

Leukocyte Ig-Like receptor B1 restrains dendritic cell function through increased expression of the NF- κ B regulator ABIN1/TNIP1

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ABSTRACT

Inhibitory receptors of the human leukocyte immunoglobulin-like receptor family are constitutively expressed on all myeloid cell types and regulate their functional activity. We demonstrate that ligation of the human leukocyte antigen class I-specific receptor *LILRB1*, during the differentiation of monocytes to dendritic cells *in vitro*, results in increased expression of the nuclear factor κ B inhibitor protein ABIN1 (also known as TNIP1). Similarly increased expression of ABIN1/TNIP1 was observed in the “immunosuppressive” monocyte populations of patients with non-Hodgkin lymphoma *ex vivo*. Reducing expression of ABIN1/TNIP1 using small interfering ribonucleic acid allows dendritic cells and immunosuppressive monocytes to respond to stimulation by allowing nuclear factor κ B translocation to the nucleus ($P < 0.001$), increasing cell surface expression of antigen presentation and costimulatory molecules ($P < 0.01$), increasing phagocytic capacity ($P < 0.001$), secreting proinflammatory cytokines ($P < 0.01$), and an increasing ability to stimulate T cell responses ($P < 0.05$). Our study, therefore, identifies an important functional role for ABIN1/TNIP1 in mediating the effects of *LILRB1* ligation-induced inhibitory effects on immune responses. Our findings suggest that inhibiting the *LILRB1*-ABIN1/TNIP1 pathway in antigen-presenting cells could be a therapeutic approach to stimulate antitumor immune responses. Conversely, stimulation of the pathway may also ameliorate autoimmune diseases in which *TNIP1* is a susceptibility gene. *J. Leukoc. Biol.* 100: 737–746; 2016.

Abbreviations: ABIN1 = A20-binding inhibitor of NF- κ B, DC = dendritic cell, LAT = linker for activation of T cells, LILR = leukocyte Ig-like receptors, MDSC = myeloid-derived suppressor cell, NHL = non-Hodgkin lymphoma, siRNA = small interfering ribonucleic acid, SNP = single-nucleotide polymorphisms, TNIP1 = tumor necrosis factor α -induced protein 3 interacting protein 1

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

Introduction

DCs have a central role in the initiation, regulation, and maintenance of immune responses. Recognition of “danger” signals by a variety of pattern-recognition receptors expressed by DCs initiates a program of cellular maturation, creating potent antigen-presenting cells, which are capable of stimulating antigen-specific, naïve T lymphocytes and establishing adaptive, antigen-specific immune responses. Conversely, immature DCs are thought to be involved in the prevention of inappropriate immune responses against “self” antigens by secreting immunosuppressive cytokines and interacting with regulatory T cell populations [1]. The regulation of DC maturation is controlled by several levels of molecular control to ensure that DCs respond appropriately and that immune homeostasis is maintained [2].

Most myeloid lineage cells, including DCs, constitutively express transmembrane cell surface receptors of the *LILR* gene family. The *LILR* genes comprise part of the leukocyte receptor cluster on human chromosome band 19q13.4 and include isoforms with either inhibitory or activating functions, dependent on their possession of ITIM motifs within their cytoplasmic tail [3]. ITIM motifs recruit phosphatase enzymes, such as src homology region 2 domain-containing phosphatase 1 (SHP1), which diminish the intracellular-signaling phosphorylation events initiated by the activating stimuli.

We have previously demonstrated that the HLA class I-specific inhibitory receptor *LILRB1* is involved in maintaining human monocyte-derived DCs in a quiescent state, regulating the capacity of DCs to increase levels of cell-surface antigen presentation and costimulatory molecules, controlling the secretion of cytokines, conferring resistance to FAS-mediated apoptosis and influencing T cell reactivity by interacting with a population of CD4⁺ CD25⁺ CD127⁻ regulatory T cells [4]. Other reports have described similar functions for additional members

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of the *LILR* family [5–8], suggesting an important role for this family of receptors in establishing activation thresholds and regulating myeloid lineage induction of immune responses. The murine homolog of inhibitory *LILR*, *Pirb*, has also been shown to control the survival and function of MDSCs [9], a label applied to a diverse range of immature myeloid-lineage cells with regulatory properties [10], often associated with reduced immune responsiveness to tumor transformation. Mice transgenic for both *LILRB1* and its ligand HLA-G have an expanded population of MDSCs [11], which can prolong skin allograft survival.

In an effort to delineate the molecular mechanisms underlying the quiescent nature of monocyte-derived DCs by the ligation of *LILRB1*, we have performed genome-wide mRNA expression analysis on human monocyte derived DCs cultured with *LILRB1*-specific mAb or isotype control. mRNA expression studies on human monocyte-derived DCs cultured with *LILRB1*-specific mAbs (henceforth, referred to as *LILRB1* DC) revealed the up-regulation of mRNA that encodes NF- κ B-inhibiting proteins—*ABIN1* (also known as *TNIP1*) and *ABIN3* (also known as *TNIP3*) (unpublished data). Here, we describe our analysis of the *ABIN1*/*TNIP1* protein (A20-binding inhibitor of NF- κ B/TNF- α -induced protein 3 interacting protein 1) [12] after ligation of *LILRB1* during DC differentiation and show that altering the expression level of *ABIN1*/*TNIP1* by siRNA-mediated knockdown of gene expression has a significant influence on the monocyte-derived DC phenotype and function *in vitro*. We also examine the expression and function of *ABIN1*/*TNIP1* in “immunosuppressive” monocytes [13] from *ex vivo* analysis of patients with NHL. Our results suggest that *LILRB1* is a potential target to manipulate *ABIN1*/*TNIP1* protein levels and modulate DC responsiveness in lymphomas and immune-mediated diseases.

MATERIALS AND METHODS

Isolation of monocytes from peripheral blood and nodal biopsies

Healthy donors were identified at the Institute of Medical Sciences (Aberdeen, United Kingdom). Patients with suspected follicular lymphoma, mantle cell lymphoma, or nodular lymphocyte-predominant Hodgkin lymphoma undergoing a lymph node biopsy were identified through the Haematology Outpatient Clinic, Aberdeen Royal Infirmary (Aberdeen, United Kingdom), and samples were obtained after full informed consent was given (North of Scotland Ethics Committee, Integrated Research Application System, project 9412). Peripheral blood obtained from the healthy controls, as well as lymph node biopsies obtained from patients, were subjected to negative selection using Depletion MyOne SA Dynabeads (Thermo Fisher Scientific, Waltham, MA, USA) to obtain a single-cell suspension of monocytes.

