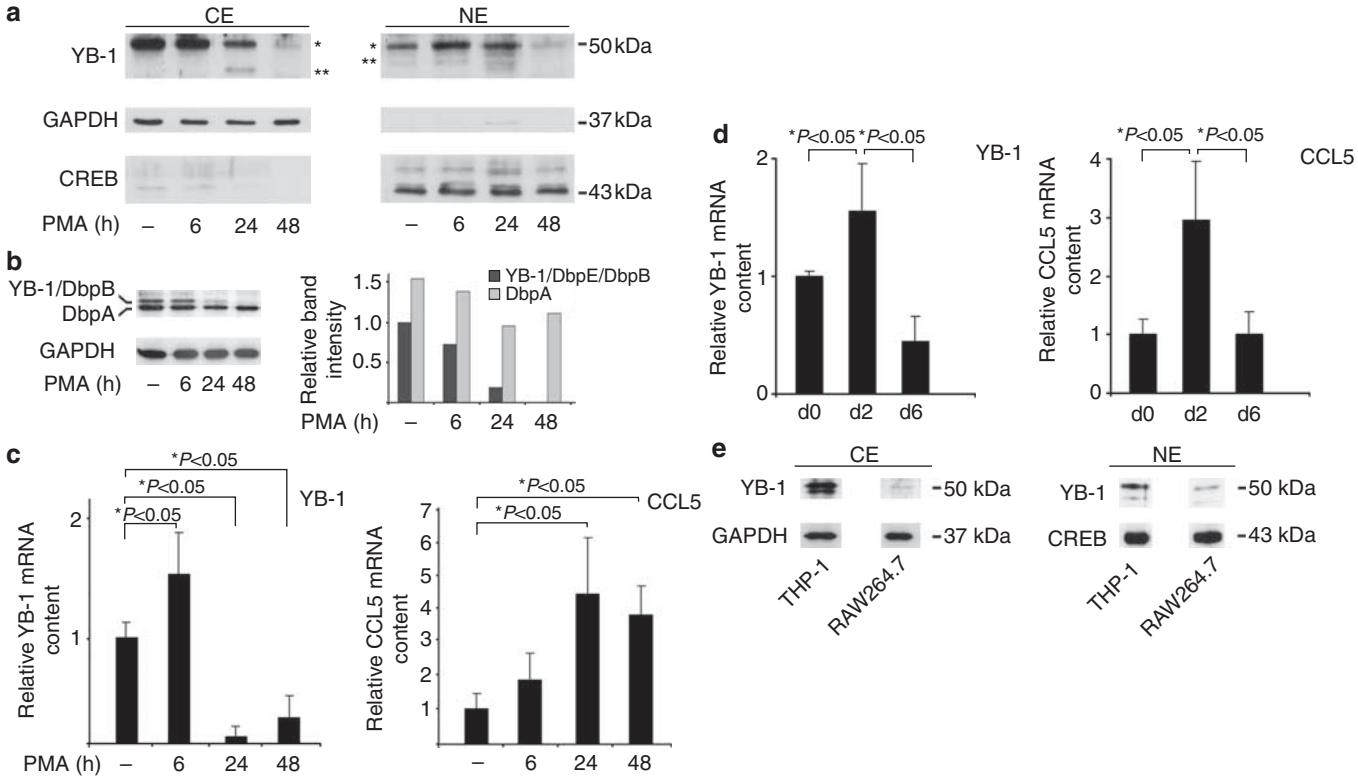

Figure 5 | Transient nuclear shuttling and subsequent degradation/downregulation of YB-1 expression in differentiating THP-1 cells. (**a**) Cytoplasmic (CE) and nuclear proteins (NE) of THP-1 cells were analyzed for YB-1 content in the course of PMA-dependent monocyte differentiation using a YB-1-specific polyclonal antibody. Separation of cell compartments and equal protein loading were ensured by determining GAPDH and CREB levels. (**b**) Protein content of cold shock proteins YB-1 and DbpA during PMA-induced monocyte differentiation. Band intensities were quantified and are shown as bar graph. (**c**) YB-1 and CCL5 transcript numbers were quantified by quantitative TaqMan analyses at different time points following PMA incubation of THP-1 cells and (**d**) during serum-induced differentiation of primary human monocytes on days 2 and 6. The data represent three independent experiments performed as triplicates. Primary cultures from four different blood donors produced similar results. (**e**) A comparison of YB-1 protein content in monocytic THP-1 cells and RAW264.7 macrophages was performed. Equal protein loading was ascertained by testing for GAPDH and CREB protein content.

