Stat1 Nuclear Translocation by Nucleolin upon Monocyte Differentiation

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Introduction

Human blood monocytes are able to differentiate into morphologically and functionally heterogeneous effector cells, including macrophages. The precise molecular mechanisms responsible for differentiation of circulating monocytes into tissue macrophages are, however, incompletely defined. Recent studies highlight the role of transcription factors and other nucleocytoplasmic shuttling proteins in these processes, which require dynamic changes in gene expression [1,2].

Nucleolin is an ubiquitous multifunctional nucleolar shuttle phosphoprotein in eukaryotic cells. Its tripartite domain structure, with an acidic histidine-like N-terminus, a central domain containing four RNA binding domains, and an arginine and glycine rich C-terminus, reflects the diverse roles of nucleolin in cell growth, proliferation, and cell death [reviewed in [3–5]]. Nucleolin has been implicated in many cellular activities, including pre-ribosomal RNA transcription and ribosome biogenesis [6], replication and recombination of DNA, cell cycle progression [7], viral infection [8,9], and apoptosis [10–13]. One remarkable characteristic of nucleolin is that it shuttles constantly between the nucleus and the cytoplasm [14] and additionally serves in some cell types as a cell surface receptor [12,15–17]. For the nucleocytoplasmic translocation, nucleolin uses its bipartite nuclear localization signal (NLS) located between the N-terminal and central domains, and thereby acts as a carrier for karyophilic proteins [18–20]. A growing body of evidence shows interactions of nucleolin with transcription factors [6,21–23].

Members of the signal transducer and activator of transcription (Stat) family of transcription factors are activated during the myeloid differentiation and may play an important role in the differentiation program, including those of monocyte-to-macrophages [24–26,2,27,28–30]. In response to ligand binding of cytokines and growth factors to cell surface receptors, the cytoplasmically located Stats become phosphorylated, form dimers, enter the nucleus, and bind to specific DNA sequences [31]. The exact role of Stat proteins in the regulation of proliferation and terminal cell differentiation of myeloid cells remains to be elucidated.

To enter the nucleus, the Stats have to traverse the nuclear membrane through a specialized structure, called the nuclear pore complex (NPC), which represents a selective filter for the import of proteins. Karyophilic molecules can bind directly to a subset of proteins of the NPC, collectively called nucleoporins. Alternatively, the transport is mediated via a carrier molecule belonging to the importin/karyopherin superfamily, which transmits the import into the nucleus through the NPC.

Abstract

Background: Members of the signal transducer and activator of transcription (Stat) family of transcription factors traverse the nuclear membrane through a specialized structure, called the nuclear pore complex (NPC), which represents a selective filter for the import of proteins. Karyophilic molecules can bind directly to a subset of proteins of the NPC, collectively called nucleoporins. Alternatively, the transport is mediated via a carrier molecule belonging to the importin/karyopherin superfamily, which transmits the import into the nucleus through the NPC.

Methodology/Principal Findings: In this study, we provide evidence for an alternative Stat1 nuclear import mechanism, which is mediated by the shuttle protein nucleolin. We observed Stat1-nucleolin association, nuclear translocation and specific binding to the regulatory DNA element GAS. Using expression of nucleolin transgenes, we found that the nuclear localization signal of nucleolin is responsible for Stat1 nuclear translocation. We show that this mechanism is utilized upon differentiation of myeloid cells and is specific for the differentiation step from monocytes to macrophages.

Conclusions/Significance: Our data add the nucleolin-Stat1 complex as a novel functional partner for the cell differentiation program, which is uniquely poised to regulate the transcription machinery via Stat1 and nuclear metabolism via nucleolin.
belonging to the importin/karyopherin superfamily, which binds to the NLS of the macromolecular cargoes, and transmits the import into the nucleus through the NPC. For Stat proteins both, the carrier-independent and the carrier-dependent nucleocytoplasmic shuttling have been described [35,36].

The aim of the present work was to analyze a possible involvement of the multifunctional shuttle protein nucleolin in myeloid differentiation of monocytes to macrophages. We report that during the monocyte-to-macrophage differentiation, nucleolin associates with the transcription factor Stat1. This association is specific for cells of monocytic origin and is involved in the monocyte-to-macrophage differentiation program. Using expression of nucleolin transgenes, we found that the NLS sequence of nucleolin is responsible for Stat1 nuclear translocation and formation of a ternary complex of nucleolin, Stat1 and the Stat1 target DNA. Our study provides evidence that in addition to so far known Stat1 nuclear import mechanisms an alternative pathway exist, which involves nucleolin-mediated Stat1 transport to the nucleus.

Results

Nucleolin and Stat1 Associate during Monocyte-to-Macrophage Differentiation

Nucleolin plays an important role in the differentiation program of hematopoietic cells [37,38]. As a nucleocytoplasmic shuttle protein, nucleolin binds to transcription factors and modulates gene expression during differentiation. To identify binding partners of nucleolin involved in monocytes differentiation, a nucleolin affinity matrix precipitation assay was performed. For this purpose, we used the N-terminal domain lacking nucleolin construct (ΔN-Ncl) generated from human cDNA clones, bacterially expressed as a GST fusion protein and bound to glutathione agarose. Human myeloid leukaemia THP-1 cells were stimulated up to four days with the phorbol ester PMA to induce the differentiation process, and whole cell lysates were used for the pull-down assay. It has been reported previously that Stat proteins are activated during myeloid differentiation [2]. Therefore, we addressed first these transcription factors and analyzed eluates of our pull-down assays using antibodies against Stat proteins. We found in these experiments that the transcription factor Stat1, but not other Stats (Stat2, Stat3, Stat4, Stat5), was specifically bound to the nucleolin-GST matrix (Fig. 1A and data not shown). This binding was time-dependent peaking at 72 hrs of PMA treatment. The macrophage differentiation was monitored by the expression of specific cell surface proteins (Fig. 1B). In the phase contrast images and after Wrights-Giemsa staining, PMA treated cells showed the characteristic macrophage morphology, namely, larger cell size, increased membrane ruffles, and large cytoplasm pseudopodia of the adherent cells (Fig. 1C).

