IMMUNOMODULATORY EFFECTS OF DRUGS FOR TREATMENT OF IMMUNE-RELATED DISEASES

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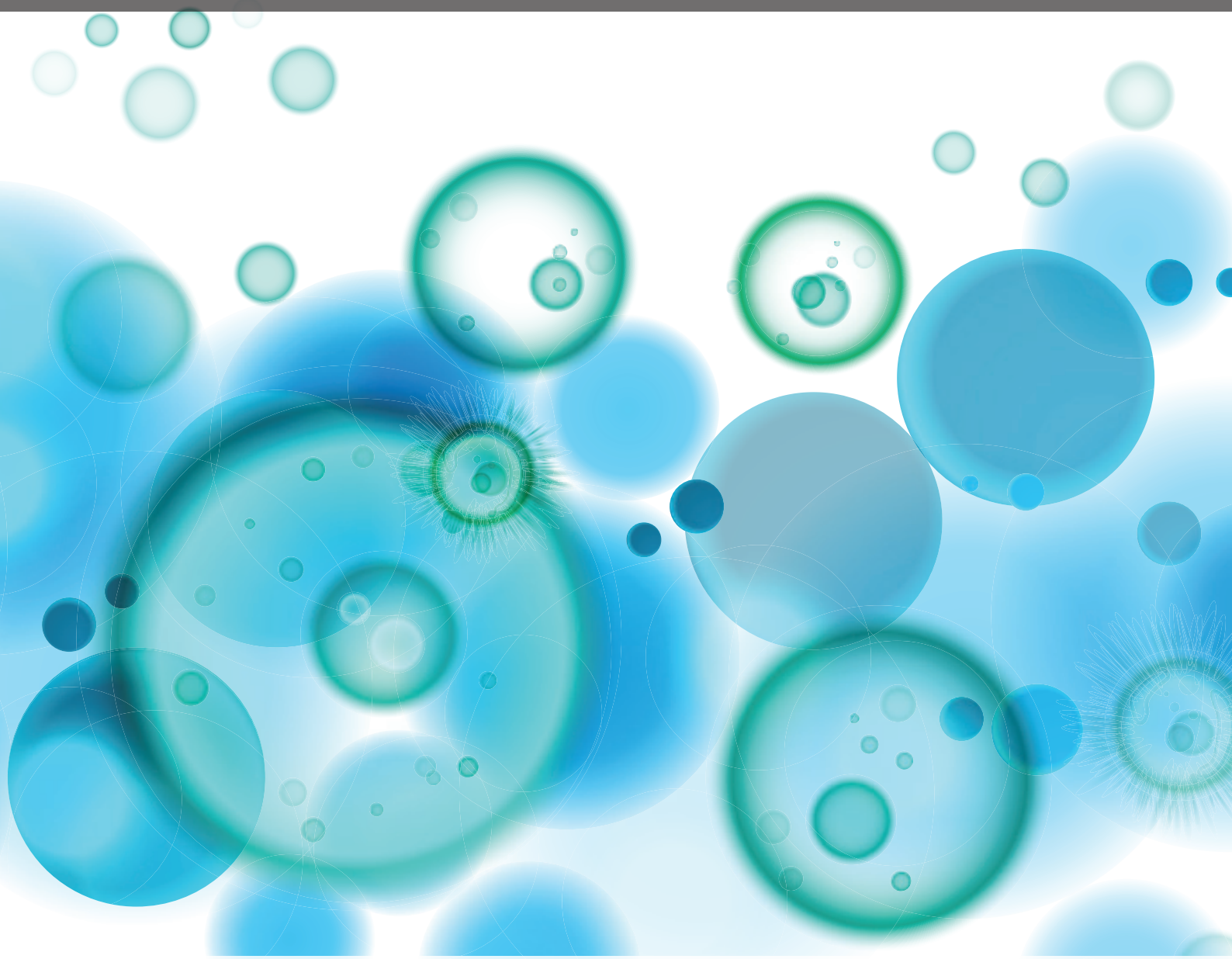

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IMMUNOMODULATORY EFFECTS OF DRUGS FOR TREATMENT OF IMMUNE-RELATED DISEASES

Topic Editor:
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More than 90% of diseases possess immunological abnormalities. Disorders such as inflammation, hypersensitivity, autoimmunity and immunodeficiency are simple examples of how the immune system misinterprets its surroundings and goes awry. Multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, inflammatory bowel diseases, among many others are manifestations of immune cells attacking normal tissues. On the other hand, damping the immune system leads to diseases such as cancer, AIDS, and severe combined immunodeficiency. The last ten years witnessed an explosion in developing drugs that target the immune system. Several novel monoclonal antibodies have been approved for treatment of various diseases confirming that personalized medicine approach is robust in combating diseases. Hence, the future holds great promise for using personalized and targeted medicine rather than generalized medications that, in most circumstances have proven to be ineffective and characteristically exert side effects. Approaches such as generating novel adjuvants that can stimulate the immune system without harmful side effects, targeting inflammatory cytokines and chemokines, harnessing and activating innate immune cells such as natural killer cells or dendritic cells, are examples of future approaches to treat autoimmune diseases, AIDS, and various forms of cancer resulting from chronic inflammation. More recently, targeting immune checkpoint molecules have shown therapeutic response against lung cancer and melanoma. Identifying molecules involved in autophagy is another example of how personalized medicine might help treat patients with refractory asthma and autoimmune diseases.

This topic introduces the reader to these novel approaches of manipulating the immune system and developing targeted therapeutic strategies for treatment of various diseases.

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EDITORIAL
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Editorial: Immunomodulatory Effects of Drugs for Treatment of Immune-Related Diseases

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Keywords: cancer, multiple sclerosis, chemokines, autophagy, adjuvants, natural killer cells

Editorial on the Research Topic

Immunomodulatory Effects of Drugs for Treatment of Immune-Related Diseases

The last 10 years have witnessed robust development of several drugs as a consequence of intense collaboration among academics, clinicians, scientists, and pharmaceutical companies. This has led to approval of these drugs for treatment of autoimmune diseases, cancer and AIDS, among many other diseases. This research topic focuses on the mechanism of action (MOA) of these drugs and reports new findings related to autophagy and adjuvanticity of drugs.

In an article by Kim et al., the function of the chemical 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) was examined. This chemical has been previously used to facilitate the delivery of hydrophobic drugs and recently used as an adjuvant. The authors reported that HP-β-CD induced the maturation of dendritic cells (DCs), including upregulating the expression of co-stimulatory molecules and MHC class II molecules (Kim et al.). This maturation promoted these cells to become professional antigen-presenting cells inducing the proliferation of T cells. The adjuvanticity of the chemical was further examined in a mouse footpad immunization model, and it was concluded that HP-β-CD functions as a potent adjuvant inducing DCs maturation, a process mediated through lipid draft formation.

The theme of new adjuvants was further discussed in an article by Miccadei et al., who reviewed the potential effects of ω3-polyunsaturated fatty acids (PUFA) as an adjuvant for chemotherapy and/or radiotherapy regiments to treat colorectal cancer. Interest has been recently generated in the effects of diets on cancer, obesity, diabetes, and many more metabolic disorders. Administration of PUFA-enriched diets may have several advantages in controlling inflammatory responses that may benefit patients with inflammatory disorders. Further, PUFA-enriched diets might also be considered for preventing colorectal cancer, provided that the MOA of this adjuvant is clearly understood.