Differentiation of monocytes into DCs

Monocytes isolated from peripheral blood of healthy donors were differentiated into DCs for a period of 7 d using a 50 ng/ml concentration of recombinant human IL-4 and GM-CSF (PeproTech, Inc, Rocky Hill, NJ, USA). Half of the medium was replaced every 2 d with fresh cytokines, and on day 6, 1 ng/ml of bacterial LPS (Sigma-Aldrich, St. Louis, MO, USA) was added for 24 h where required. Ligation of the *LILRB1* receptor was mediated by the addition of purified anti-*LILRB1* (clone 292305, R&D Systems, Minneapolis, MN, USA; or clone HPP1, eBioscience, San Diego, CA, USA) at a final

concentration of 5 μ g/ml in the presence of 1 μ g/ml Protein G (Sigma-Aldrich). Mouse IgG1 κ (MOPC21; Sigma-Aldrich) was used as an isotype control at the same concentration as previously described [4].

siRNA-mediated knockdown of *ABIN1*/*TNIP1* expression

The cells of interest were seeded into 6-well plates at 2×10^5 cells/well in 2 ml of antibiotic-free RPMI 1640 medium supplemented with 10% heat-inactivated human AB serum for 24 h to ensure confluence. For each transfection, 60 pM *ABIN1* siRNA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was used along with 100 μ l siRNA transfection medium (Santa Cruz Biotechnology) and 100 μ l siRNA transfection reagent (Santa Cruz Biotechnology). The cells were then incubated at 37°C, 5% CO₂ for an additional 5 h; following which, the medium in each well containing the siRNA-treated cells was changed to RPMI 1640 supplemented with 10% heat-inactivated human AB serum and 50 U/ml of penicillin-streptomycin and was incubated for an additional 24 h at 37°C, 5% CO₂.

Flow cytometry

The following antibodies were used for flow cytometry: anti-CD3-PE (clone SK7), anti-CD4-AF700 (clone RPA-T4), anti-CD8-PE-Cy5 (clone RPA-T8), anti-CD80-FITC (clone L307), anti-CD86-APC (clone 2331 FUN-1), anti-HLA-DR-PE-Cy5 (clone TU36), anti-HLA-ABC-PE (clone G46-2.6), anti-pERK1/2-PE (clone 25/MEK1), anti-pLAT-AF488 (clone 158-1169), anti-IFN- γ -AF700 (clone B27), anti-IL-12p70-PE (clone 20C2) (all BD Biosciences, Franklin Lakes, NJ, USA); anti-IL-10-PE-Cy7 (clone JES3-9D7; BioLegend, San Diego, CA, USA); anti-IFN- α -PE (clone 1-D1K; Mabtech, Cincinnati, OH, USA). Anti-*TNIP1* (*ABIN1*) antibody (clone 5C4, Abcam, Cambridge, MA, USA) was used in conjunction with goat anti-mouse IgG (heavy and light chain)-PE (Beckman Coulter, Brea, CA, USA). Monocytes, DCs, or PBMCs were washed and incubated at room temperature for 30 min with the above antibodies. The cells were then fixed with 4% paraformaldehyde, washed and analyzed on a BD LSR II flow cytometer (BD Biosciences) using FlowJo software (Tree Star, Ashland, OR, USA). DCs and monocytes were identified using forward and side light scatter characteristics. A minimum of 10,000 events were acquired on all samples.

To assess the macropinocytic capacity of different DC populations, the cells were incubated with FITC-conjugated dextran molecules at a concentration of 50 μ g/ml for 24 h before fixation and flow cytometric analysis.

To quantify cytokine production, DCs, monocytes or PBMCs were treated with 1 μ g/ml monensin (BD Biosciences), fixed with 4% paraformaldehyde, and permeabilized with methanol before staining for flow cytometry. PBMCs were stained with T cell-specific markers to analyze cytokine production from T cell that were stimulated with different DC populations.

To assess T cell proliferation when stimulated by different DC populations, CFSE (Thermo Fisher Scientific)-stained PBMCs were incubated with DCs at a ratio of 10:1 at 37°C for 5 d; following which, the cells were stained with T cell-specific markers, fixed, washed, and analyzed on a BD LSR II flow cytometer using FlowJo software. A minimum of 100,000 events were acquired on all samples.

Fluorescence and light microscopy

To assess the expression levels of NF- κ B p65 in the nucleus of different DC populations, DCs were seeded into poly-L-lysine-treated wells of a 48 well plate and left to adhere at 37°C for 2 h. The cells were then fixed with 4% formaldehyde, permeabilized with Triton X-100, and blocked with 1% BSA in PBS containing 0.05% Tween 20, all at room temperature. The cells were stained with an anti-NF- κ B p65 antibody (Abcam) for 1 h at room temperature; after which, they were washed and incubated with a goat anti-mouse heavy and light chain secondary antibody conjugated to FITC (Abcam) for 2 h at room temperature. The nucleus of the cells was stained with Hoechst stain (ImmunoChemistry Technologies, Bloomington, MN, USA) for 5 min. The cells were then washed with PBS and analyzed on a Zeiss Axio Observer Z1 inverted microscope (Carl Zeiss Microscopy, Thornwood, NY, USA). Nuclear expression of NF- κ B p65 was analyzed using the ImageJ software (U.S.

National Institutes of Health, Bethesda, MD, USA) that quantified the fluorescence emitted by fluorochrome-conjugated antibodies specific to the NF-κB subunit p65. A maximum of 50 cells were analyzed per treatment per donor, set up in triplicates.

To assess the phagocytic population of different DC populations, cells were incubated with 6 μm polystyrene beads at a ratio of 1:3 for 3 h. The cells were washed with PBS, fixed with 4% formaldehyde, and analyzed on an EVOS ×1 transmitted light microscope (Thermo Fisher Scientific).

Statistical analysis

Differences in ABIN1 protein expression, NF-κB/p65 translocation, phagocytic/macropinocytic capacity, cytokine production, expression of antigen presentation, TLRs, and costimulatory molecules in DC populations, and phosphorylation of intracellular molecules, and proliferation of T cells was analyzed using 1-way ANOVA test, followed by the Bonferroni multiple comparison test. Differences in ABIN1 expression, expression of antigen presentation and costimulatory molecules, and cytokine production between monocytes obtained from healthy donors and those obtained from patients with NHL were analyzed using unpaired *t* tests. Differences in cytokine production and expression of antigen presentation and costimulatory

molecules in control monocytes and monocytes obtained from patients with NHL after treatment with ABIN1 siRNA were analyzed using paired *t* tests.