To provide further evidence for the nucleolin-Stat1 binding, several approaches were used. Since the C-terminal glycine/arginine-rich domain of nucleolin (RGG) was reported to serve for nucleolin interactions [3], the corresponding nucleolin constructs (C(Ncl) and ΔN(Ncl)) were generated, as well as a full-length Stat1 (Fig. 2A). Pull-down assays using both ΔN(Ncl)-GST and C(Ncl)-GST, as well as Stat1-GST, confirmed nucleolin- Stat1 binding in THP-1 cells stimulated for differentiation. These results suggest that the RGG region of nucleolin is involved in this interaction. The Stat1-nucleolin binding was additionally found in cells of the human U937 promyelocytic cell line and in the M1 mouse myeloid leukemia cells stimulated for differentiation (Fig. 2B). To assess whether nucleolin and Stat1 might associate in living cells, co-immunoprecipitation experiments using anti-nucleolin and

![Figure 1: Stat1 and nucleolin bind in THP-1 cells in a time-dependent manner.](https://example.com/figure1.png)
anti-Stat1 antibodies were performed. Indeed, both proteins were co-immunoprecipitated. Remarkably, nucleolin-Stat1 association was only observed in cells stimulated for differentiation, but not in the unstimulated cells (Fig. 2C). As a third approach investigating Stat1-nucleolin interference, we performed cross-linking studies (Fig. 2D). Cytosolic proteins of PMA stimulated THP-1 cells were subjected to cross-linking using chemical cross-linker BS3, followed by immunoprecipitation with anti-nucleolin (left panel) or anti-Stat1 antibodies (right panel). An additional high molecular mass band over 250 kDa was revealed in both experimental settings. From the molecular mass value, we assume that the complex contains a Stat1 dimer and nucleolin. These findings implicate a direct interaction between Stat1 and nucleolin.

Since usage of cell lines and cell stimulation with PMA reflects a model system, we were interested whether association of nucleolin with Stat1 might have a physiological relevance. For this purpose, a full-length human macrophage colony stimulating factor receptor (M-CSFR), which is a physiological receptor to mediate monocyte differentiation, was expressed in THP-1 cells by means of a lentiviral gene transfer. The expressed M-CSFR was functionally competent and revealed an autophosphorylation in response to stimulation with its natural ligand, CSF-1 (Fig. 3A). Pull-down assay demonstrated binding of nucleolin and Stat1 in M-CSFR expressing THP-1 cells stimulated with CSF-1 (Fig. 3B). The same results were obtained in highly purified human peripheral blood derived monocytes (Fig. 3C), but not in cells of non-monocytic origin like primary vascular smooth muscle cells, endothelial cells or cells of the fibrosarcoma HT1080 cell line (data not shown). The increased association of Stat1 with nucleolin upon cell stimulation was not related to changes in their expression. As shown in control experiments, neither Stat1 nor nucleolin protein expression was affected upon the differentiation process (Fig. 3C).

Together, these data indicate that Stat1-nucleolin association is a physiological phenomenon specific for cells of monocytic origin.

**Nucleolin-Stat1 complex Translocates into the Nucleus and Binds to the Stat1 Specific DNA Sequence GAS**

Nucleolin can shuttle between the nucleus and the cytoplasm [14]. Activated Stat proteins form dimers, enter the nucleus, and bind to specific DNA sequences to affect gene transcription [31]. To investigate the cellular localization of nucleolin and Stat1 during the differentiation process, immunocytochemical studies were performed. Human primary monocytes were stimulated with CSF-1 for indicated time points to induce monocyte-to-macrophage differentiation. The staining patterns in the confocal images demonstrated that though Stat1 and nucleolin are present in both the cytoplasm and the nucleus, their maximal colocalization was observed after 72 hours CSF-1 treatment. At this time point, the majority of the cells showed the characteristic macrophage-like morphology.
morphology (Fig. 4A, lower panel). Cell differentiation was additionally controlled by the expression of the cell surface macrophage-mannose receptor (CD206) by FACS analysis (Fig. 4B). The observed nucleolin-Stat1 colocalization was transient and decreased with longer stimulation.

We next asked whether or not the nucleolin-Stat1 complex binds to the Stat1 specific DNA sequences after translocation into the nucleus. In our EMSA, 32P-labeled GAS oligonucleotide was used as a probe to analyze nuclear extracts from THP-1 cells activated with PMA for up to 72 hours. Cells stimulated with interferon-gamma (IFN-γ), which is one of the most effective Stat1-activating cytokines, were used as a positive control. The results of these experiments are presented in Fig. 5. Cell stimulation with PMA led to the induction of one specific DNA-binding protein complex migrating in gel more slowly than complex formed in response to IFN-γ. The kinetics of the DNA binding activity correlated with those for the Stat1-nucleolin nuclear translocation (Fig. 4A). No binding to the GAS was detected in the presence of excess of unlabeled GAS, whereas an unrelated oligonucleotide did not affect the DNA-protein binding. To examine the presence of Stat1 and/or nucleolin in the observed complex, a supershift assay was performed. The corresponding band was inhibited by both anti-Stat1 and anti-nucleolin antibody.

These data implicate a role of the nucleolin-Stat1 complex for the regulated gene expression upon the monocyte differentiation program.