Musters et al. advocated the use of off-label drugs to treat immune-mediated diseases that failed all other possible treatments. The authors described a case of multicentric Castleman's disease (MCD) with disseminated polyclonal B cell proliferation throughout multiple lymph nodes. This patient was treated in the authors' center with tocilizumab, a humanized monoclonal antibody against human IL-6 receptor, which was approved for treatment of rheumatoid arthritis (RA) and juvenile RA patients, but not for Castleman's disease patients. The patient showed a remarkable response to the drug including lower C-reactive protein level, improved sedimentation rate, and normalization of hemoglobin level. The authors concluded that off-label prescription drugs can be used after carefully evaluating the efficacy and safety of these biologic drugs.

The effects of latency-reversing agents (LRAs) on natural killer (NK) cells during clearance of HIV infection was explored in this issue (Garrido et al.). NK cells are the predominant innate immune cells that are important to fight against HIV infection (1), as well as many other viral infections.

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However, the effects of LRAs on NK cell activities have not been previously investigated in details, which are the subjects of this report (Garrido et al.). The authors utilized several drugs including histone deacetylase (HDAC) inhibitors such as SAHA or vorinostat, romidepsin, and panobinostat, which are standard chemotherapeutic agents for several cancer patients. They also used protein kinase C (PKC) agonists including prostratin and ingenol. Intriguingly, exposure to romidepsin and panobinostat was detrimental for NK cell activity, whereas exposure to the PKC agonists may be beneficial. Hence, caution should be exercised before these drugs can be considered to eradicate HIV infection, particularly in relation to their effects on the antiviral/antitumor effectors NK cells.

The potential effects of NK cells in cancer immunotherapy were also discussed (Maghazachi et al.). It was observed that NK cells incubated *in vitro* with two drugs; one approved for treatment of multiple sclerosis (MS), namely, dimethyl fumarate (DMF), and the second known as monomethyl fumarate (MMF), upregulated the expression of chemokine receptor 10 (CCR10) on NK cell surfaces. This was corroborated with increased chemotaxis of these cells toward the concentration gradients of the ligands for CCR10 such as CCL27 and CCL28, as well as enhanced NK cell cytotoxicity against tumor cell lines. These results might have clinical implication for harnessing the antitumor effector cells *in vitro* in order to induce their migration toward the sites of tumor growth, particularly those secreting CCL27 and CCL28. Further, the potential therapeutic effects of DMF and MMF for various disorders including MS, neurodegenerative diseases, cancer, and gastrointestinal ulcers were reviewed in this issue (Al-Jaderi and Maghazachi). The authors described the effects of DMF and MMF on various immune cells including T cells, B cells, NK cells, DCs, macrophages, and neutrophils, as well as on keratinocytes, endothelial cells, microglia, astrocytes, and neurons (Al-Jaderi and Maghazachi).

The topic of autophagy was discussed in two separate publications. Poon et al. described a novel effect of collagen type V alpha 1 (COL5A1) in exacerbating asthma. The authors suggested that autophagy-related 5 (ATG5) gene expression was positively correlated with the expression of COL5A1, and the subsequent deposition of collagen in the lungs of refractory asthmatic patients. These results indicate that dysregulation of autophagy may correlate with the fibrosis in the airway of asthmatic patients, suggesting that ATG5 might be a target for therapy in refractory asthmatic patients. On the other hand, Li et al. described beneficial effects for autophagy in autoimmune hepatitis, an autoimmune disorder characterized by abnormal Th1 activation and reduced apoptosis. It was observed that mice rich in n-3 PUFA resist liver damage after the administration of concanavalin-A, which induced hepatitis in these mice. The authors sought to examine the MOA of n-3 PUFA and observed that this fatty acid enhanced T cell autophagy and reduced T cell activation resulting in protection against severe liver injury. Collectively, these observations might form a basis for the potential therapeutic application of n-3 PUFA as well as other related fish products such as eicosapentaenoic acid or docosahexaenoic acid, toward liver damage and autoimmune hepatitis.

Hematological disorders were described in two papers by the same authors. In the first, Brenner et al. studied the effects of chemokine receptor expression in 79 patients with acute myeloid leukemia (AML) an aggressive form of leukemia and reported that CCL28 is constitutively released by primary human AML cells. Therefore, this chemokine may play a role in the cross talk among leukemic cells and neighboring bone marrow cells. However, the effect can be either inhibitory for the growth of leukemia if stem cell factor or GM-CSF factor is present, whereas the final effect of CCL28 can be stimulatory when Flt3 ligand is exogenously released. Although it was suggested that blocking chemokine receptor in this model might be beneficial for AML patients, caution should be exercised as CCR10, the receptor for CCL28, has been found to be up-regulated on the surface of the anti-tumor NK cells, which may facilitate their recruitment toward the sites of tumor growth (Maghazachi et al.). Therefore, a balance between the stimulatory and inhibitory effects of chemokine/chemokine receptor expression must be carefully weighed before regiments of cancer therapy are developed based on this concept. In a subsequent study, the same authors examined cytokine-mediated cross talk among AML and mesenchymal stem cells (MSCs), and observed that MSCs release cytokines that increase the viability and proliferation of leukemic cells, despite the heterogeneity of AML (Brenner et al.). These findings may have implications in targeting the relevant cytokines or their receptors for therapeutic purposes of aggressive leukemia.

In summary, the topics described in this issue should form the basis for understanding the MOA of drugs that may facilitate the development of personalized medicine to treat solid tumors, blood lymphoproliferative disorders, autoimmune diseases, and inflammatory conditions.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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1. Maghazachi AA, Al-Aoukaty A. Chemokines activate natural killer cells through heterotrimeric G-proteins: implications for the treatment of AIDS and cancer. *FASEB J* (1998) 12(11):913–24.

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ORIGINAL RESEARCH
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Induction of Dendritic Cell Maturation and Activation by a Potential Adjuvant, 2-Hydroxypropyl-β-Cyclodextrin

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2-Hydroxypropyl-β-cyclodextrin (HP-β-CD) is a chemically modified cyclic oligosaccharide produced from starch that is commonly used as an excipient. Although HP-β-CD has been suggested as a potential adjuvant for vaccines, its immunological properties and mechanism of action have yet to be characterized. In the present study, we investigated the maturation and activation of human dendritic cells (DCs) treated with HP-β-CD. We found that DCs stimulated with HP-β-CD exhibited a remarkable upregulation of costimulatory molecules, MHC proteins, and PD-L1/L2. In addition, the production of cytokines, such as TNF-α, IL-6, and IL-10, was modestly increased in DCs when treated with HP-β-CD. Furthermore, HP-β-CD-sensitized DCs markedly induced the proliferation and activation of autologous T lymphocytes. HP-β-CD also induced a lipid raft formation in DCs. In contrast, filipin, a lipid raft inhibitor, attenuated HP-β-CD-induced DC maturation, the cytokine expression, and the T lymphocyte-stimulating activities. To determine the *in vivo* relevance of the results, we investigated the adjuvanticity of HP-β-CD and the modulation of DCs in a mouse footpad immunization model. When mice were immunized with ovalbumin in the presence of HP-β-CD through a hind footpad, serum ovalbumin-specific antibodies were markedly elevated. Concomitantly, DC populations expressing CD11c and MHC class II were increased in the draining lymph nodes, and the expression of costimulatory molecules was upregulated. Collectively, our data suggest that HP-β-CD induces phenotypic and functional maturation of DCs mainly mediated through lipid raft formation, which might mediate the adjuvanticity of HP-β-CD.