RESULTS

Increased expression levels of ABIN1/TNIP1 protein in monocyte-derived DCs after LILRB1 ligation

mRNA expression studies and intracellular flow cytometry in monocyte-derived DCs, in which LILRB1 had been ligated throughout in vitro differentiation, demonstrated enhanced expression of ABIN1/TNIP1 on an mRNA level (data not shown) and protein level (Fig. 1A and C) when compared with DCs cultured with the isotype control antibody. ABIN1/TNIP1 protein levels were significantly increased after exposure to the TLR4 ligand bacterial lipopolysaccharide ($P < 0.001$) (Fig. 1A and C). Significant differences ($P < 0.05$) were also noted after transfection of LILRB1 DCs with siRNA specific for ABIN1/TNIP1, where a reduction in protein expression was observed

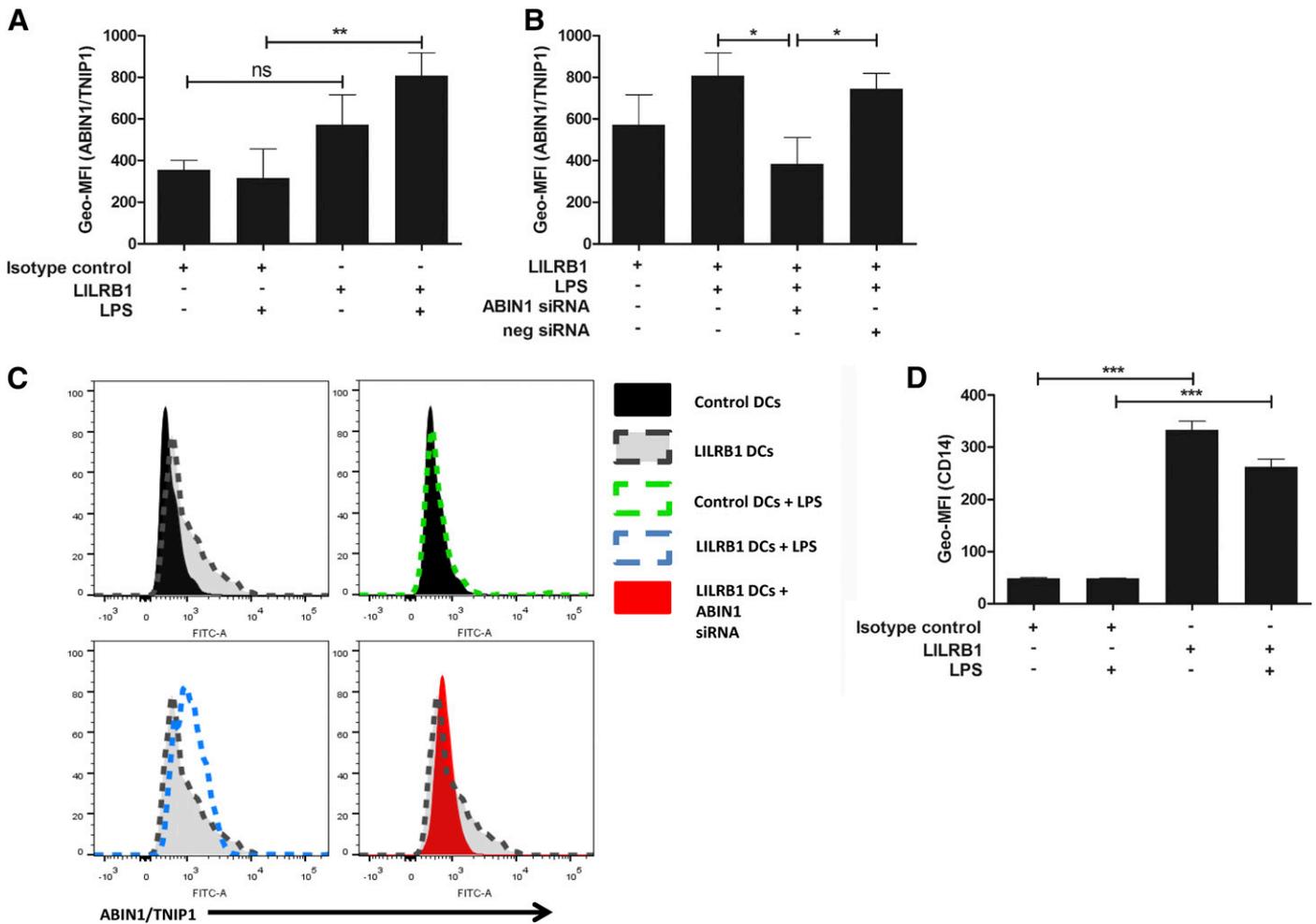


Figure 1. LILRB1 ligation during in vitro DC differentiation results in higher levels of ABIN1/TNIP1 protein levels. (A and C) Higher ABIN1/TNIP1 protein levels are evident in LILRB1 DCs and can be significantly increased ($P < 0.01$) after LPS exposure. (B and C) ABIN1/TNIP1 levels can be significantly reduced in LILRB1 DCs using siRNA. (D) LILRB1 DCs display significantly elevated levels of CD14 when compared with control DCs. In all experiments ($n = 4$ individual donors), bars depict means \pm SEM. CI, confidence interval; Geo-MFI, geometric mean fluorescence intensity; Gep-MFI, granulin-epithelin precursor mean fluorescence intensity; neg, negative. * $P < 0.05$ (95% CI), ** $P < 0.01$ (99% CI), *** $P < 0.001$ (99.9% CI).

(Fig. 1B and C). LILRB1 DCs expressed significantly elevated levels of CD14 when compared with the isotype control DCs (Fig. 1D).

ABIN1/TNIP1 influences NF-κB nuclear translocation and macropinocytic/phagocytic functions of LILRB1 DCs

In accordance with ABIN1/TNIP1's known cellular function, NF-κB translocated to the nucleus less efficiently in LILRB1 DCs than it did in isotype control DCs ($P < 0.001$), but translocation significantly ($P < 0.001$) increased after siRNA-mediated reduction in ABIN1/TNIP1 expression levels (Fig. 2A).

DCs are characterized by their antigen sampling abilities and their capacity to secrete cytokines rapidly after recognition of pathogens or damage-associated molecular patterns. After incubation with FITC-labeled dextran as a surrogate for a fluid phase antigen, LILRB1 DCs dramatically increased their uptake capacity after the reduction in ABIN1/TNIP1 levels, as measured by flow cytometry ($P < 0.001$; Fig. 2B, upper panel), whereas phagocytic uptake of 6 μM polystyrene beads, measured by light microscopy, also significantly increased ($P < 0.05$, Fig. 2B, lower panel).

ABIN1/TNIP1 influences expression levels of antigen-presentation molecules and cytokine secretion in LILRB1 DCs

Flow cytometric analysis of cell surface levels of important ligands in DC antigen presentation and T cell costimulation demonstrated significant increases in amounts of HLA-ABC ($P < 0.001$) and HLA-DR ($P < 0.05$) when ABIN1/TNIP1 levels were reduced by siRNA-mediated knockdown. This finding was particularly strong for HLA-ABC because LILRB1 DCs did not increase expression after exposure to LPS, unlike isotype control DCs, but HLA-ABC levels almost quadrupled after reduction in ABIN1/TNIP1 levels, even in immature LILRB1 DCs (Fig. 3A, lower panel). Although CD80 and CD86 levels also increased, this relationship did not reach statistical significance (Fig. 3A, upper panel).

Likewise, siRNA-mediated knockdown of ABIN1/TNIP1 expression significantly increased LILRB1 DC production of IL-12p70 ($P < 0.001$) after stimulation with the TLR4 ligand LPS (Fig. 3B, upper panel). Significant increases were also observed in LILRB1 DC's capacity to produce IL-10 ($P < 0.05$) and IFN-α ($P < 0.05$) (Fig. 3B, upper and lower panels) following ABIN1/TNIP1 knockdown.