Nucleolin Serves as a Carrier for the Nuclear Translocation of Stat1

Molecular mechanisms of Stat1 nuclear translocation have been intensively studied [39] However, an involvement of nucleolin in this process has not been reported yet. Therefore, we next examined whether the observed association of nucleolin and Stat1 is necessary for the transport of this transcription factor into the nucleus. We specifically inhibited nucleolin expression in THP-1 cells by RNA silencing using a lentiviral RNA interference vector constructed for this purpose. Up to 70% of infection rate was achieved by this way (Fig. 6), whereas conventional transfection reagents showed only low efficiency in cells of monocytic origin [40].

To elucidate whether nucleolin is required for the nuclear transport of Stat1, we performed cell fractionation and examined nuclear extracts isolated from Nclsi-THP-1 cells, non-treated or treated with PMA for 72 hrs. Specific markers were used to control the purity of obtained fractions (Fig. 7A). Stat1 enrichment in nuclear fractions was strongly impaired in PMA stimulated Nclsi-THP-1 cells, but not in control infected cells. Interestingly, we found no impact of nucleolin on the Stat1 nuclear transport in response to IFN-γ. Thus, although IFN-γ elicited Stat1 enrichment in nuclear fractions, there was no difference between the IFN-γ stimulated Nclsi-THP-1 cells and control infected cells. In addition, longer stimulation with IFN-γ did not show any influence of nucleolin on the Stat1 nuclear distribution (data not shown). These data favor a specificity of the nucleolin-dependent Stat1 nuclear translocation for the monocyte-to-macrophage differentiation, which is independent of IFN-γ.

To verify these findings further, we used Nclsi-THP-1 cells under the same experimental design for confocal microscopy studies (Fig. 7B). The staining patterns for Stat1 were consistent with the results of immunoblotting of the nuclear extracts (Fig. 7A). Stat1 enrichment in nuclear fractions was strongly impaired in PMA stimulated Nclsi-THP-1 cells, but not in control infected cells. In addition, longer stimulation with IFN-γ showed no influence of nucleolin on the Stat1 nuclear distribution (data not shown). These data favor a specificity of the nucleolin-dependent Stat1 nuclear translocation for the monocyte-to-macrophage differentiation, which is independent of IFN-γ.

Together, these findings demonstrate the requirement of nucleolin for the nuclear transport of the transcription factor Stat1 upon the monocyte differentiation process.
Figure 4. Stat1 and nucleolin are translocated into the nucleus. (A) Primary blood-derived human monocytes were fixed, permeabilized, and stained using polyclonal anti-nucleolin antibody, monoclonal anti-Stat1 antibody, and the corresponding Alexa coupled secondary antibodies; simultaneous DNA labelling with DRAQ5 was performed to visualize the nuclear compartment. The merged confocal images of monocytes stimulated with CSF-1 for indicated time points are shown. The lower panel shows phase contrast pictures. The images were acquired with a resolution of 1024x1024 pixels with a MRC1024 confocal microscope (BioRad, Hercules, CA) attached to a Nikon Diaphot. All images were taken with oil-immersed x63 objective, and were recorded for triple staining sequentially with detection wavelengths range for Alexa488, Alexa568 and DRAQ5 (Ex$_{\text{max}}$ 646 nm). Pictures were merged using the Lasersharp software (BioRad). (B) The monocyte-to-macrophage differentiation was monitored by FACS analysis. Cell surface expression of the macrophage-mannose receptor (CD206) in CSF-1 stimulated (dark grey curve) and non-stimulated monocytes (open line) are shown. The broken line represents the FITC-isotype control.
doi:10.1371/journal.pone.0008302.g004
The Stat1-Nucleolin Interference Is Specific for the Monocyte-to-Macrophage Differentiation Step and Regulates the Expression of the Macrophage Scavenger Receptor CD36

Nucleolin is highly expressed in cells of the hematopoietic system and its important role for basic biological functions in hematopoietic stem/progenitor cells has been recently demonstrated [38]. To determine whether nucleolin-mediated Stat1 nuclear translocation is induced in a stage specific manner during myelopoiesis, we performed experiments using primary human bone marrow-derived CD34-positive cells. Cells were stimulated with CSF-1 to induce differentiation to the monocyte/macrophage specific unilineage [41]. Differentiation was monitored by expression of specific markers in FACS analysis. Though both, nucleolin and Stat1 were constantly expressed over the whole process of CD34+ cells differentiation, we observed nucleolin-Stat1 binding and nuclear translocation only at the stage of monocyte-to-macrophage differentiation; nucleolin silencing impaired Stat1 nuclear accumulation (Fig. 8A). To further investigate the stage-dependent specificity of this effect, we triggered PLB-985 cells to differentiate to neutrophils and analyzed the nucleolin-Stat1 binding in pull-down assays. A high level of Stat1 expression was detected in these cells, however, no binding of Stat1 with nucleolin was observed (Fig. 8B).

To investigate functional consequences of this association for the monocyte-to-macrophage differentiation process, we analyzed the expression of genuine macrophage specific markers after nucleolin silencing. As shown in Fig. 8C, in THP-1 cells, the expression of CD11b and CD36 was significantly decreased by about 50% in Nclsi- compared with control cells. The revealed dependency of the scavenger receptor CD36 on nucleolin is of special interest, because it has been reported recently, that CD36 serves as a specific Stat1 target gene regulating CD36-directed foam cell formation in macrophages [42].

Collectively, these data indicate that nucleolin-Stat1 interaction is specific to the monocyte-to-macrophage step and that intact nucleolin is required to promote the monocytic scavenger receptor CD36.