Keywords: 2-hydroxypropyl-β-cyclodextrin, vaccine adjuvants, dendritic cells, maturation, lipid raft

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INTRODUCTION

Cyclodextrins are cyclic oligosaccharides composed of sugar molecules. These oligosaccharides consist of 6, 7, or 8 α-D-glucopyranose units bound *via* α-1,4-glycosidic linkages, namely, α-, β-, or γ-cyclodextrin, respectively. Cyclodextrins exhibit a bucket-shaped structure with a hydrophobic central cavity and a hydrophilic exterior (1). Cyclodextrins can efficiently form water-soluble inclusion complexes with hydrophobic molecules, which enhances the solubility and bioavailability of many insoluble compounds (2, 3). In addition, cyclodextrins improve and prolong the medicinal

effects of drugs by controlling compound release, increasing their stability, and regulating the metabolism of the incorporated molecules (4). Due to these physicochemical properties, cyclodextrins are commonly utilized as excipients of pharmaceutical agents, food products, and cosmetics.

β-Cyclodextrin and some of its derivatives are widely used additives of commercial drugs because they are easy to produce, belong to generally recognized as safe (GRAS) materials for humans, and have improved solubility compared with the other cyclodextrins (4, 5). 2-Hydroxypropyl-β-cyclodextrin (HP-β-CD) is a chemically modified derivative of β-cyclodextrin that exhibits an enhanced safety profile compared with its naturally occurring parent compound (4). HP-β-CD is used as an excipient for cardiac dysrhythmia, inflammation, and fungal disease medications (6). Furthermore, HP-β-CD has been proposed as a vaccine adjuvant because it markedly enhances humoral immune responses to an influenza vaccine without any adverse effects (7). However, the immunological properties and action mechanism of HP-β-CD need to be further characterized for the human use.

Dendritic cells (DCs) are professional antigen-presenting cells that bridge the innate and adaptive immunities. Immature DCs are characterized by high endocytic activity coincident with a low expression of costimulatory molecules and cytokines (8). When immature DCs meet microbial antigens or damage-associated molecular patterns, they begin the process of maturation (8, 9). This process is accompanied by upregulation of (i) MHC associated with the antigen; (ii) costimulatory molecules including CD40, CD80, and CD86; and (iii) inflammatory cytokines such as IL-12, IL-6, and TNF-α (10). These phenotypic changes optimize conditions for T lymphocyte activation and differentiation (11, 12). Since mature DCs potently stimulate adaptive immunity better than immature DCs, many vaccine adjuvants currently under development are designed to efficiently induce functional maturation and activation of DCs (13–15). In the present study, we investigated immunological function of HP-β-CD by determining its ability to mature and activate DCs leading to the induction of adaptive immunity.

MATERIALS AND METHODS

Reagents and Chemicals

2-Hydroxypropyl-β-cyclodextrin was purchased from Sigma-Aldrich (Saint Louis, MO, USA). Ficoll-Paque PLUS was obtained from GE Healthcare (Uppsala, Sweden). Fetal bovine serum (FBS) was purchased from GIBCO (Grand Island, NY, USA). RPMI-1640 medium and HyClone™ penicillin–streptomycin solution were from HyClone (Logan, UT, USA). Anti-human CD14 magnetic beads (clone: MΦP9) and anti-human CD3 magnetic beads (clone: HIT3a) were purchased from BD Biosciences (San Diego, CA, USA). Recombinant human granulocyte macrophage-colony stimulating factor (GM-CSF) and IL-4 were purchased from R&D Systems (Minneapolis, MN, USA) and CreaGene (Sungnam, Korea), respectively. Recombinant murine GM-CSF was obtained from CreaGene. 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate and enzyme-linked immunosorbent assay (ELISA) kits for the quantification of human TNF-α, IL-6, IL-12p70, and IL-10, and mouse TNF-α and IL-6 were purchased from BioLegend (San Diego, CA, USA). 5,6-Carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) and the Vybrant® Alexa Fluor® 594 lipid raft-labeling kit were obtained from Molecular Probes (Eugene, OR, USA). Filipin, bovine serum albumin (BSA), 2,2,2-tribromoethanol, 2-methyl-2-butanol, ovalbumin (OVA), and red blood cell (RBC)-lysis buffer were purchased from Sigma-Aldrich. Luria Bertani broth and Bacto™ Agar were purchased from BD Biosciences. FITC-labeled anti-human CD80 (clone: 2D10), PE-labeled anti-human CD83 (clone: HB15e), APC-labeled anti-human CD86 (clone: IT2.2), APC-labeled anti-PD-L1 (clone: 29E.2A3), PE-labeled anti-human PD-L2 (clone: 24F.10C12), APC-labeled anti-human CD25 (clone: BC96), PE-labeled anti-human CD4 (clone: RPA-T4), and PE-labeled anti-human CD8 (clone: RPA-T8) antibodies were purchased from BioLegend. FITC-labeled anti-human HLA-DR, DP, DQ (clone: Tu39) antibody for MHC class II, FITC-labeled anti-mouse CD86 (clone: GL-1) antibody, PE-labeled anti-mouse CD80 (clone: 16-10A1) antibody, PerCP-labeled anti-mouse CD11c (clone: N418) antibody, and FITC-labeled anti-mouse I-A^b (clone: 25-9-17) antibody for MHC class II were obtained from BD Biosciences. All isotype-matched antibodies were purchased from BD Biosciences or BioLegend. Horseradish peroxidase (HRP)-conjugated anti-mouse total IgG, anti-mouse IgG1, and anti-mouse IgG2a were purchased from Southern Biotech (Birmingham, AL, USA).

Preparation of Human Monocyte-Derived DCs

Human peripheral blood samples donated by healthy adult male subjects ($n = 15$) were provided from the Korean Red Cross (Seoul, Korea) after obtaining informed consent. All experiments using human blood were conducted under the approval of the Institutional Review Board of Seoul National University. The peripheral blood was diluted in phosphate-buffered saline (PBS) and overlaid on the Ficoll-Paque PLUS, and peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation as previously described (16, 17). PBMCs were washed with PBS three times to remove platelets and the remaining Ficoll. To isolate $CD14^+$ monocytes, PBMCs were incubated with anti-human CD14 magnetic beads for 30 min at room temperature. The cells were separated on a magnetic field, and then $CD14^+$ cells were enriched by positive selection. $CD14^+$ monocytes were suspended at a concentration of 2×10^6 cells/ml in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin–streptomycin solution. The isolated monocytes were differentiated into immature DCs in the presence of human recombinant GM-CSF (5 ng/ml) and IL-4 (10 ng/ml) for 6 days. Cell culture medium supplemented with GM-CSF and IL-4 was added every 3 days.

Preparation of HKEC

Escherichia coli BL21 (DE3) strain obtained from Stratagene (La Jolla, CA, USA) was cultured in Luria Bertani broth at 37°C until reaching mid-log phase and then harvested by centrifugation. The harvested bacterial cells were resuspended in PBS and killed

by heating at 60°C for 1 h. To confirm the complete killing, the heat-treated *E. coli* was plated onto Luria Bertani agar plates and cultured overnight at 37°C. No bacterial colonies were observed.