ABIN1/TNIP1 influences the capacity of LILRB1-ligated DCs to activate allogeneic T lymphocytes

The prime function of DCs is to stimulate specific T lymphocytes to initiate an adaptive, antigen-specific immune response. We investigated the role that ABIN1/TNIP1 has in regulating the function of LILRB1 DCs by coculturing the isotype control or LILRB1 DCs with allogeneic CD4⁺ and CD8⁺ T lymphocytes after siRNA-mediated reduction in ABIN1/TNIP1 expression. T lymphocyte responses were assessed by flow cytometric analysis of phosphorylation of key T cell signaling molecules ERK1/2 and

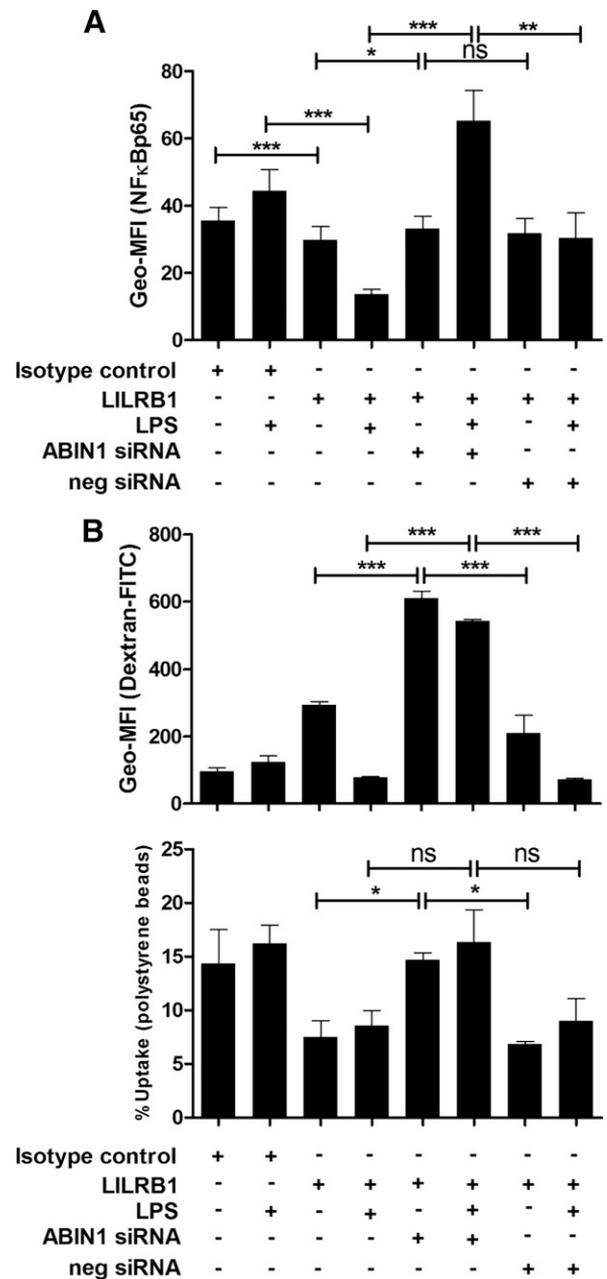


Figure 2. Reducing ABIN1/TNIP1 expression significantly increases NF-κB nuclear translocation and the macropinocytic and phagocytic capacity of LILRB1 DC. Fluorescence microscopy was used to determine nuclear expression levels of NF-κB/p65, as described in the Materials and Methods section. (A) Nuclear expression of NF-κB/p65 was significantly lower in LILRB1 DCs than it was in isotype control DCs ($P < 0.001$) and was significantly restored on siRNA knockdown of ABIN1/TNIP1 ($P < 0.001$) in semimature LILRB1 DCs. Following ABIN1/TNIP1 knockdown, LILRB1 DCs increased their capacity for macropinocytic/phagocytic uptake of fluorescently labeled dextran (B, upper panel) ($P < 0.001$), as determined by flow cytometry and 6 μm polystyrene beads (B, lower panel) ($P < 0.05$), as determined by light microscopy. In all experiments ($n = 6$ individual donors), bars depict means \pm SEM. CI, confidence interval; Geo-MFI, geometric mean fluorescence intensity; neg, negative; ns, not significant. * $P < 0.05$ (95% CI), ** $P < 0.01$ (99% CI), *** $P < 0.001$ (99.9% CI). Representative examples of images used to obtain data depicted in (A) are provided in Supplemental Fig. 1.