Stat1 Nuclear Import Requires the Nuclear Localization Signal of Nucleolin

Nucleolin shuttles between the cytoplasm and the nucleus, and its NPC-related carrier function for karyophilic proteins depends
on the intact NLS [18–20]. Our next question was to investigate a role of the nucleolin NLS for the nucleolin-transmitted Stat1 nuclear transport in hematopoietic cells. We used lentiviral gene transfer to overexpress in a dominant-negative fashion a NLS containing proteins are transported into the nucleus by importins. The classic mono- or bipartite NLS typically contains a cluster of basic residues that is recognized and bound by the NLS receptor importin-α, which is further associated through a separate domain containing proteins are transported into the nucleus by importins. The classic mono- or bipartite NLS typically contains a cluster of basic residues that is recognized and bound by the NLS receptor importin-α, which is further associated through a separate domain

**Discussion**

In this study, we identified a novel molecular mechanism for Stat1 nuclear transport. This finding represents an alternative pathway to the already reported Stat1 nuclear translocation mechanisms, which is mediated by the shuttle protein nucleolin. The NLS sequence of nucleolin is necessary for the correct import into the nuclear compartment. The important result of our experiments is that this mechanism is induced in a stage-specific manner along the monocyte/macrophage pathway. Nucleolin deficiency is associated with impaired expression of monocyte differentiation markers, one of which, the scavenger receptor CD36, is a Stat1 target gene and plays an important role in pathophysiological events related to lipid uptake and inflammation.

Many different mechanisms orchestrate the myeloid developmental program, including cooperative gene regulation, protein-protein interactions, and induction of cell cycle arrest. We addressed a role of nucleolin in cell differentiation and identified the transcription factor Stat1 as a novel and specific binding partner of nucleolin in hematopoietic cells. Increasing evidence point to multiple functions of Stat1 in myeloid cells mediating inflammatory, proapoptotic and antiproliferative events [2,43,44]. These studies suggest that Stat1 may be an early transcription factor activated during the monocyte maturation process upon migration into extravascular tissues. They further indicate that Stat1 does not trigger per se the differentiation process but rather is a part of a developmental program leading to the regulation the transcription of genes specific of mature macrophages. Our findings are along these lines of evidence. We observed the formation of the Stat1-nucleolin complex and its nuclear translocation in the later phase of the monocyte-to-macrophage differentiation. Our results support the studies of others reporting Stat1 constitutive activation at day five of monocyte cultivation [2], as well as changes in activation and DNA binding capacity of LIL-Stat during monocyte maturation [45]. These data suggest that the effects of Stats are tightly controlled according to the status of cell differentiation. The underlying molecular events are, however, sparsely explored.

Specificity of Stat-mediated cellular reactions is a complex multistep process that explains the variety of Stat-mediated cellular functions. This molecular machinery includes the primary stimuli, activation of specific receptors and transactivation of co-receptors, a cross-talk of intracellular signaling pathways and finally, Stat nuclear translocation and DNA binding. Our study provides evidence that Stat1 nuclear transport and DNA binding in a stage-specific manner along the monocyte/macrophage pathway is mediated by the shuttle protein nucleolin. Our results also suggest that nucleo-cytoplasmic shuttle may play a significant role in the modulation of gene expression that occur during monocyte differentiation. The revealed requirement of nucleolin for Stat1 nuclear translocation was surprising, since the molecular mechanisms of Stat1 nuclear transport have been extensively studied before. Thus, Stat1 can associate with specific transport factors of the importin family [46–48] or directly interact with the NPC [36]. Also an unusual nuclear import signal that specifically regulates the nuclear entry of tyrosine-phosphorylated dimeric Stat1, termed dimer-specific NLS (dsNLS), was identified [49]. Our findings add to these schemes a novel mechanism for Stat1 nuclear transport. This finding represents an alternative pathway to the already reported Stat1 nuclear translocation mechanisms, which is mediated by the shuttle protein nucleolin. The NLS sequence of nucleolin is necessary for the correct import into the nuclear compartment. The important result of our experiments is that this mechanism is induced in a stage-specific manner along the monocyte/macrophage pathway.

**Figure 7. Nucleolin gene silencing prevents nuclear Stat1 translocation.** (A) THP-1 cells were infected with nucleolin-si lentiviruses (Nclsi) or empty viruses (con). Three days post infection, cells were cultivated for further 72 hrs without or with 2 nM PMA or stimulated with INF-γ for 2 hrs. Nuclear extracts were prepared, separated by SDS-PAGE, and Stat1 nuclear translocation was visualized by Western blotting using monoclonal anti-Stat1 antibody (upper panel). The purity of cell fractionation was proved by reprobing the membrane with anti-histone antibody (nuclear marker, middle panel) and anti-β-tubulin IgG (cytoplasmic marker, lower panel). (B) Inhibition of the Stat1 nuclear translocation is shown by confocal microscopy studies in Nclsi infected THP-1 cells stimulated as indicated. Cells were fixed and permeabilized, and monoclonal anti-Stat1 antibody and corresponding Alexa 488-coupled secondary IgG were used for staining (green colour). Nuclear compartments were visualized by simultaneous DNA labelling with DRAQ5 (red colour). The fluorescence cell images were captured using a Leica TCS-SP2 AOBS confocal microscope (Leica Microsystems). All images were taken with oil-immersed x63 objective, NA = 1.4. Resolution 1024×1024.