Analysis of DC Phenotypes

Mouse BM-DCs (5×10^5 cells/ml) were stimulated with either HP-β-CD (0, 0.1, or 1 mg/ml) or LPS (100 ng/ml) in the presence of murine GM-CSF (10 ng/ml) for 24 h. Human monocyte-derived DCs (2.5×10^5 cells/ml) were stimulated with either HP-β-CD (0, 0.1, 0.3, or 1 mg/ml) or HKEC (1×10^7 CFU/ml) in the presence of GM-CSF (2.5 ng/ml) and IL-4 (5 ng/ml) for 24 h. The unstimulated or stimulated DCs were then stained with fluorochrome-conjugated monoclonal antibodies specific to CD80, CD83, CD86, MHC class II, PD-L1, or PD-L2 for 30 min on ice, and then the cells were washed once with PBS. The geometric mean fluorescence intensity (MFI) of each group of DCs was obtained by flow cytometric analysis. More than 8,500 events were acquired for each group, and cell debris and dead cells were gated out. Phenotypes of DCs were analyzed using flow cytometry with FACSCalibur (BD Biosciences) and FlowJo software (TreeStar, San Carlos, CA, USA).

Cytokine Quantification

The levels of cytokines produced by DCs were quantified by ELISA, as previously described (18). Briefly, mouse BM-DCs (5×10^5 cells/ml) were stimulated with either HP-β-CD (0, 0.1, or 1 mg/ml) or LPS (100 ng/ml) in the presence of murine GM-CSF (10 ng/ml) for 24 h. Human monocyte-derived DCs (2.5×10^5 cells/ml) were stimulated with either HP-β-CD (0, 0.1, 0.3, or 1 mg/ml) or HKEC (1×10^7 CFU/ml) in the presence of GM-CSF (2.5 ng/ml) and IL-4 (5 ng/ml) for 24 h. The levels of TNF-α, IL-6, IL-12p70, and IL-10 in the culture supernatants were measured by ELISA kits according to the manufacturers' instructions.

Coculture of DCs with Autologous T Lymphocytes

To isolate $CD3^+$ T lymphocytes, $CD14^+$ monocyte-depleted PBMCs were incubated with anti-human CD3 magnetic beads for 30 min, and then $CD3^+$ T lymphocytes were enriched by positive selection. The isolated $CD3^+$ T lymphocytes were labeled with 10 μM CFDA-SE for 15 min at 37°C and washed with PBS. Immature DCs (2.5×10^5 cells/ml) were stimulated with either HP-β-CD (0, 0.1, 0.3, or 1 mg/ml) or HKEC (1×10^7 CFU/ml) in the presence of GM-CSF (2.5 ng/ml) and IL-4 (5 ng/ml) for 16 h. After removal of the culture supernatant, the DCs (5×10^4 cells) were cocultured with carboxyfluorescein succinimidyl ester (CFSE)-labeled autologous $CD3^+$ T lymphocytes (5×10^4 cells) for 4–5 days. To analyze DC-mediated proliferation and activation of T lymphocyte subsets, the cells were stained with anti-human CD4, anti-human CD8, or anti-human CD25 antibodies and then analyzed by flow cytometry.

Analysis of Lipid Raft Formation

Dendritic cells (2.5×10^5 cells/ml) were stimulated with HP-β-CD (1 mg/ml) in the presence or absence of filipin (30 μg/ml) for 45 min. Cell staining was performed with the lipid raft-labeling kit according to the manufacturer's instructions. Briefly, unstimulated or HP-β-CD-stimulated DCs were washed once with ice-cold PBS and stained with Alexa Fluor® 594-conjugated CTB conjugate for 10 min on ice. The DCs were washed once with ice-cold PBS and incubated with rabbit serum containing anti-CTB antibodies for 10 min on ice to crosslink lipid rafts on the surface of the DCs. Formation of lipid raft on the DCs was analyzed by confocal laser scanning microscopy (Carl Zeiss MicroImaging GmbH, Jena, Germany). Fluorescence intensity of DCs was analyzed by ZEN software, Lite Edition (Carl Zeiss, Oberkochen, Germany).

Immunization with OVA Plus HP-β-CD in Mice

Seven-week-old male C57BL/6 mice were purchased from Orient Bio (Sungnam, Korea) and maintained in a specific pathogen-free animal facility. All experiments using animals were conducted under the approval of the Institutional Animal Care and Use Committee of Seoul National University. Care and treatment of the animals were carried out in accordance with the approved guidelines. Mice were anesthetized by intraperitoneal injection of Avertin (2,2,2-tribromoethanol and 2-methyl-2-butanol) and administered with 20 μg OVA with or without 3 mg HP-β-CD through a hind footpad. The mice were maintained for 24 h or 7 days and sacrificed to obtain the draining lymph nodes and the blood, respectively.

Analysis of DC Activation in the Draining Lymph Nodes

Twenty-four hours after the immunization with OVA in the presence or absence of HP-β-CD, draining lymph nodes, including popliteal and inguinal lymph nodes, were harvested and dissociated into a single cell suspension on a cell strainer (BD Biosciences). To analyze DC populations in the lymph nodes, the cells were stained with fluorochrome-conjugated antibodies specific to CD11c, MHC class II, CD86, and CD80 at 4°C for 30 min. $CD11c^+$ MHC class II^+ cells in the lymph nodes and their phenotypic changes upon the administration of OVA in the presence or absence of HP-β-CD were analyzed by flow cytometry using FACSVerse (BD Biosciences).

Preparation of Mouse Bone Marrow-Derived DCs

Bone marrow-derived DCs (BM-DCs) were generated as previously described (19), with minor modifications. Briefly, BM cells were isolated from mouse femurs and tibias, and the RBCs were lysed with the RBC lysis buffer. Isolated BM cells were suspended in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin–streptomycin solution and plated in petri dishes at 4×10^5 cells/ml. The BM cells were differentiated into immature DCs in the presence of murine recombinant GM-CSF (20 ng/ml) for 7 days. Cell culture medium supplemented with GM-CSF was added every 4 days.

Statistical Analysis

The statistical significance of differences between the experimental groups and the control group was analyzed using Student's *t*-test. *P*-values less than 0.05 were considered statistically significant. Results are presented as mean value ± SD or SEM.

RESULTS

HP-β-CD Induces Maturation of Human Monocyte-Derived DCs

Maturation of DCs is an essential process for activating antigen-specific adaptive immunity and includes the upregulation of costimulatory molecules, MHC class I/II, and certain cytokines (12). Thus, we first examined the effects of HP-β-CD on the phenotypic maturation and cytokine production in human monocyte-derived DCs. Notably, HP-β-CD was not cytotoxic to DCs at concentrations up to 1 mg/ml (Figure S1A in Supplementary Material). Stimulation with HP-β-CD remarkably augmented the expression of costimulatory molecules, such as CD80, CD83, and CD86 (**Figures 1A,D**), together with MHC class II (**Figures 1B,E**). Additionally, HP-β-CD-treated DCs exhibited increased expression of inhibitory molecules, such as PD-L1 and PD-L2 (**Figures 1C,F**). Moreover, HP-β-CD weakly increased the expression of proinflammatory cytokines, including TNF-α and IL-6, in a dose-dependent manner (**Figures 1G,H**). Furthermore, HP-β-CD treatment slightly increased IL-10 expression in DCs (**Figure 1I**), and IL-12p70 was not detected in the culture supernatant of HP-β-CD-treated DCs (data not shown). These results suggest that HP-β-CD upregulates the expression of maturation markers coincident with weak induction of cytokines in human monocyte-derived DCs.