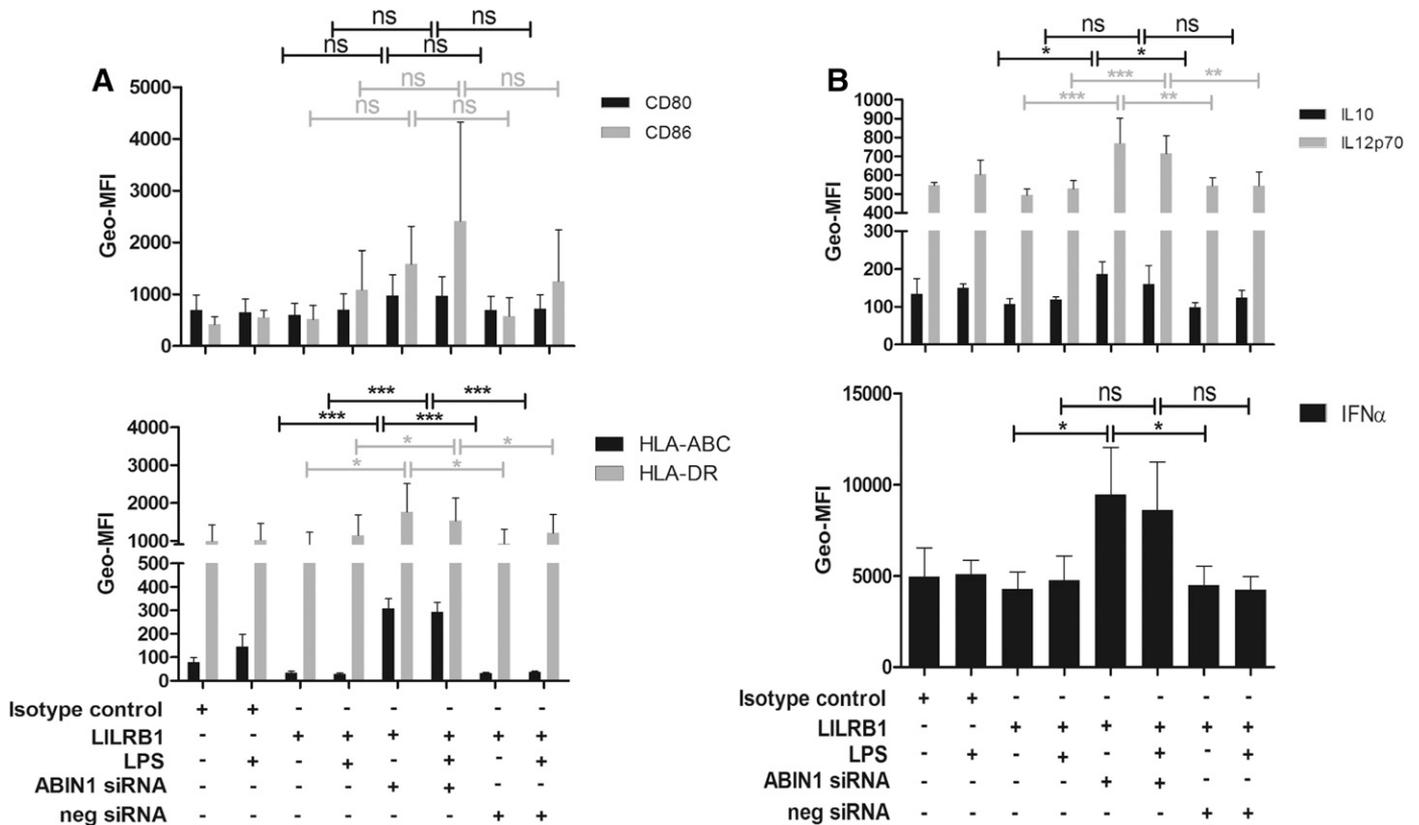


Figure 3. Reducing ABIN1/TNIP1 expression in LILRB1 DCs increases expression of cell-surface antigen presentation and costimulatory molecules, with increased expression of proinflammatory cytokines. CD80 (ns) and CD86 (ns) (A, upper panel) and HLA-ABC ($P < 0.001$) and HLA-DR ($P < 0.05$) (A, lower panel) were increased in LILRB1 DCs following siRNA-mediated knockdown of ABIN1/TNIP1 expression. Production of proinflammatory cytokines IL-12 p70 (B, upper panel) ($P < 0.001$) and IFN- α (B, lower panel) ($P < 0.05$) were also increased in LILRB1 DCs following treatment with ABIN1 siRNA. In all experiments ($n = 6$ individual donors), bars depict means \pm SEM. Geo-MFI, geometric mean fluorescence intensity; neg, negative; ns, not significant. * $P < 0.05$ (95% CI), ** $P < 0.01$ (99% CI), *** $P < 0.001$ (99.9% CI). Representative examples of the combined data are provided in Supplemental Fig. 2.

LAT using phosphospecific antibodies. T cell proliferation was also measured by the loss of CFSE fluorescence from proliferating T cells after 5 d of culture.

We observed no significant differences in level of phosphorylation of ERK1/2 (pERK1/2) in both responding CD4 (Fig. 4A, left panel) and CD8 T lymphocytes (Fig. 4B, left panel) when stimulated by control or wild-type LILRB1-ligated DCs. However, a significant ($P < 0.05$) increase in pERK1/2 was seen in both T cell subsets (Fig. 4A and B, left panel) when stimulated by LILRB1 DCs carrying the ABIN1/TNIP1 expression knockdown. A similar result was observed for the phosphorylation of LAT residue 171 after ABIN1/TNIP1 expression knockdown in LILRB1 DCs (Fig. 4A and B, middle panel). Analysis of phosphorylation of LAT residue 226 did not show any specific differences (data not shown).

LILRB1 ligated DCs were poor stimulators of allogeneic T cell proliferation in mixed lymphocyte cultures in comparison to isotype control DCs, and this was reversed by a reduction in ABIN1/TNIP1 levels. LILRB1 DCs after ABIN1/TNIP1 expression knockdown stimulated a significant increase in the proliferation of CD4⁺ T cells (Fig. 4A, right panel). No statistically significant changes were observed with respect to CD8⁺ T cell proliferation (Fig. 4B, right panel). Furthermore, no statistically

significant changes were observed with respect to T cells capacity to produce IFN- γ when treated with the different DC populations (Fig. 4C).

ABIN1/TNIP1 is expressed at high levels in monocytes isolated from lymph nodes involved by NHL

Several studies have reported the presence of an immunosuppressive monocyte population in patients with NHL. Patients with NHL presenting with increased percentages of these suppressive monocytes usually display a more progressive disease. We examined expression levels of ABIN1/TNIP1 in monocytes obtained from patients with NHL (follicular, mantle cell, or nodular lymphocyte predominant Hodgkin lymphoma) ($n = 12$). Intracellular levels of ABIN1/TNIP1 were significantly higher ($P < 0.01$) in monocytes obtained from patient samples than they were from peripheral population of monocytes of healthy donors and increased after stimulation with TLR4 agonists (Fig. 5A, left panel). ABIN1/TNIP1 levels were significantly reduced in monocytes obtained from patients with NHL after siRNA treatment (Fig. 5A, right panel). Patient-derived monocytes expressed lower cell surface levels of CD80 ($P < 0.01$), CD86 (not significant, [ns]), and HLA-DR ($P < 0.001$) than did those of healthy controls (Fig. 5B). Similarly, monocytes from