doi:10.1371/journal.pone.0008302.g007
Figure 8. Stat1-nucleolin complex is specific for the differentiation step of monocytes to macrophages and regulates expression of the macrophage scavenger receptor CD36. (A) Peripheral blood CD34+ cells were stimulated with M-CSF, to induce differentiation to the monocyte/macrophage specific unilineage. Nucleolin downregulation in the CD34+ cells was achieved by lentiviral gene silencing. Cells were fixed, permeabilized, and stained using polyclonal anti-nucleolin antibody (green colour), monoclonal anti-Stat1 antibody (red colour), and the corresponding Alexa-coupled secondary antibodies; simultaneous DNA labelling with DAPI (blue colour) was performed to visualize the nuclear compartment. The merged confocal images of Ncl-Stat1-DAPI triple stained CD34+ cells taken by a Leica TCS-SP2 AOBS confocal microscope are shown in the right panel. (B) Nucleolin-GST pull-down assay with whole cell extracts from monocytic THP-1 cells and PLB-985, differentiated to a neutrophilic phenotype by DMSO treatment. Eluates were analyzed by Western blotting; GST glutathione agarose was used as control. Crude cell lysates show equal Stat1 expression in PLB-985 cells. (C) THP-1 cells, lentiviral Nclsi-pLVTHM (Nclsi) infected or with empty lentiviruses (con) treated, were analyzed by FACS after induction of monocyte/macrophage differentiation by PMA. Cell surface expressions of CD11b and CD36 are shown.
doi:10.1371/journal.pone.0008302.g008
evidence that nucleolin, beyond mediating Stat1 import into the nucleus, is a part of the Stat1-DNA binding complex. Transcription factors can act as multiprotein complexes, whose components may be involved in different aspects of transcriptional regulation. Adapter proteins and co-activators may serve for bridging the specific transcription factor to the basic transcription machinery or to facilitate the contact with histone acetylases and deacetylases, which are required for the chromatin remodeling [22,58]. Interestingly, the N-terminal portion of nucleolin can bind to DNA and histone H1 [59] and the C-terminus shows a helicase activity [60]. Both could be responsible for the capacity of nucleolin to remodel the chromatin structure, an important process for the activation or repression of gene expression [61].

Our results show that association and nuclear translocation of Stat1 and nucleolin is specific for the stage of monocyte/macrophage differentiation. This association was found in different human and murine cell lines and primary cells of monocyte origin including hematopoietic progenitor cells stimulated for monocyte-to-macrophage differentiation. Though being expressed, Stat1 and nucleolin did not associate when cells were stimulated to differentiate to neutrophils and in cells of non-myeloid lineages. Furthermore, in our experiments using nucleolin transgenes we show that IFNγ-induced Stat1 nuclear translocation does not utilize nucleolin and in particular its NLS sequence. Our data support the possibility that the nucleolin-mediated Stat1 nuclear transport might represent a specialized pathway for the time-coordinated control of Stat1-regulated gene expression in myelopoiesis. From a clinical perspective, it would be interesting to identify factors, which specifically induce this pathway in vivo. Our results indicate that this pathway is most likely regulated via the CSF-1/M-CSFR system. Conversely, we need to know whether a paracrine or an autocrine mechanism is involved.

With the exception for some IFN-stimulated genes, the cellular genes dependent on cytokine-activated Stat proteins are poorly defined. Recent studies point to a novel function of Stat1 in the pathogenesis of numerous diseases beyond tumorigenesis and host defenses, such as atherosclerosis and other cardiovascular disorders [62,63]. It has been shown that myocardial ischemia and reperfusion induced a rapid activation of Stat1 [63,64]. This functional outcome from Stat1 activation was related to promoting apoptotic cell death upon ischemia/reperfusion injury. A critical role of Stat1 as a sensor responding to cellular stress was further demonstrated in vivo and in vitro in endoplasmic reticulum stress-induced macrophage apoptosis and atherosclerotic plaque progression [63]. Stat1 may have an additional role in the early lesions that is independent of macrophage cell death but rather related to regulation of CD36 scavenger receptor expression and foam cell formation [42]. These observations may explain, at least in part, why Stat1 deficiency in apolipoprotein E-/- mice blocks foam cell formation and early lesion development. Our results suggest that nucleolin-directed Stat1 nuclear transport during the course of macrophage maturation might represent a specific pathway to regulate CD36 expression. Thus, we observed that nucleolin silencing resulted in abrogation of CD36 expression. The regulation of CD36 by Stat1 may be important in other pathophysiological events involving CD36-dependent lipid uptake and inflammation, such as diabetes mellitus and the metabolic syndrome. Therefore, Stat1 inhibition could represent a target to reduce inflammation and to prevent progression of these diseases. Elucidation of the underlying molecular mechanisms of Stat1 regulation could lead to enhanced understanding of these physiological and pathophysiological processes. Our study indicates Stat1-nucleolin interference as one of these mechanisms.

Figure 9. NLS sequence of nucleolin is necessary for the nuclear translocation of Stat1. (A) For infection of monocytic murine M1 cells, a full-length (FL) and a NLS sequence deleted (ΔNLS) nucleolin cDNA from murine origin were used to generate lentiviral particles. (B) Three days post infection with FL(Ncl), ΔNLS(Ncl), or empty lentiviruses (con), M1 cells were stimulated for differentiation with TCM or left non-stimulated. Cells were fixed and permeabilized, and stained for Stat1 (monoclonal anti-Stat1 antibody and corresponding Alexa488-coupled secondary antibody, green colour). Simultaneous DNA labelling with DRAQ5 (red colour) was performed to visualize the nuclear compartment. The translocation of Stat1 into the nucleus was analyzed with a Leica TCS-SF2 AOBS confocal microscope. doi:10.1371/journal.pone.0008302.g009

with importin-β [50,51]. Although it cannot be excluded that the nucleolin-Stat1 complex interacts with importins, our cross-linking experiments do not support this implication. Nucleolin, especially the aminoterminal third, is highly phosphorylated [52,53], and the efficiency of nucleolin shuttling depends on its phosphorylation state [54]. There is a direct link between the grade of nucleolin phosphorylation and the function of NLS for the nuclear membrane passage through the NPC [18,19]. Therefore, phosphorylation/dephosphorylation reactions might be a regulatory element of nucleolin localization. Stat1 phosphorylation as a result of its activation upon monocyte differentiation has been demonstrated [27,30,55]. Though we confirmed these observations of others, no specific requirement for phosphorylated Stat1 for nucleolin binding was determined (data not shown). Our experiments with INFγ suggest that Stat1 phosphorylation of itself is not sufficient for Stat1 nuclear transport via nucleolin. In agreement with this observation, nuclear translocation of other Stats independently of tyrosine phosphorylation was recently documented [56]. These intriguing issues need further studies.