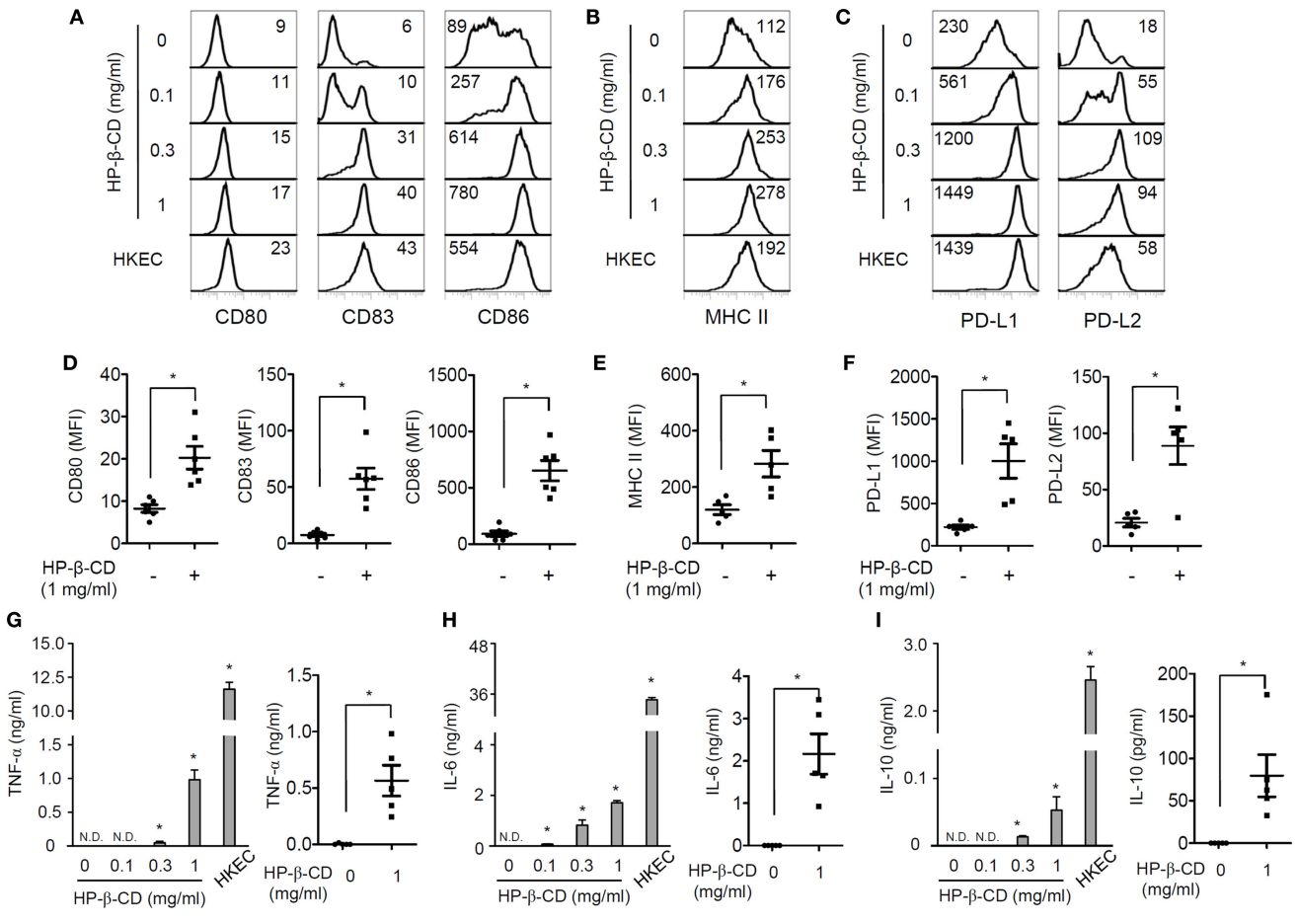

FIGURE 1 | HP-β-CD induces maturation of human monocyte-derived DCs. Human CD14$^+$ monocytes (2 × 10^6 cells/ml) were differentiated into immature DCs in the presence of GM-CSF and IL-4 for 6 days. The monocyte-derived DCs (2.5 × 10^5 cells/ml) were stimulated with HP-β-CD (0, 0.1, 0.3, or 1 mg/ml) in the presence of GM-CSF and IL-4 for 24 h. **(A–F)** Expression of **(A,D)** CD80, CD83, and CD86, **(B,E)** MHC class II, and **(C,F)** PD-L1 and PD-L2 was analyzed by flow cytometry. The number on each histogram indicates MFI of the DCs. **(D–F)** The scatter plots below the histograms indicate the average MFI of DCs ± SEM for each molecule (n = 5). The levels of **(G)** TNF-α, **(H)** IL-6, and **(I)** IL-10 in the DC culture supernatants were measured by ELISA. The scatter plots on the right side of each bar graph indicate the mean concentrations ± SEM of the cytokines (n = 5). HKEC (1 × 10^7 CFU/ml) was used as a positive control. Statistical differences between compared groups were analyzed by paired Student's *t*-test. N.D., not detected; *P < 0.05.

HP-β-CD-Sensitized DCs Elicit Autologous T Lymphocyte Proliferation and Activation

To examine whether HP-β-CD potentiates the T lymphocyte-activating ability of DCs, unstimulated or HP-β-CD-stimulated DCs were cocultured with autologous T lymphocytes, and the extents of T lymphocyte proliferation and activation were analyzed. HP-β-CD-sensitized DCs significantly induced T lymphocyte proliferation and CD25 expression, an activation marker of T lymphocytes (**Figures 2A,B**). The enhancement of proliferation and CD25 expression was observed in $CD4^+$ T lymphocytes but not in $CD8^+$ T lymphocytes (**Figures 2C,D**). However, HP-β-CD did not directly enhance the proliferative activity or CD25 expression of the T lymphocytes, indicating that HP-β-CD requires the help of DCs to activate T lymphocytes (Figures S2A,B in Supplementary Material). Therefore, the results suggest that HP-β-CD potentiates the ability of DCs to induce $CD4^+$ cells.

HP-β-CD Triggers Lipid Raft Formation on the DC Plasma Membrane

Lipid rafts are hydrophobic microstructures that play an important role as signal transduction platforms in many eukaryotic cells (20). Since HP-β-CD interacts with cellular cholesterol (21), an essential component of lipid rafts, we examined whether HP-β-CD induces lipid raft formation on the plasma membrane based on the hypothesis that lipid rafts are involved in the HP-β-CD-induced DC maturation. DCs were stimulated with HP-β-CD in the presence or absence of filipin, which disrupts