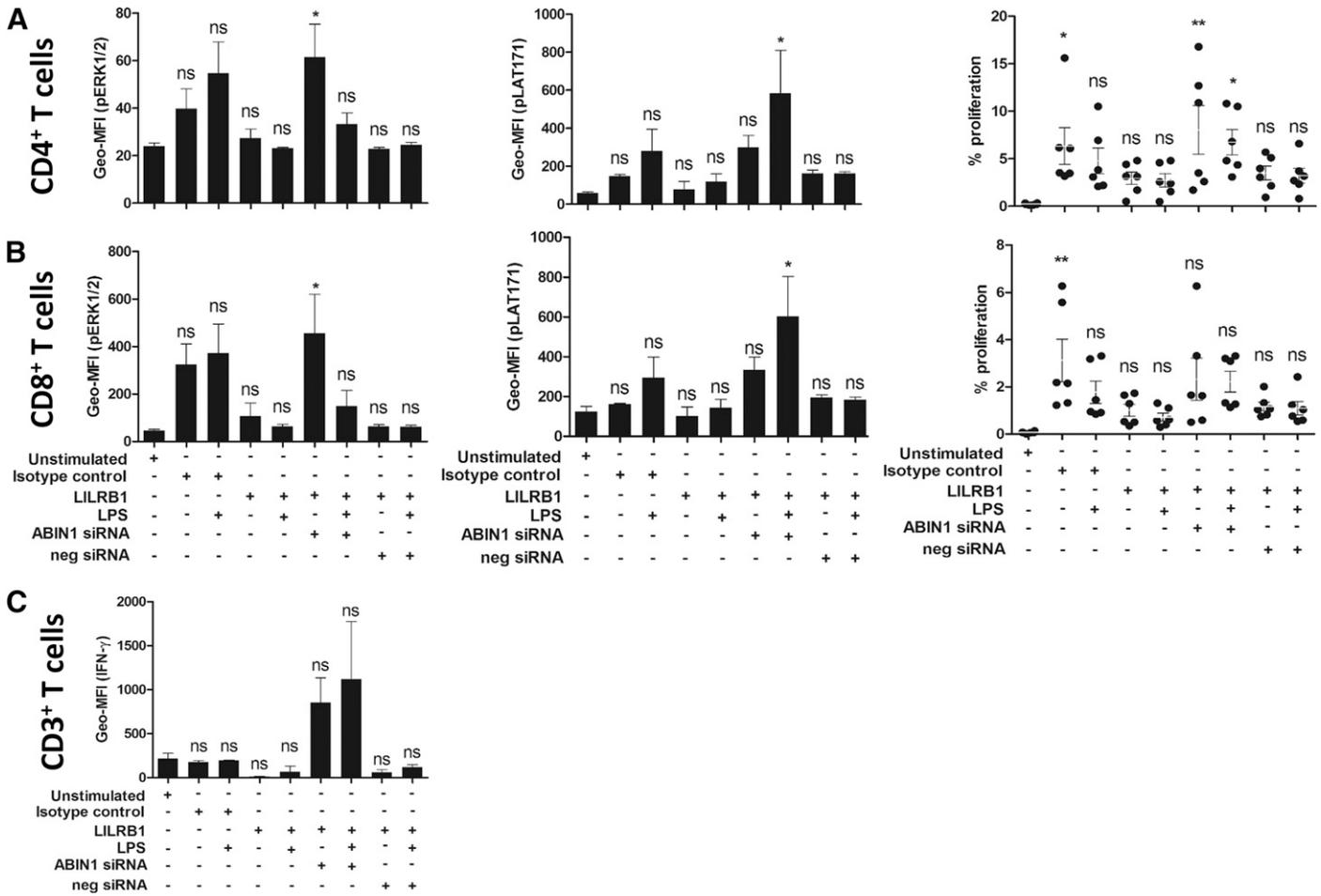


Figure 4. Reducing ABIN1/TNIP1 expression restores the ability of LILRB1 DCs to stimulate allogeneic CD4⁺ and CD8⁺ T lymphocytes. No statistically significant difference were observed in the phosphorylation of ERK1/2 in CD4⁺ (A) and CD8⁺ (B) T cells when control or LILRB1 DCs were used as stimulators. Following ABIN1/TNIP1 knockdown in LILRB1 DCs, immature LILRB1 DCs stimulated significantly elevated phosphorylation of ERK1/2 ($P < 0.05$) in allogeneic T cells (A and B, left panel), whereas the semimature LILRB1 DC population stimulated significantly elevated phosphorylation of LAT171 ($P < 0.05$) in allogeneic T cells (A and B, middle panel). Knockdown of ABIN1/TNIP1 resulted in a significantly increased capacity of both immature ($P < 0.01$) and semimature ($P < 0.05$) LILRB1 DCs to stimulate CD4⁺ T cell proliferation (A, right panel). Results for CD8⁺ T cell proliferation were not significant (B, right panel). (C) Reducing ABIN1/TNIP1 levels in LILRB1 DCs had no statistically significant effect in stimulating production of IFN- γ in CD3⁺ T cells. In all experiments, statistical comparisons were made between unstimulated T cells and T cells stimulated with different monocyte-derived DC populations. In all experiments ($n = 6$ individual donors), bars depict means \pm SEM. Geo-MFI, geometric mean fluorescence intensity; CI, confidence interval; neg, negative; ns, not significant. * $P < 0.05$ (95% CI), ** $P < 0.01$ (99% CI). Representative examples of the combined data are provided in Supplemental Fig. 3.

patients produced significantly less IL-12p70 ($P < 0.01$) and IFN- α ($P < 0.05$) (Fig. 5C). Significant differences were not observed in IL-10 production of monocytes between those of patients with NHL and those from healthy controls (Fig. 5C).

ABIN1/TNIP1 regulates the phenotype and function of monocytes from patients with NHL

Monocytes obtained from healthy controls after LPS stimulation significantly increased their expression of CD80 ($P < 0.01$), CD86 ($P < 0.05$), HLA-DR ($P < 0.05$), IL-12p70 ($P < 0.05$), and IFN- α ($P < 0.05$) (Fig. 6A and B). Monocytes obtained from patients with NHL after LPS stimulation did not significantly increase expression of CD80, CD86, HLA-DR, IL-12p70, or IFN- α possibly because of elevated levels of ABIN1/TNIP1. siRNA-

mediated reduction in ABIN1/TNIP1 expression levels and LPS stimulation produced statistically significant increases in expression levels of CD80 ($P < 0.05$), CD86 ($P < 0.01$), and HLA-DR ($P < 0.05$) in the monocytes isolated from patients with NHL (Fig. 6A). Additionally, significant increases ($P < 0.05$) in production of IL-12p70 and IFN- α were observed in monocytes of patients with NHL, after stimulation with LPS, and reduction in ABIN1/TNIP1 levels. No significant changes were observed for IL-10 (Fig. 6B).

DISCUSSION

The plasticity of DCs, with respect to its evolving phenotype or function as an initiator of immune responses or an inducer of