Nuclear transport mechanisms play a fundamental role in regulating the activity of transcription factors [57]. We provide evidence that nucleolin, beyond mediating Stat1 import into the nucleus, is a part of the Stat1-DNA binding complex. Transcription factors can act as multiprotein complexes, whose components may be involved in different aspects of transcriptional regulation. Adapter proteins and co-activators may serve for bridging the specific transcription factor to the basic transcription machinery or to facilitate the contact with histone acetylases and deacetylases, which are required for the chromatin remodeling [22,58]. Interestingly, the N-terminal portion of nucleolin can bind to DNA and histone H1 [59] and the C-terminus shows a helicase activity [60]. Both could be responsible for the capacity of nucleolin to remodel the chromatin structure, an important process for the activation or repression of gene expression [61].

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**Materials and Methods**

**Materials**

High-quality commercial grade chemicals were purchased from Sigma (St. Louis, MO), Merck (Darmstadt, Germany), and Roth (Karlsruhe, Germany). Chemiluminescent signal enhancer was obtained from NEN™ Life Science Products, Inc. (Boston, MA). The far-red fluorescent DNA dye DRAQ5 was from Biostatus Limited Ltd. (Shepshed, UK). Aqua-Poly/Mount mounting media was purchased from Polysciences, Inc. (Warrington, PA). Oligonucleotides were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

**Antibodies**

Monoclonal antibodies for Stat1 protein were from BD Transduction Laboratories (Lexington, KY). Monoclonal anti-nucleolin antibodies were purchased from Medical and Biological Laboratories, Co. Ltd. (Nagoya, Japan) and from Santa Cruz Biotechnology (sc-8031). Polyclonal antibodies for Stat1 (sc-345), M-CSFR, histone H1 and β-tubulin were from Santa Cruz Biotechnology. Polyclonal anti-phospho-M-CSFR (Tyr723) antibody was obtained from Cell Signaling Technology, Inc. (Danvers, MA). FITC- or PE-conjugated specific antibodies or isotype-matched controls for FACS analysis were obtained from Immunotech (Marseilles, France), BD Pharmingen (San Diego, CA), and Serotec (Oxford, UK). Fluorescent Alexa 488- and Alexa 594-conjugated secondary antibodies were from Molecular Probes, Inc. (Eugene, OR). Horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson Immunoresearch Laboratories (West Grove, PA) and Santa Cruz, Inc.

**Cell Culture**

Peripheral blood mononuclear cells (PBMC) were isolated from healthy volunteers using Biocoll Separation Solution (Biochrom KG Seromed, Berlin, Germany) according to the standard protocol. After density gradient centrifugation, pure monocyte fraction (purity approx. 92%) was obtained by using an indirect magnetic labeling system (Monocyte Isolation Kit II, Miltenyi Biotec Inc., Auburn, CA), as advised by the manufacturers. Monocytes were cultured in RPMI 1640 medium (Biochrom) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. For stimulation, 50 ng/ml CSF-1 (R&D Systems). For CD34+ cells, the CliniMACS system (Miltenyi Biotec, Bergisch Gladbach, Germany) was used. The purity of the isolated cells for CD34+ was >99%. The majority of the CD34+ cell population (~80%) was double positive for the common leukocyte antigen CD45 and the stem cell marker CD133. Cells were cultivated in StemSpan™ SFEM Serum-free medium for expansion and culture of hematopoietic cells from StemCell Technologies Inc. supplemented with 100 ng/ml Stem Cell Factor (Cell Systems), 50 ng/ml Flt3 ligand (Cell Systems), 20 ng/ml TPO (thrombopoietin, Peprotech EC Ltd). Differentiation to the monocytic lineage was initiated by 10 ng/ml human CSF-1 (M-CSF) (R&D Systems).

**Plasmid Construction**

The nucleolin constructs (ΔC[Ncl] aa284-aa707, and C[Ncl] aa538-aa707) were generated from a human nucleolin cDNA clone [53] and subcloned into the pSOG vector (Stratagene, La Jolla, CA). To generate the GST fusion proteins, the corresponding constructs were cloned into the bacterial expression vector pGEX-2T (Amersham Pharmacia Biotec Inc.) and expressed after Isopropyl-1-thio-b-D-galactopyranoside (IPTG) induction in DH5α E.coli strain.

Mouse nucleolin cDNA was obtained from the American Type Culture Collection (ATCC; Manassas, VA). The sequence encoding full-length mouse nucleolin was subcloned into pMT/BiP/5′-His expression vector (Invitrogen, Carlsbad, CA). The coding sequence for a FLAG epitope was introduced at the N-terminus of nucleolin (pMT/BiP-FLAG-m-ncl) using QuickChange® site-directed mutagenesis kit (Stratagen, La Jolla, CA). pMT/BiP-FLAG-m-ncl plasmid served as a template for further cloning procedures. Constructs encoding N-terminus FLAG-tagged wild-type mouse nucleolin and a mutant, in which the NLS (encoded by nt 841 to 897) was deleted (ΔNLS) were constructed and cloned in pcDNA3.1(+) vector (Invitrogen) using standard PCR-mediated cloning procedures. For overexpression of mouse nucleolin, lentivirus transfer vector pWPTS-adaptor was generated from the pWPTS-GFP vector by BamHI and SalI cloning duplex. Full length mouse nucleolin cDNA and NLS nucleolin mutant were transferred from pcDNA3.1(+) into pWPTS-adaptor in Sall and SpeI sites. Final construct were designated as pWPTS-FL(Ncl) and pWPTS-ΔNLS(Ncl) accordingly.