down that was observed after 6, 24 and 48 h (Figure 4e), with a threefold induction of the *CCL5* promoter activity and subsequent protein secretion (Figure 4f) after 48 h. Thus, in accord with the results obtained with the macrophage cell line RAW264.7, YB-1 has a suppressive effect on the *CCL5* promoter in 'differentiated' THP-1 cells. The suppressive effect of YB-1 in differentiated cells seems to be maximal, as further overexpression of YB-1 was not able to suppress *CCL5* gene transcription markedly. On the other hand, YB-1 knockdown was very effective in increasing CCL5 transcription rates.

Endogenous cellular YB-1 protein levels are downregulated during PMA-induced monocyte cell differentiation

As YB-1 overexpression influenced the *CCL5* promoter in monocytes and macrophages in opposing directions, we were interested to determine whether endogenous YB-1 protein levels change in the differentiation process. By immunoblotting using a YB-1-specific antibody a transient increase of nuclear YB-1 was detected following PMA incubation for 6 h. Subsequently, YB-1 protein content in the cytoplasm as well as in the nucleus was lower, starting around 24 h (Figure 5a). After 48 h YB-1 protein was only detectable in both compartments following extended exposure of the immunoblots. When the same blot was probed with an antibody specific for DNA-binding protein-A, another member of the cold shock protein family, it became apparent that the cellular YB-1 protein content was specifically downregulated in the course of monocytic cell differentiation (Figure 5b, bar diagram).

Following PMA incubation for 6 h, YB-1 transcript numbers were upregulated (Figure 5c, left). Thereafter, a biphasic response was apparent with downregulation within 24 h and minor upregulation after 48 h. At the same time points, CCL5 transcript numbers gradually rose over the time period of 48 h (Figure 5c, right). These results suggest that not only protein degradation, for example, through N-terminal protein cleavage indicated by ** in Figure 5a, may be of relevance for the downregulated cellular YB-1 content in 'differentiated' THP-1 cells but may also be that *YB-1* gene transcription rates and/or the mRNA half-life is markedly decreased.

To confirm these findings in primary cells, human monocytes were harvested from human volunteers and

analyzed. YB-1 and CCL5 mRNA levels were determined in these nondifferentiated cells and compared to cells incubated for 2 or 6 days with human serum to induce differentiation to macrophage-like cells. In accordance to the results in THP-1 cells, a transient increase and subsequent downregulation of YB-1 was demonstrated during differentiation of primary cells (Figure 5d, left). The CCL5 transcript numbers were transiently upregulated threefold at day 2 of serum-induced differentiation, whereas after 6 days of serum incubation CCL5 expression was lowered to values of undifferentiated cells.

To directly compare the endogenous YB-1 protein levels in monocytic THP-1 cells and the macrophage cell line RAW264.7, cytoplasmic and nuclear protein extracts were compared by immunoblotting. The results indicate an approximately fivefold lower YB-1 content in the macrophage cell line compared to monocytic cells (Figure 5e).

Mapping of YB-1-binding sites within the human CCL5 promoter

Previous studies performed with human smooth muscle cells demonstrated the physical and functional interaction of YB-1 with the distal-binding site (denoted I in the schematic overview Figure 4c) located at −205/−174 bp of the human *CCL5* promoter.[24] In the following, we evaluated the two more proximal-binding sites for YB-1 binding.