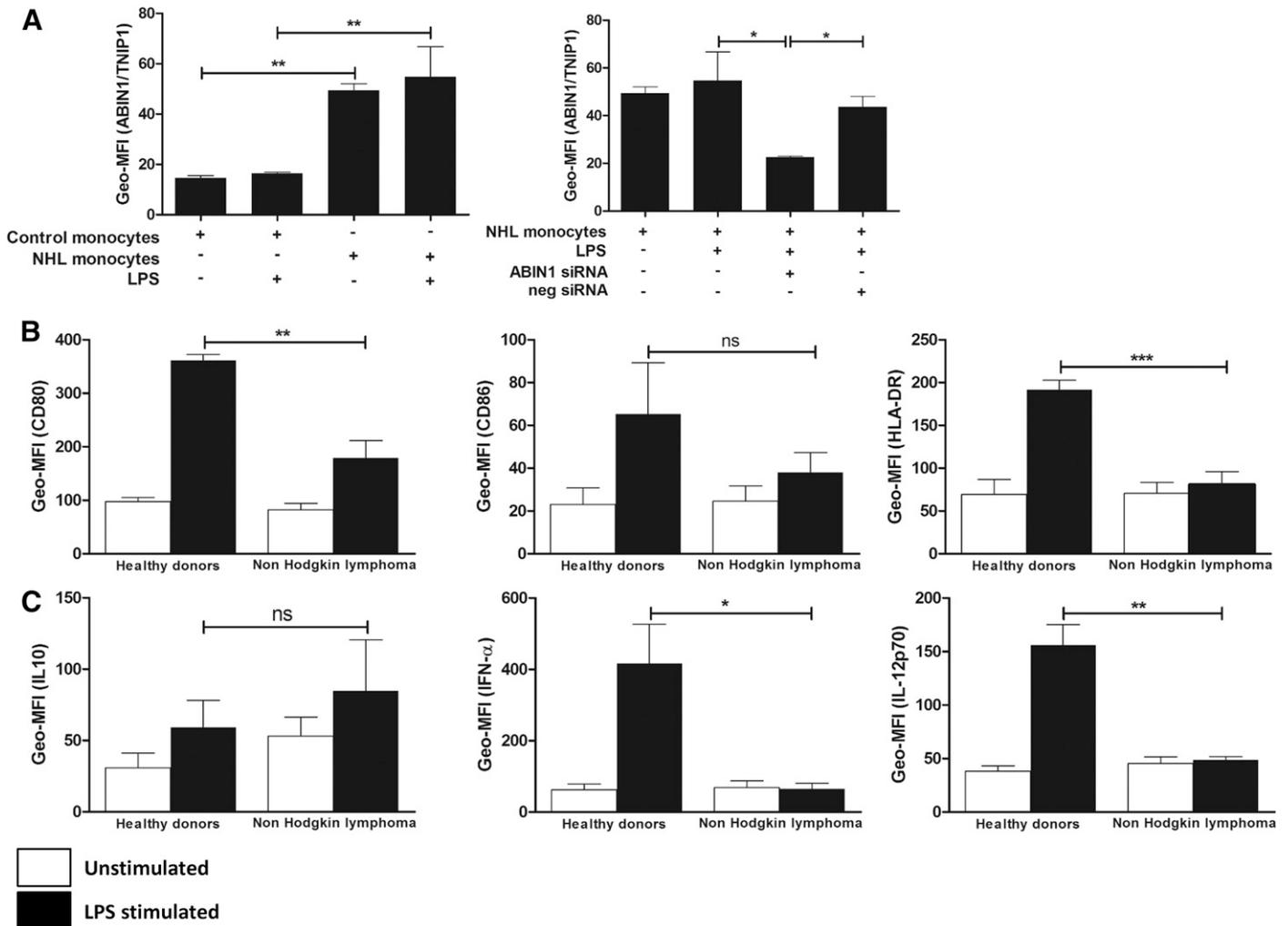


Figure 5. Monocytes from patients with lymphoma express higher levels of intracellular ABIN1/TNIP1 ex vivo, poorly express cell-surface antigen presentation and costimulatory molecules, and produce limited amounts of proinflammatory cytokines. (A, left panel) Monocytes obtained from patients with NHL expressed ABIN1/TNIP1 at significantly ($P < 0.01$) higher levels than did monocytes obtained from healthy controls. (A, right panel) ABIN1/TNIP1 levels in patient monocytes could be significantly reduced following treatment with ABIN1 siRNA. Patient monocytes had lower cell-surface levels of CD80 ($P < 0.01$), CD86 (ns), and HLA-DR ($P < 0.001$) (B) and produced significantly less IFN- α ($P < 0.05$) and IL-12p70 ($P < 0.01$) (C). Results for IL-10 were not significant. (B and C) Black bars indicate samples were stimulated with 1 ng/ml LPS, whereas white bars are unstimulated samples. In all experiments ($n = 12$ individual donors), bars depict means \pm SEM. Geo-MFI, geometric mean fluorescence intensity; CI, confidence interval; HC, healthy control; neg, negative; ns, not significant. * $P < 0.05$, ** $P < 0.01$ at 95% CI, *** $P < 0.001$ at 99.9% CI. Representative examples of the combined data are provided in Supplemental Fig. 4.

tolerance, has made it an attractive cellular target for immunotherapy in cancer and autoimmunity. A key component of DC activation and maturation, after pattern recognition, is activation of the transcription factor NF- κ B, which is released from a complex, regulated control system in the cytoplasm and relocates to the nucleus, where it initiates the expression of hundreds of genes encoding proinflammatory proteins. Inhibition of canonical NF- κ B signaling has been shown to reduce the functionality of both CD34⁺ myeloid DCs and monocyte-derived DCs by regulating the survival, differentiation, and maturation of both DC populations [2, 14, 15].

We have demonstrated higher expression levels of the NF- κ B regulator ABIN1/TNIP1 in DCs in which the HLA class I-specific inhibitory receptor LILRB1 has been ligated during in vitro differentiation from PBMCs. We have confirmed previous

findings that LILRB1 DCs retain surface expression of CD14 after their in vitro differentiation from monocytes [4]. In this study, we showed that modulating the expression levels of the ABIN1/TNIP1 protein reversed the functional effects of LILRB1 ligation, allowing the monocyte-derived DCs to partially up-regulate cell surface expression of antigen-presenting and costimulatory molecules, to produce cytokines, and to stimulate T cell activation in response to challenge with LPS. The reduction of ABIN1/TNIP1 in LILRB1-ligated, monocyte-derived DCs leave the resultant DC population with a phenotype that bares resemblance to the "semimature" DC population that is extensively reviewed in the following study [16]. Our results suggest ABIN1/TNIP1 is a potential regulator of monocyte-derived DC activation, and the increased ABIN1/TNIP1 expression after self-HLA recognition by LILRB1 is a mechanism