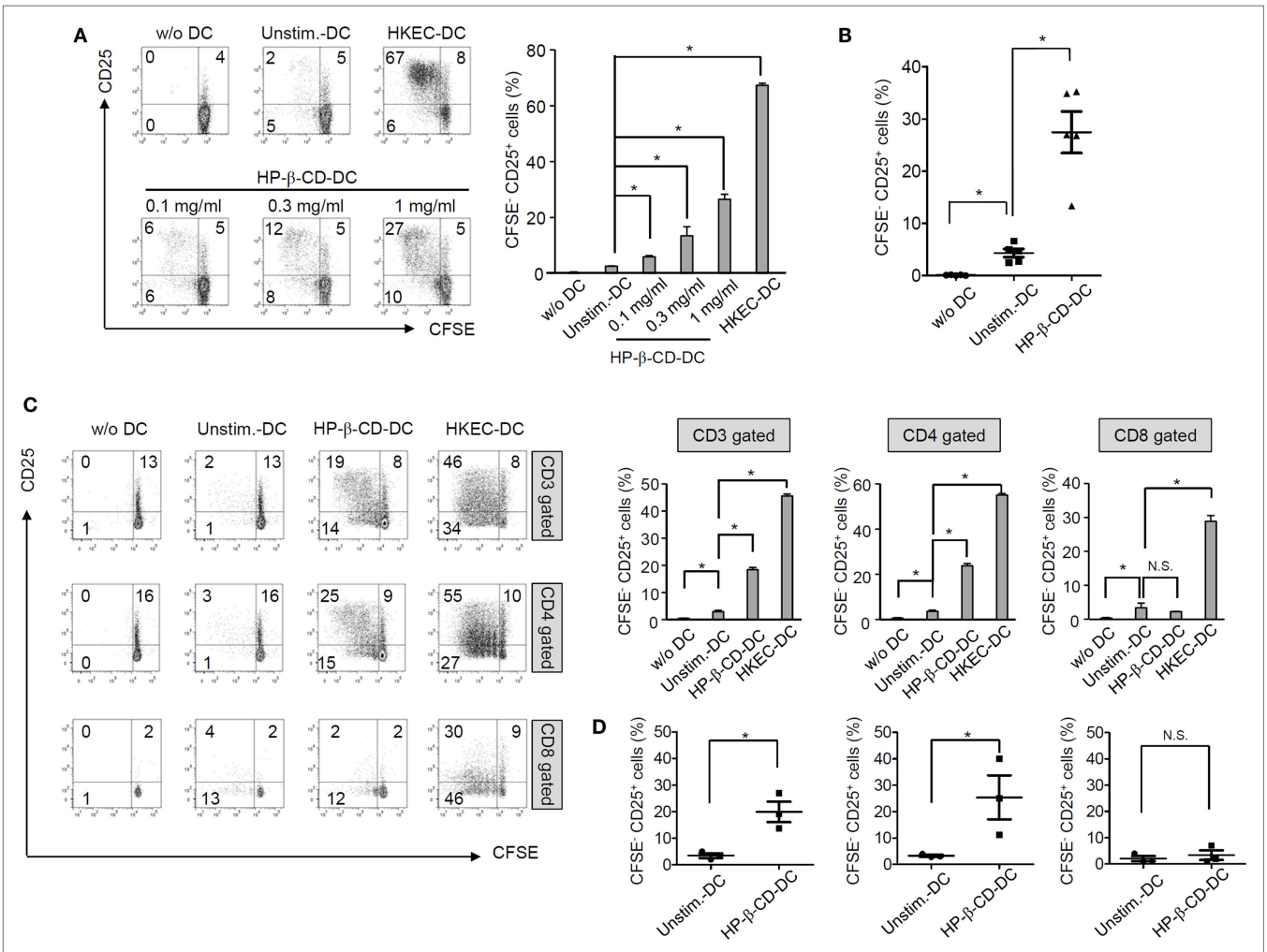

FIGURE 2 | HP-β-CD-sensitized DCs elicit the proliferation and activation of autologous T lymphocytes. Human monocyte-derived DCs (2.5×10^5 cells/ml) were stimulated with HP-β-CD (0, 0.1, 0.3, or 1 mg/ml) for 16 h. Then, the cells (5×10^4 cells) were cocultured with CFSE-labeled autologous $CD3^+$ T cells (5×10^4 cells) for 4 days. **(A)** T lymphocyte proliferation and CD25 expression were analyzed by flow cytometry. The bar graph beside the histograms displays the average of triplicate measurements for the proliferation of $CD25^+$ T lymphocytes. **(B)** A scatter plot indicates activated T lymphocytes by DCs ($n = 5$). **(C)** The proliferation and CD25 expression levels of $CD3^+$, $CD4^+$, or $CD8^+$ T lymphocytes were analyzed by flow cytometry. The bar graphs on the right side of the histograms exhibit the average of triplicate measurements for the proliferation of $CD25^+$ T lymphocytes. **(D)** Scatter plots exhibit activated $CD3^+$, $CD4^+$, or $CD8^+$ T lymphocytes by DCs ($n = 3$). The immune responses of T lymphocytes cultured with HKEC-stimulated DCs are shown as positive controls. The numbers on the histograms indicate the percentages of T lymphocytes in each quadrant. *$P < 0.05$. Unstim.-DC indicates T lymphocytes cocultured with unstimulated DCs.

a lipid raft formation, and fluorochrome-conjugated CTB was used to detect the formation of lipid rafts. As shown in **Figure 3**, HP-β-CD triggered lipid raft formation on the surface of DCs, whereas such effect was blocked by preexposure to the lipid raft inhibitor filipin.

Inhibition of Lipid Raft Formation Reduces HP-β-CD-Induced DC Maturation

Next, we further determined the role of lipid rafts in HP-β-CD-mediated DC maturation by blocking the lipid raft formation using filipin. Of note, treatment with filipin and/or HP-β-CD was not cytotoxic to DCs (Figure S1B in Supplementary Material). As shown in **Figures 4A–C**, treatment with filipin remarkably attenuated HP-β-CD-elicited expression of surface costimulatory molecules, PD-L1/L2, but not MHC class II. In addition, HP-β-CD-mediated induction of TNF-α, IL-6, and IL-10 in DCs was significantly reduced upon the inhibition of lipid raft (**Figure 4D**). Furthermore, lipid raft inhibition in DCs stimulated with HP-β-CD abrogated their ability to activate T lymphocytes (**Figure 4E**). These results suggest that HP-β-CD requires the formation of lipid rafts to trigger DC maturation and further to activate T lymphocytes.

HP-β-CD Potentiates Humoral Immune Responses to Coadministered Antigens and DC Activation in the Draining Lymph Nodes in Mice

Next, we determined whether HP-β-CD has an adjuvanticity with a mouse footpad immunization model (22). To immunize mice, OVA with or without HP-β-CD was injected through a hind footpad, and titers of serum antibodies specific to OVA were determined. Coadministration with HP-β-CD and OVA efficiently increased OVA-specific total IgG in the blood (Figure S3A in Supplementary Material). IgG1 was the major antibody subtype induced following immunization (Figure S3B in Supplementary Material), and no IgG2a antibodies were detected (data not shown). As DCs are crucial in the mediation of naive T cell responses, we subsequently analyzed DC populations in the draining lymph nodes, including popliteal and inguinal lymph nodes upon administration of OVA with or without HP-β-CD. Mice administered with OVA with HP-β-CD showed an increase in the size and cell number of the draining lymph nodes (**Figure 5A**). OVA administration with HP-β-CD markedly augmented the number of $CD11c^+$ MHC class II^+ cells in the popliteal and inguinal lymph nodes (**Figures 5B,C**). In addition, the DCs showed an upregulation in the expression of costimulatory molecules, including CD80 and CD86 (**Figures 5D,E**). To determine whether DC activation in the draining lymph nodes of mice administered with OVA and HP-β-CD is directly attributed to stimulatory functions of HP-β-CD, we examined HP-β-CD effects on the maturation of BM-DCs generated *in vitro*. HP-β-CD markedly upregulated the expression of surface costimulatory molecules, such as CD80, CD83, and CD86, and MHC class II (**Figure 5F**). In addition, HP-β-CD-treated BM-DCs modestly increased the production of proinflammatory cytokines including TNF-α and IL-6 in BM-DCs compared to LPS-stimulated BM-DCs (**Figure 5G**). Collectively, these results suggest that HP-β-CD-induced DC maturation and activation *in vivo* that might be necessary for the adjuvanticity.