Under the chosen conditions, YB-1 binding was not present to site II (−185/−163), neither the sense nor the

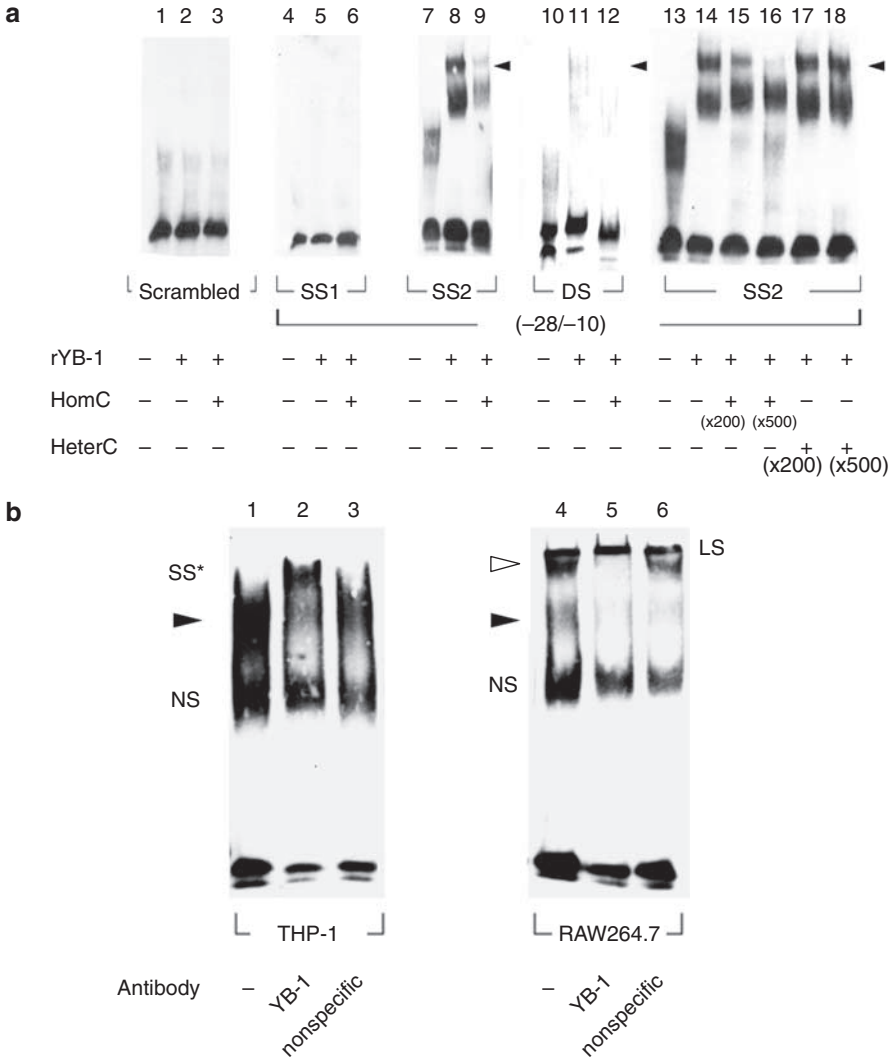

Figure 6 | YB-1 binds to a sequence motif within the proximal CCL5 promoter. (**a**) DNA-binding analyses of the −28/−10 bp element was performed with recombinant YB-1 protein. Scrambled, sense (SS1), anti-sense (SS2), and double-stranded (DS) probes were tested for binding with recombinant YB-1 protein. HomC, homologous competitor DNA; HeterC, heterologous competitor. (**b**) Nuclear protein extracts from monocytic THP-1 cells and RAW264.7 cells were prepared and complex formation with the anti-sense (SS2, −28/−10 bp) probe was assessed. A strong nucleoprotein complex appeared with THP-1 protein extract (filled arrowhead), whereas it was markedly weaker with RAW264.7 cell extracts. Participation of YB-1 in the complex formation was confirmed by supershift analyses (SS*) using a YB-1-specific, but not nonspecific rabbit IgG antibody. With RAW264.7 cell extracts an additional, low-mobility complex is detected (empty arrowhead). This complex is absent in the presence of YB-1-specific antibody. NS, nonspecific complexes; LS, loading slot.

anti-sense strands, using recombinant protein or nuclear extracts from THP-1 cells (data not shown).