For nucleolin silencing, the target sites in human nucleolin mRNA (Acc. #NM005381) for RNAi were determined using the siRNA Selection Server (http://jura.wi.mit.edu/bioc/siRNAext/home.php)41 and designed as oligonucleotides encoding short hairpin RNAs (shRNAs). The following complimentary sequences were selected:

- Nuc 64
  - G AGG TAG AAG AAG ATA GTT
  - Nuc 719
  - A CGC TAA AGA AGC TTT AAT
  - For cloning in pLVTHM vector, the following duplexes were used:
    - Nuc 64
    - CGCGTCGCCAGGTTAGAAAGATAGTTTTTCAAGAAGAAATATCCTCTTACCTCTTCTTGTGAAAT
    - CGATTTCGAAAAAGGGTTAAGAAAGATATGCCTCTTCACGTAGGGGA
    - Nuc 719
    - CGCGTCGCCAGGTTAGAAAGATAGTTTTTCAAGAAGAAATATCCTCTTACCTCTTCTTGTGAAAT
    - CGATTTCGAAAAAGGGTTAAGAAAGATATGCCTCTTCACGTAGGGGA

This work was supported by the Deutsche Forschungsgemeinschaft (DFG) grants (AoF 255/1-3 and Br 1208/1-2) to H.K. and B.B. and the EU (LIFE-2006-245241) to E.H. and R.O. This study was supported by the Medical University of Vienna IZ-175. The authors are grateful to B. Huhn (IMF, Vienna) and T. Asscher-Velthuis (University of Amsterdam) for providing cell lines and helpful discussions.
For downregulation of nucleolin expression by a lentivirus containing nucleolin-specific siRNA, pLVTH-Ncsi plasmid was generated by ligation of the oligonucleotide duplex Ncsi in MluI and ClaI sites of pLVTHM (Tronolab, Switzerland). Nuc 64 showed more extended silencing property and was therefore selected for the next experiments.

For M-CSFR overexpression, the lentiviruses pDEST-lenti transfer vector was generated by blant ligation Gateway Cassette rfa-verB (Invitrogen) in PmeI and Smal sates of the pLV-VRKRAB-Red vector (Tronolab). Entry clones for the transfer of the M-CSFR were produced by cloning the PCR products in the pENTR/D TOPO plasmid (Invitrogen). For M-CSFR overexpression, pEXPR clones were generated by site-specific recombinatn between pDEST-lenti and pENTR/D TOPO-M-CSFR by Gateway LR Clonase Enzyme mix (Invitrogen).

Lentiviral Vector Production and Cell Infection

For experiments with M-CSFR constructs, pCMV-dR8.74, pMD.2G (Tronolab) and pEXPR plasmids were co-transfected (using ratio pLVTH-MpCMV-dr8.74mpMD.2G = 5:2:1) into 293T cells by PerFectin transfection reagent (Genlantis, San Diego, CA) as recommended by manufacturer. After 48 h post transfection, the viral particles containing cell supernatants were harvested, filtered, concentrated, and stored at -70°C for future use. pLVTH-Ncsi, pWPT5-FL/Nci, pWPT5-ANL5/Nci vectors were used instead pEXPR for nucleolin silencing or overexpression as stated above.

For infection, 0.5-0.7 x 10^9 cells/0.6 ml in the presence of 16 g/ml leupetin, 0.3 mM sodium orthovanadate), and put -TCGCGAAGGCAGGTAAAAA-3 (antisense), 5'-GAAGATGGTGATGGGATTC-3 (sense), 6-FAM-AAG-GTGAAGGTCGGAGTC-3 (antisense), 6-FAM-CATCCTCCTCCGTGATTCCGCTCAGC-TAMRA (probe) (sense), 5'-CGACCTCTTTTCCTATCA-3 (antisense), 5'-6-FAM-AAG-GTGAACCCAAGAATGGGTCCTC-CACAGG-TAMRA (probe).

Cell Lysis and Immunoblotting

Non-stimulated cells were washed twice with PBS [phosphate buffered saline], stimulated, adherent cells were detached by adding 5 mM EDTA [ethylene diamine tetraamine acetate] in PBS. Cells were washed twice with ice cold PBS, resuspended in lysis buffer (20 mM Tris-HCl, pH 8.0, 138 mM NaCl, 10% glycerol, 2 mM EDTA, 1% Triton X-100), and protease inhibitors 1 mM PMSF [phenylmethylsulfonyl fluoride], 10 μg/ml aproitin, 10 μg/ml leupetin, 0.3 mM sodium orthovanadate], and put on ice for 10 min. Lysates were clarified by centrifugation at 13,000 rpm for 10 min. Western blotting was performed as described elsewhere [67].

Fusion Protein Precipitation Assay (Pull Down Assay)

Nucleolin and/or Stat1 GST fusion proteins immobilized on glutathione-agarose beads (Sigma) were used for affinity precipitation. Cell lysates containing 800 to 1,500 μg protein were incubated for 1 hr at RT or overnight at 4°C with immobilized GST fusion protein. GST- matrix was used as control affinity matrix. Precipitates were washed three times with Tris-buffed saline containing 0.1% Tween20 (TBS-T). Precipitated proteins were eluted with Laemmli sample buffer containing 20 mM dithioeritol (DTT), and were used for SDS-PAGE and Immunoblotting with corresponding antibodies.