DISCUSSION

Dendritic cells play a pivotal role in the induction of antigen-specific adaptive immune response by presenting the antigens to

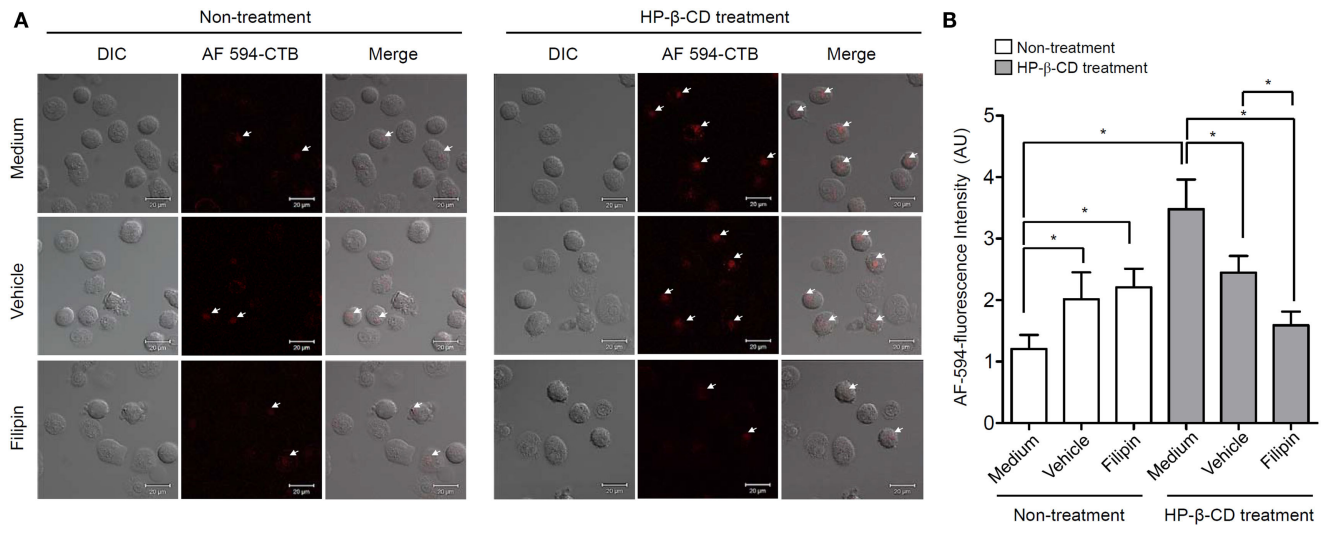

FIGURE 3 | HP-β-CD triggers lipid raft formation in DCs. Human monocyte-derived DCs (2.5×10^5 cells/ml) were stimulated with HP-β-CD (1 mg/ml) in the presence or absence of filipin (30 μg/ml) or methanol as a vehicle control for 45 min. **(A)** Stimulated DCs were stained with Alexa Fluor® 594-conjugated CTB, and the formation of lipid rafts on the cell surface was examined by confocal microscopy. **(B)** Fluorescence intensity of DCs was analyzed by ZEN software. DIC, differential interference contrast; AU, arbitrary unit. Images shown are representative of three similar results.