In contrast to the above results, two distinct complexes were detected with the anti-sense oligonucleotide (SS2) containing the proximal site (III, −28/−10 bp) together with recombinant YB-1 (Figure 6a, lanes 8 and 14). As described for numerous other target genes,[20,27] a very weak interaction was seen with the double-stranded oligonucleotide (lane 11), whereas no complexes appeared with the sense strand (SS1; lane 5) or with a scrambled control oligonucleotide (lane 2).

To further confirm specificity of the protein:DNA interaction, a titration experiment with inclusion of homologous and heterologous competitors DNA was performed. As expected, a concentration-dependent competition was observed with homologous competitor DNA, whereas even in the presence of high concentrations of scrambled oligonucleotide no change of complex formation was seen (Figure 6a, lanes 15–18).

Differences of DNA:protein complexes at the site III of the *CCL5* promoter in monocytes and macrophages

As YB-1 exerts opposing effects on the *CCL5* promoter in monocytes and macrophages, we compared the binding to the anti-sense strand encoding for site III with nuclear protein extracts from THP-1 and RAW264.7 cells. A strong complex was detected with THP-1 nuclear protein (Figure 6b, lane 1, filled arrowhead) that corresponds in its mobility to the one observed with recombinant YB-1 protein (Figure 6a, lanes 8 and 14). Inclusion of YB-1-specific antibody resulted in a supershift (lane 2, SS*), whereas an irrelevant antibody (lane 3) had no effect. These results emphasize the binding specificity of YB-1 with this promoter region.

In contrast to THP-1 cells, the DNA:protein complex was only weak with nuclear proteins from RAW264.7 cells (Figure 6b, lane 4, filled arrowhead). A distinct low mobility band was present (open arrowhead), that just entered the gel and separated from the loading slot (LS). This complex also includes YB-1 protein, as its formation was abrogated in the presence of a YB-1 specific (lane 5), however not a nonspecific antibody (lane 6).

DISCUSSION

Chemokines, especially CC-chemokines such as CCL5, MIP-1α, MIP-1β, and MCP-1, as well as CXC-chemokines such as IL-8 and IP-10 are known to contribute to renal transplant rejection.[28–30] The kinetics and molecular control mechanisms involved in CCL5 transcription/expression vary among cell types[31,32] and the blockade of chemokine receptors by Met-CCL5 diminishes early infiltration and activation of mononuclear cells into grafts.[13] Thus, understanding mechanisms of *CCL5* gene regulation may provide important insights that can be utilized to prevent or ameliorate transplant rejection.

There are a few reports on the regulative role of YB-1 in inflammatory diseases, such as allergic asthma, mesangioproliferative glomerulonephritis, and thrombin-dependent endothelial cell activation.[33–35] Otherwise the focus of most YB-1 studies has been on its role in cancerogenesis[36,37] as well as tumor progression.[38,39] YB-1 has been originally cloned with a regulatory element of the *HLA-DR* gene promoter[40] and further investigations demonstrated a repressive effect of YB-1 on interferon-γ-dependent major histocompatibility protein expression.[41] Furthermore, YB-1 regulates the expression of granulocyte-macrophage colony-stimulating factor[25] and markedly prolongs the half-life of IL-2 mRNA in T cells and thereby its translation efficacy.[22] Similar posttranscriptional regulation events have also been described for CCL5 mRNA, for example by RFLAT-1, regulating CCL5 synthesis in T lymphocytes in response to stress situations.[42] These finding indicate that YB-1 may also be relevant in the immunologic response of allotransplant rejection. Analyses from our group recently demonstrated that YB-1 serves as a transcriptional regulator of CCL5 expression in human arterial smooth muscle cells, thereby contributing to neointimal and medial hyperplasia in an experimental model for accelerated atherosclerosis in *apoE*−/− mice.[24] As CCL5 is prominently expressed during cell-mediated renal allograft rejection,[28] we extended our study to human kidney transplants and investigated the impact of YB-1 on CCL5 expression in MCs and immune cells typically present in cellular infiltrates during transplant rejection.