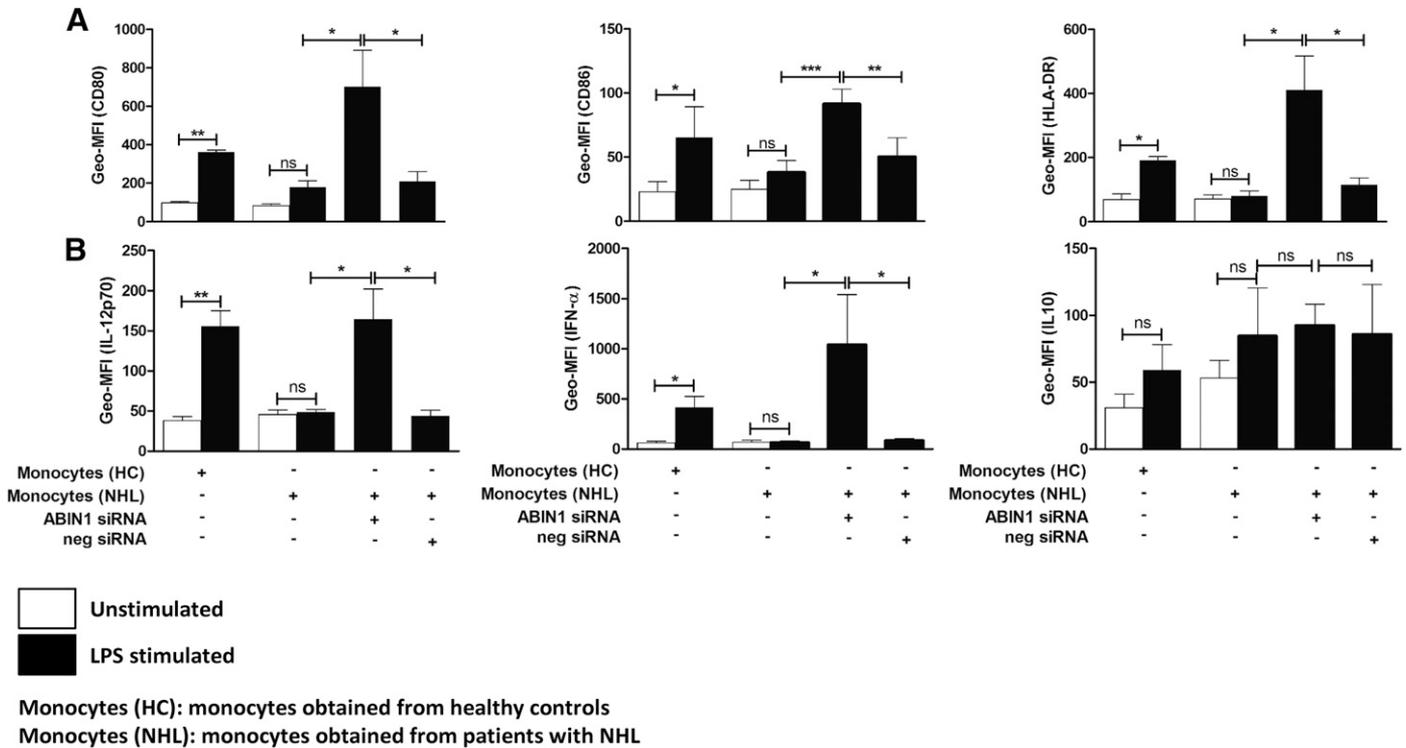


Figure 6. Reducing expression of ABIN1/TNIP1 in monocytes from patients with lymphoma restores cell-surface expression of CD80 and HLA-DR and significantly increases expression of IL-12p70 and IFN-α. (A) Lowering ABIN1/TNIP1 expression resulted in significantly increased expression of CD80 ($P < 0.05$), CD86 ($P < 0.01$), and HLA-DR ($P < 0.05$) in the monocytes of patients with lymphoma after stimulation with LPS. (B) After ABIN1/TNIP1 knockdown and LPS stimulation, the monocytes of patients with NHL produced significantly greater amounts of IL-12p70 ($P < 0.05$) and IFN-α ($P < 0.05$), whereas results for IL-10 were not significant. Black bars indicate samples were stimulated with 1 ng/ml LPS, whereas white bars are unstimulated samples. In all experiments ($n = 12$ individual donors), bars depict means \pm SEM. Geo-MFI, geometric mean fluorescence intensity; CI, confidence interval; HC, healthy control; neg, negative; ns, not significant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ at 95% CI. Representative examples of the combined data are provided in Supplemental Fig. 4.

establishing a threshold for NF-κB translocation and DC maturation in response to triggering of pattern recognition receptors. The increased production of IFN-α we detected is consistent with a report of ABIN1/TNIP1 involvement in regulating cellular antiviral responses [17].

ABIN1/TNIP1 functions in cooperation with the ubiquitin-sensing regulatory protein TNFAIP3 (TNF-α-induced protein 3), also known as A20, to regulate the ubiquitination of the NF-κB inhibitory protein IκB kinase-γ [18], thus controlling its proteasomal degradation. ABIN1/TNIP1 also functions to prevent TNF-α-induced apoptosis [12]. Genetic knockout of ABIN1/TNIP1 is lethal in embryonic mice, but hemizygous litter mates [19] or mice with an introduced, nonfunctioning mutation of the polyubiquitin-binding domain [20] are highly susceptible to the development of autoimmune conditions [21]. In addition, many genetic studies of autoimmune conditions in human populations have identified SNPs in *TNIP1* and *TNFAIP3* genes as susceptibility factors for the development of systemic lupus erythematosus [22, 23], psoriasis [24, 25], systemic sclerosis [26, 27], and myasthenia gravis [28]. One hypothesis suggests that these SNPs influence expression levels of the *TNIP1* gene, as was shown for lower ABIN1/TNIP1 protein levels in a limited cohort of systemic sclerosis patients [26], thus potentially lowering immune activation thresholds. This hypothesis is consistent with

our findings that lowering ABIN1/TNIP1 protein levels in monocyte-derived DCs allows them to achieve a “semimature” state and to activate T cell responses.

Previously, we demonstrated that LILRB1 ligation during differentiation of DCs in vitro resulted in a phenotype in which the cells remain CD14⁺ and express low levels of HLA-DR, which is not up-regulated on LPS exposure [4]. This cellular phenotype resembles that of a population of “immunosuppressive monocytes” detected in patients with a variety of hematologic [13, 29] or other malignancies [30–32]. These immunosuppressive monocytes are sometimes referred to as MDSC, and their numbers correlate with a poorer prognosis in several studies [32, 33], with their poor stimulatory capacity for T cell activation often attributed to contribute to this effect. When comparing the monocytes obtained from biopsies of patients with NHL to circulating monocytes from healthy controls, we observed that patient monocytes had lower surface expression of antigen presentation and costimulatory molecules. Patient monocytes also expressed intracellular levels of ABIN1/TNIP1 protein approximately twice that of monocytes obtained from healthy donors. Reducing ABIN1/TNIP1 expression levels, followed by LPS stimulation, allowed these patient cells to increase expression of relevant cell surface molecules, secrete cytokines, and potentially activate T cell responses.

LILRB1 is expressed by >99% of monocytes, and it is plausible that HLA class I-mediated inhibition of monocyte differentiation is the cause of the increased ABIN1/TNIP1 levels in the immunosuppressive monocyte population, as suggested by previous studies of murine MDSC [9, 11]. Although tumor cells often down-regulate specific HLA antigen expression to escape recognition by class I-restricted cytotoxic T lymphocytes [34], LILRB1 recognizes all HLA-A, HLA-B, and HLA-C antigens and is unlikely to be affected overall by specific HLA antigen loss. In addition, many tumors express the nonclassical HLA-G protein [35], an efficient ligand for LILRB1 [36].

Further investigation of the regulatory mechanism we have identified is warranted in view of the novel functions of ABIN1/TNIP1 [37] in control of cellular functions. However, our findings in this study suggest the LILRB1-ABIN1/TNIP1 pathway is a potential target for manipulation of immune activity, perhaps by blocking LILRB1 interactions with HLA class I in lymphoma and other malignancies to reduce ABIN1/TNIP1 levels, which may allow maturation and differentiation of immunosuppressive monocytes into functional antitumor antigen-presenting cells. Alternatively, the use of antibody or recombinant protein therapies [38] to ligate LILRB1 should increase ABIN1/TNIP1 levels and activation thresholds in autoimmune disease and organ transplantation.

AUTHORSHIP

R.C.K, M.K, A.R, and N.T.Y performed experiments and collected and analyzed data; R.C.K and N.T.Y planned the experiments; A.L and M.A.V contributed patient samples; and R.C.K and N.T.Y wrote the manuscript, with valuable contributions from A.L and M.A.V.

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DISCLOSURES

The authors declare no conflicts of interests.

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