Immunoprecipitation and Cross-Linking

Immunoprecipitation studies were performed as described [67,68]. For cross-linking, 2 mM of the bifunctional chemical cross-linker bis(sulfosuccinimidyl) suberate (BS^2, Pierce, Rockford, IL) was added to cytosolic fractions for 30 min at RT. The reaction was stopped by adding Tris to a final concentration of 50 mM and incubated for further 15 min. Cross-linked samples were used for immunoprecipitation and Western blotting.

Nuclear Extract Preparation

The cell pellet of approx. 3.5 x 10^7 cells was washed, resuspended in 1 ml hypotonic buffer (10 mM HEPES pH 7.9, 1.5 mM MgCl2, 10 mM KC1, 0.5 mM DTT) and incubated on ice for 30 min. After sonication and centrifugation (600 ×g, 5 min, 4°C), the pellet was resuspended in 600 μl 0.25 M sucrose in buffer A (50 mM Tris-HCl, pH 7.4, 5 mM MgSO4, 2 mM DTT), laid over 400 μl 0.25 M sucrose in buffer A and centrifuged (700 ×g, 7 min, 4°C). The pellet was resuspended in 6 ml 1.4 M sucrose in buffer A and laid between 4 ml 2.2 M sucrose and 2 ml 0.25 M sucrose in buffer A. After ultracentrifugation (100,000 ×g for 45 min at 4°C, Beckmann Sw40Ti), the pure nuclei were resuspended in 40 μl of a low salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 20 mM KC1, 0.2 mM EDTA, 0.5 mM DTT). 30 μl high salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM MgCl2, 800 mM KC1, 0.2 mM EDTA, 0.5 mM DTT), were added and incubated on ice for 20 min, followed by centrifugation at 13,000 ×g (5 min, 4°C). The supernatant was dialyzed against 20 mM HEPES, pH 7.9, 20% glycerol, 100 mM KC1, 0.2 mM EDTA, 0.5 mM DTT).

For crude nuclear extracts, the cell pellet of 1.5 x 10^7 monocytes was resuspended in 400 μl ice cold extract buffer (20 mM HEPES, pH 7.9, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT) containing 10 mM KC1, placed on ice for 20 min and lysed in 0.5% Nonidet P-40. After centrifugation, the pellet was resuspended in 50 μl ice cold extract buffer containing 20% glycerol and 400 mM NaCl and incubated at 4°C for 30 min. After centrifugation, crude nuclear extracts were microdialyzed against PBS, used immediately or stored at -80°C.

Electric Mobility Shift Assay (EMSA)

Electrophoretic mobility shift assays were performed as described previously [69]. Binding reaction was performed for 30 min at 25°C with 2–4 μg of whole cell extract. For antibody-supershift and competition analysis protein extracts were pre-incubated with 3 μg of Stat1 polyclonal or nucleolin monoclonal antibodies for 30 min on ice. For oligonucleotide competition 5-fold excess of unlabeled oligonucleotides were added to the reaction. The following double-stranded oligonucleotides were used:

- GAS sense, 5’-CATGTTATGCATAATCTCTGTAAAGT-3’
- GAS anti-sense, 5’-CATTGCATTACAAGAATATGCATATA-3’
- H2K sense 5’-GATCAGGCGCTGGAGTCCCACATCTC-CACAGG-3’

Stat1-Nucleolin Complex
H2K anti-sense 5'-GTCCGGACCCCTAAAGGGTGAGG-TGTCCTAG-3'

Immunofluorescence Confocal Microscopy

Suspension cells, primary human blood monocytes or monocytic cells (THP-1, U937, M1, CD34+), were pelleted on microscope slides by using a cytospinner (Hettich, Tuttingen, Germany; 200×g, 7 min). Stimulated, adherent cells were seeded and cultured on glass coverslips. Cells were fixed, stained and mounted as described by us previously [67]. Fc receptors were blocked with Fc receptor blocking reagents (Miltenyi Biotech or BD Biosciences). DNA staining was performed with far-red fluorophore DRAQ5 (Biostatus Limited Ltd., 1:100, 15 min at RT) or with DAPI [4',6-Diamidino-2-phenylindoldihydrochlorid] (Invitrogen, 300 nM in PBS, 5 min at RT).

FACS Analysis

Phenotypic expression of CD11b, CD36, and CD206 was quantified by FACS (FACScan; Becton Dickinson, Heidelberg, Germany). Briefly, adherent cells were detached with 5 mM EDTA in PBS. Cells were collected by centrifugation, incubated with Fc receptor blocking reagents and stained with the corresponding FITC- or PE-conjugated specific antibody or the Alexa Fluor® 680-conjugated secondary antibody. Alexa Fluor® 680-conjugated secondary antibody was partially expressed on the surface of HepG2 cells that binds lipoproteins specifically is nucleolin. Biochemistry 29: 9708–13.

Acknowledgments

We are grateful to Jana Tretuler for excellent technical assistance, Dr. Ying Shan (Max-Delbrück-Center for Molecular Medicine, Berlin, Germany) for providing us with the nucleolin pGEX-2T vector, Dr. Didier Trono (Trombone, Department of Genetics and Microbiology, Faculty of Medicine, University of Geneva, Switzerland) for the pLV-IRKRA-B-Red, pLV-THM, pCMV-δR8.74, pMD.2G, and pWPTs-GFP vectors, Dr. Marine Rousset (St. Jude Children’s Research Hospital, Memphis, TN) for the M-CSFR plasmids and Dr. Karsten Grote (Hannover Medical School, Hannover, Germany) for providing us with the purified CD34+ cells.

Author Contributions

Conceived and designed the experiments: UJ ID. Performed the experiments: UJ ST JK AK MH. Analyzed the data: UJ. Contributed reagents/materials/analysis tools: ST VS BF. Wrote the paper: UJ ID. Approved the paper: AK, RD, HH, BF.

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