Intrinsic kidney cells, such as MCs, are known to fulfill numerous functions otherwise observed with immune cells, for example, phagocytosis and MHC classes I and II expression.[43,44] Furthermore, they quickly upregulate CCL5 transcript numbers in response to TNFα stimulation, a prototypic proinflammatory cytokine.[31] We found a close linear relationship between YB-1 and CCL5 expression in MCs, as well as in T lymphocytes. A 21-fold induction of YB-1 transcript numbers was apparent with allograft rejection. In comparison, in control patients without increased CCL5 expression, for example, living donor allografts without rejection and in kidneys with diagnosis of minimal change disease, YB-1 transcript numbers were largely unchanged. Therefore, YB-1 appears to be an important player in modulating gene expression in this inflammatory event. A highly significant correlation between the expression of YB-1 and CCL5 in acute and chronic rejection was determined, rendering a direct influence of YB-1 on CCL5 expression during renal transplant rejection very likely. By *in vitro* analyses a proximal-binding motif within the *CCL5* gene was mapped as binding site for endogenous and recombinant YB-1. Mutations of YB-1-binding sites en bloc or with dual or single nucleotide substitutions have been performed previously by our group for the *collagen type I* gene, providing ambiguous results.[45] These findings were in accord with the notion that this transcription factor has no core-binding motif and the reported inverted CCAAT box (denoted Y-box) is not an absolute requirement and unsufficient for binding. YB-1 interacts with extended regions of DNA, therefore mutation analyses will likely not

solve the question whether this promoter region is required for YB-1 regulation.

Notably, not all patients with transplant rejection exhibited markedly elevated YB-1 and CCL5 transcript numbers, indicating that other mechanisms for rejection may also exist. Currently, we perform gene expression profiling to determine whether these groups may be distinguished. In accordance with these findings, the delivery of chemokine receptor antagonist Met-RANTES did not completely prevent transplant rejection in a renal transplant model.[13]

Infiltration of renal allografts by leukocytes is a hallmark of acute transplant rejection. Peripheral blood monocytes and T lymphocytes recruited to sites of inflammation adhere to endothelial cells and transmigrate through vessel walls. Leukocyte infiltration is accompanied by alteration of the cellular phenotype and physiologic activities. To monitor the YB-1 and CCL5 expression levels in this process, we used the THP-1 monocytic cell line that displays macrophage-like differentiation in response to phorbol esters.[26] The expression patterns of both proteins, CCL5 and YB-1, were temporally and differentially regulated during the process. PMA stimulation induced YB-1 expression and nuclear localization early-on (6 h), thereafter the cellular protein content decreased over time during cell maturation, along with a declined *CCL5* promoter activity.

In human renal allografts, CCL5 receptors on infiltrating interstitial macrophages have been reported to be lost, most likely due to downregulation after transmigration.[46] Several transcription factor are known to control macrophage development and directly control the acquisition of a mature monocyte phenotype, for example, by induction of macrophage-specific genes, as do PU.1 and AML1.[47] Other transcription factors are involved in the maintenance of a stem cell population, such as GATA-2[48] and are repressed at early stages of myelopoiesis. Factors thought to orchestrate the intermediate state of myelopoiesis, such as Foxp1[18] or c-Myc,[49] are highly expressed during the first stages of differentiation and repressed before the end of maturation. Notably, c-Myc and YB-1 are known to promote cell proliferation, for example, by stimulating cyclin A and B1 expression.[50,51]

From studies with the *gelatinase A*[52] and *granulocyte-macrophage colony-stimulating factor*[25] genes, it is known that YB-1 may act as transcriptional activator and repressor of the same gene, depending on the cellular context. For the first time, we could demonstrate that the *CCL5* gene is regulated in a cell-type-specific manner by YB-1 and that this effect depends on the cellular differentiation status. Furthermore, the appearance of high mobility complexes in DNA-binding studies with the site III-binding motif of the *CCL5* promoter (−28/−10 bp) indicates that partnering with other transcription factors/cofactors occurs. This likely mediates the repressive effect on gene transcription and to identify the components of this complex is the focus of ongoing research. Thus, YB-1 has the potential to initiate and later on to abate the inflammation process, as depicted in the model of Figure 7. As early infiltration by monocytes is a poor prognostic sign for allograft survival and given our identification of YB-1 as a key regulator of CCL5 expression in nondifferentiated monocytes and T cells, YB-1 may be useful as early target of incipient transplant rejection.

MATERIALS AND METHODS

mRNA expression levels in human renal biopsies and cultured cells

Human renal biopsies were collected in a multicenter study (the European Renal cDNA Bank/ERCB) after informed consent was obtained according to the guidelines of the respective local ethical committees (for ERCB members, see Appendix). Five groups of

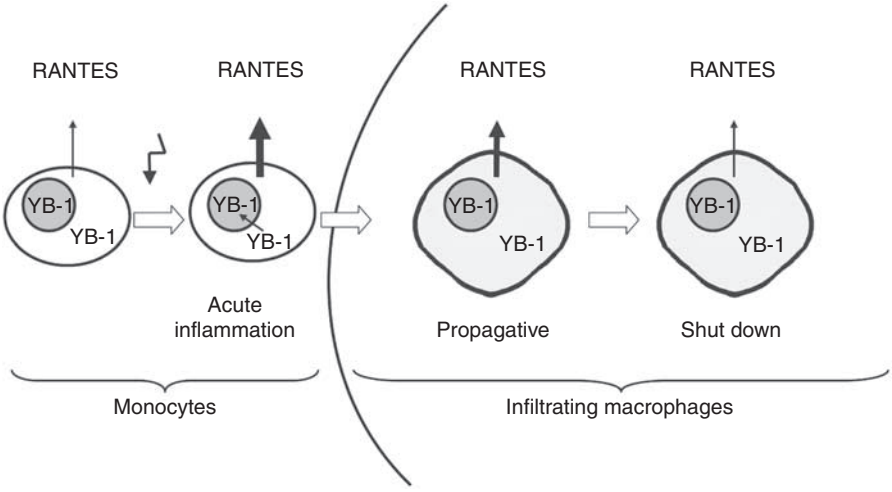

Figure 7 | Schematic model of YB-1 governing CCL5 synthesis during renal allograft rejection. Alloantigen-activated monocytes recruited to the allograft infiltrate and differentiate into macrophages. During the onset of acute inflammation, YB-1 shuttles into the nucleus and stimulates CCL5 expression that amplifies the inflammatory trigger by auto- and paracrine activity. During propagative inflammation and macrophage differentiation, YB-1 is downregulated and gains *trans*-repressive capacity on the *CCL5* promoter. After a time lag, the CCL5 expression is shut down.

renal biopsies available in ERCB were analyzed for relative expression levels of YB-1 (NM_004559) in kidney tissue obtained from human living donors without abnormalities ($n=8$), kidneys from cadaveric donors ($n=8$), patients with minimal change glomerulonephritis ($n=8$), acute ($n=7$) and chronic renal transplant rejection ($n=8$) using the CCTT modification for diagnosis of renal allograft pathology, CCTT I and CCTT II.[3] Amounts of YB-1 mRNA were determined by quantitative TaqMan analysis and normalized for the 18sRNA content, as described.[53] In samples obtained from patients with acute ($n=7$) or chronic transplant rejection ($n=8$), the relative content of CCL5 mRNA (AF043341) was quantified in the same specimen and correlation analysis was performed using Spearman's equation.

Student's *t*-test was used for comparisons between experimental groups. Significant differences were defined at $P<0.05$.

Plasmids, cell lines, and culture conditions

A full-length YB-1 expression plasmid (pSG5-YB-1) was kindly donated by J. Ting (Lineberger Comprehensive Center, University of North Carolina, North Carolina, USA),[27] plasmids encoding for CCL5 promoter fused to the luciferase gene extended up to −974 bp. CCL5 promoter deletion construct (−168/+1) has been described.[31,54] Knockdown of endogenous YB-1 by small-interfering RNA was performed with pSuper vector harboring the sequence 5′-GGTCATCGCAACGAAGGTTTT-3′ (OligoEngine, Seattle, WA, USA) as a tail-to-tail tandem repeat of base pair 285–305 of the human YB-1-coding sequence as described before.[33] Rat MCs were established and characterized as previously described.[23] MC, human HUT78 T-cell line, THP-1 (human monocytic cell line), and RAW264.7 (mouse macrophage cell line) were grown in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamin, 100 μg/ml streptomycin and 100 U/ml penicillin at 37 °C in humidified 5% CO_2 in air. Cell culture materials were purchased from Gibco BRL (Gaithersburg, MD, USA).

Transient transfections, monocyte differentiation, and luciferase measurements

Rat MC and mouse RAW 264.7 cells were transiently transfected with lipid-based transfection reagent Fugene (Roche, Mannheim, Germany) according to the manufacturer's instruction. Human THP-1 and HUT78 T cells were transfected by means of electroporation. Briefly, nonadherent cells were pelleted and resuspendend in electroporation medium supplemented with 20% fetal calf serum at 2×10^7 cells per ml. Cells (250 μl; 5×10^6 cells) were added to electroporation cuvettes (0.4 cm gap, Bio-Rad, Hercules, CA, USA) together with a total amount of 20 μg plasmid DNA in various combinations as outlined (pSG5, pSG5-YB-1, pGL3, CCL5-promoter-pGL3, YB-1-short-hairpin RNA; β-GAL). The mixture was incubated for 5 min (THP-1) or 10 min (HUT78) on ice and electroporated at 250 V/975 μF (THP-1) or 200 V/1200 μF (HUT78) in a Gene Pulser II electroporation system (Bio-Rad). Cells were incubated another 5 min on ice before they were resuspended in 4 ml of RPMI-1640 media supplemented with 20% fetal calf serum and transferred to six-well tissue culture plates, and incubated at 37 °C and 5% CO_2 for 24 h.

THP-1 cells were induced to differentiate into adherent macrophage-like cells by treatment with PMA (100 nM; Sigma-Aldrich, St. Louis, MO, USA). Nontransfected cells were seeded at 2.5×10^6 cells per plate, electroporated cells (4×10^6 cells per cuvette) were divided post-transfection onto six-well plates and incubated for the indicated time periods. After treatment with PMA differentiation occurs and cells adhere, become flat, and amoeboid in shape.

Human peripheral blood mononuclear cells from healthy blood donors were isolated by density gradient centrifugation by means of buffy coats obtained from the local blood bank. Monocytes were isolated by magnetic cell sorting using anti-CD14 antibody. Separated cells were divided into three groups and stored at −80 °C (day 0) or resuspended in RPMI-1640 that contained 5% human serum to allow cells to adhere and differentiate for 2 or 6 days at 37 °C.

Luciferase activity was quantified using the Promega luciferase assay system (Mannheim, Germany) in a Sirius luminometer (Berthold Detection Systems). Results were confirmed in at least three independent experiments and calculated as fold changes relative to luciferase activity measured with promoterless pGL3 plasmid.

Nuclear and cytoplasmic cell extracts and western blot analyses

Nuclear and cytoplasmic cell extracts were prepared as described previously.[23] Protein concentrations were determined by the Bio-Rad protein assay using bovine serum albumin as standard. Extracts were stored at −80 °C until subjected to SDS-polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose and detected by suitable primary peptide-derived antibodies specific for YB-1 and DNA-binding protein-A (antibodies-online, Aachen, Germany), furthermore glyceraldehyde-3-phosphate dehydrogenase or cAMP response element-binding protein and secondary peroxidase-linked anti-mouse or anti-rabbit antibodies (Amersham Biosciences, Piscataway, NJ, USA) using ECL (Amersham Biosciences) as chemiluminescent. Band intensities were quantified by OptiQuant software, whereas the YB-1 content in cells without PMA treatment was set as 1 and relative band intensities were calculated.

Electrophoretic mobility shift analyses

Recombinant YB-1 was prepared with a pRSET vector (Invitrogen, Carlsbad, CA, USA) containing an insert encoding for a hexahistidine T7 epitope-YB-1 fusion protein as described.[52] Synthetic DNA probes corresponding to the sense as well as antisense strands of the CCL5 promoter sequences −185/−174 and −28/−10 bp were end-labeled by means of the biotin 3′ end DNA-labeling kit (Pierce, Rockford, IL, USA). Double-stranded DNA was annealed by mixing together equal amounts of complementary oligos and incubating the mixture for 1 h at room temperature.

The nucleotide sequences were:

(−185/−163 bp): 5′-CCGGTACCGGAAACTGATGAGCTCACT CACTCTAGATGAGAGATCTGC-3′

(−28/−10 bp): 5′-CCGGTACCCCCCTCAACTGGCCCTATAA AGGGCCAGCCTGAGATCTGC-3′

Biotin-labeled DNA was incubated with affinity-purified recombinant YB-1 protein or nuclear cell extract for 20 min on ice and was subjected to electrophoresis on native 6% PA gels. Following transfer to nylon membranes bands were visualized by streptavidin-horseradish peroxidase conjugate and chemiluminescent substrate (LightShift chemiluminescent EMSA kit, Pierce).

For supershift analyses, peptide-derived affinity-purified rabbit YB-1-specific antibody (antibodies-online, Aachen, Germany) was incubated with nuclear proteins 12 h prior to addition of probes. The binding reaction was performed as described above and samples were subjected to electrophoresis on 6% polyacrylamide gels.

Tetracycline-inducible YB-1 expression system in mesangial cells

The Tet-off system was established as described.[55] Briefly, stable double transfection of rat MCs were constituted with regulatory, response, and selection plasmids that are pTet-Off, pTRE-HA-YB-1, and pTK-Hyg in the presence of selection media containing G418 (Calbiochem, San Diego, CA, USA) and hygromycin (Invitrogen). Transfections were performed using effectene (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Single cell colonies were grown in the presence of 1 μg/ml doxycyclin (ICN, Aurora, OH, USA), and medium supplemented with fresh doxycyclin was replaced every other day. Expression of HA-YB-1 at reduced doxycyclin concentrations (10^{-2}, 10^{-4}, and 10^{-6} mol/l) was monitored by Western blotting using polyclonal anti-HA tag antibody (Santa Cruz Biotechnology, CA, USA). All plasmids were kindly donated by H Bujard (University Heidelberg, Germany) except for pTRE-HA (BD Biosciences, San Jose, CA, USA). Experiments have been performed as triplicates and were confirmed in three independent experiments.

Enzyme-linked immunosorbent assay

Secreted CCL5 protein was assayed in cell culture medium of THP-1, RAW264.7, and HUT78 T cells using the quantitative sandwich enzyme immunoassay technique with reagents from R&D Systems (DuoSet ELISA development kit DY278, Minneapolis, MN, USA). The optical density was measured using a microplate reader set to 450 nm. All standards (diluted 1:2 and 1:10) and samples (supernatants of THP-1 cells: 1:20, RAW264.7 cells: 1:5, HUT78 T cells: 1:30) were assayed as duplicates of at least two independent experiments.

Statistical analysis

All values are expressed as means ± s.d. Statistical significance was evaluated using the Student's *t*-test with significance accepted when $P < 0.05$.

DISCLOSURE

All the authors declared no competing interests.

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APPENDIX

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