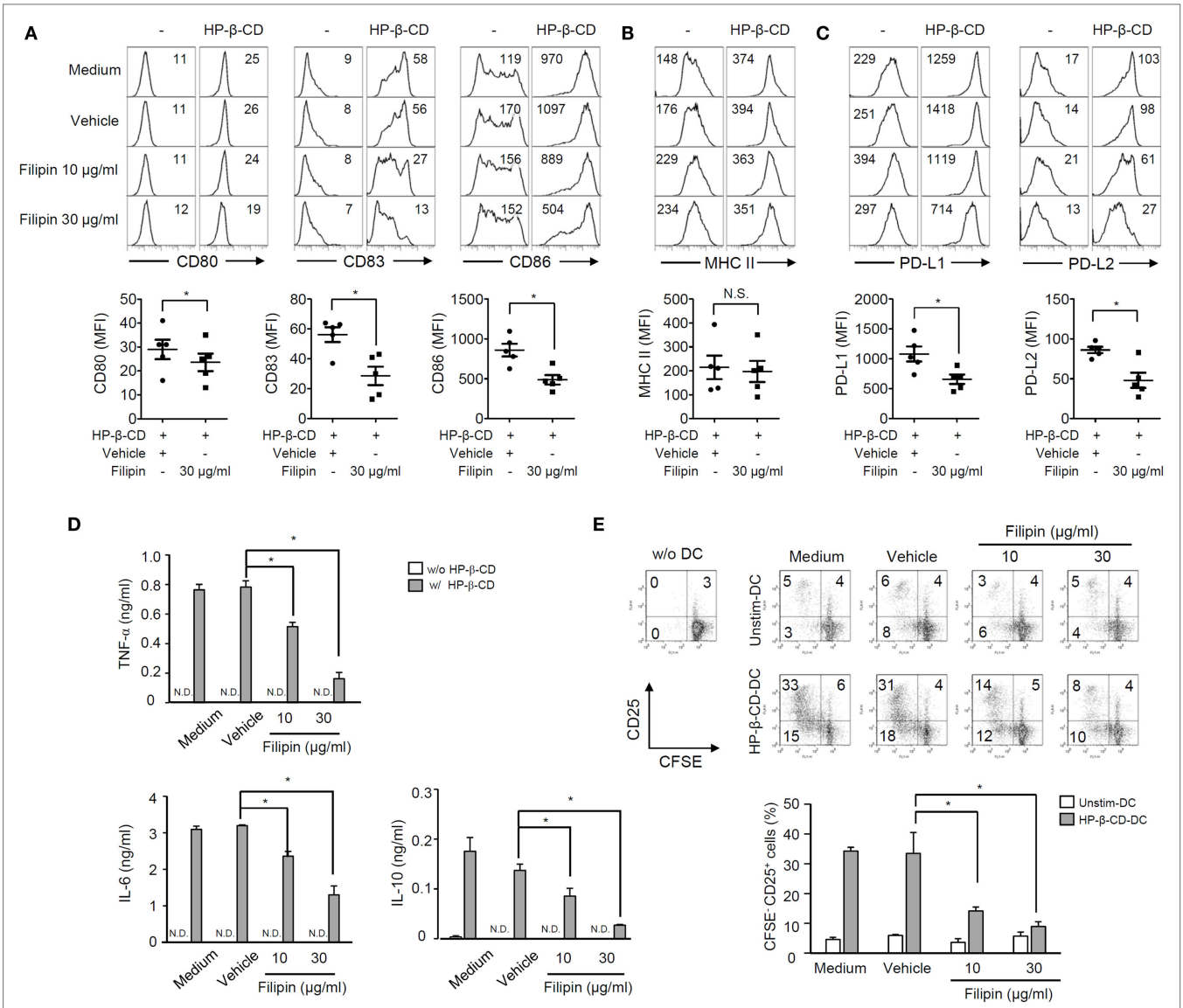

FIGURE 4 | Inhibition of lipid raft formation attenuates DC maturation, cytokine production, and autologous T cell activation induced by HP-β-CD. Human monocyte-derived DCs (2.5×10^5 cells/ml) were pretreated with filipin (10 and 30 μg/ml) or methanol as a vehicle control for 1 h, followed by stimulation with HP-β-CD (1 mg/ml) for another 24 h. **(A–C)** The expression of costimulatory molecules, MHC class II, and PD-L1/L2 was analyzed by flow cytometry. The numbers on the histograms indicate the MFIs of the cells. Scatter plots under the histogram show MFI of DCs ($n = 5$). Statistical differences between compared groups were analyzed by paired Student's *t*-test. N.S., not significant; $^*P < 0.05$. **(D)** The concentrations of TNF-α, IL-6, and IL-10 in the supernatants were measured by ELISA. **(E)** Unstimulated or HP-β-CD-stimulated DCs (5×10^4 cells) were cocultured with CFSE-labeled autologous T lymphocytes (5×10^4 cells) for 5 days. The levels of proliferation and CD25 expression of the T lymphocytes were analyzed by flow cytometry. The numbers on the histograms indicate the percentages of cells in each quadrant. The bar graph below the histograms displays the average of triplicate measurements ± SD for the frequencies of $CD25^+$ T lymphocytes with proliferative ability. Unstim.-DC indicates T lymphocytes cocultured with unstimulated DCs. Results shown are representative of three independent experiments. Statistical differences between compared groups were analyzed by Student's *t*-test. N.D., not detected; $^*P < 0.05$.

T cells and activating appropriate subtypes of T cells. HP-β-CD has long been utilized as a solubilizer and a delivery compound of hydrophobic drugs due to its physicochemical properties (6). However, recent studies have reported a novel beneficial effect of HP-β-CD on the immunogenicity of vaccines (7, 23) as well as on the progression of incurable metabolic disorders (24) and viral infections (25). Although the previous findings have suggested that HP-β-CD could be a potential vaccine adjuvant (7, 23), the effects of HP-β-CD on vaccine immunogenicity and DC properties have been poorly investigated. Here, we demonstrated that HP-β-CD has an adjuvanticity to OVA, and the maturation of DCs found in the draining lymph nodes in a mouse footpad immunization model. *In vitro* studies showed that HP-β-CD induces the maturation of DCs to induce the proliferation and activation of autologous T lymphocytes. Mechanism studies further showed that the lipid raft formation in DCs is essential for

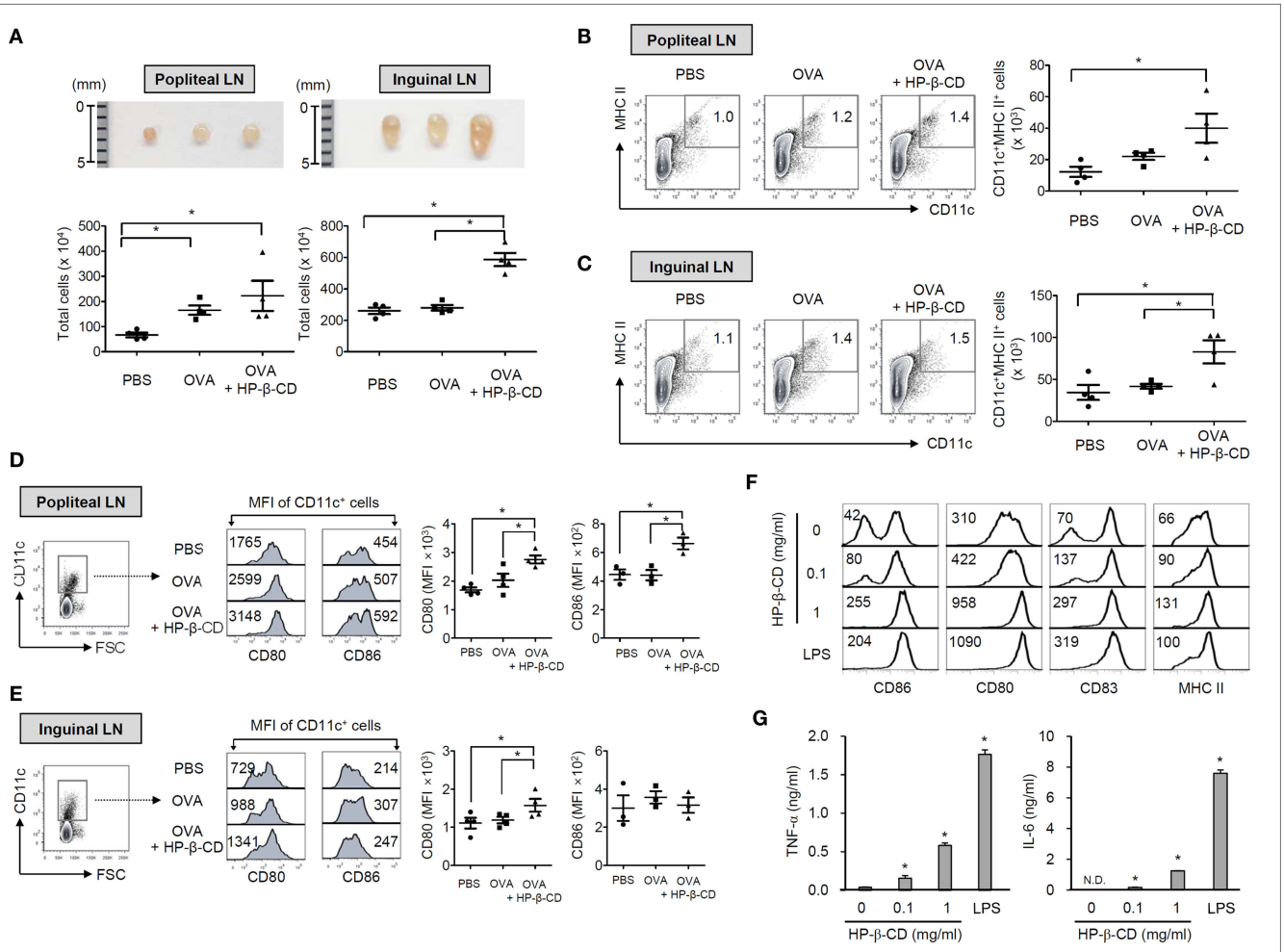

FIGURE 5 | HP-β-CD induces DC maturation and activation *in vivo* and *in vitro* mouse models. C57BL/6 mice were immunized with 20 μg OVA in the presence or absence of 3 mg HP-β-CD through a hind footpad. Twenty-four hours after the immunization, DC populations were analyzed in the draining lymph nodes including popliteal and inguinal lymph nodes. **(A)** Size of the lymph nodes and the total cell numbers were measured ($n = 4$). CD11c$^+$ MHC class II$^+$ cells in the draining lymph nodes, **(B)** popliteal and **(C)** inguinal lymph nodes were analyzed by flow cytometry ($n = 4$). Expression of CD80 and CD86 on CD11c$^+$ cells was analyzed in the draining lymph nodes, **(D)** popliteal and **(E)** inguinal lymph nodes by flow cytometry. Scatter plots on the right side the histograms indicate MFI of DCs for CD80 ($n = 4$) and CD86 ($n = 3$). **(F)** BM-DCs (5×10^5 cells/ml) were stimulated with HP-β-CD (0.1 or 1 mg/ml) or LPS (100 ng/ml) in the presence of murine GM-CSF for 24 h. The surface expression of various maturation markers, including **(F)** CD80, CD83, CD86, and MHC class II molecules on the DCs, was analyzed by flow cytometry. **(G)** The amounts of TNF-α and IL-6 in the culture supernatants of DCs were quantified by ELISA. The number on each histogram indicates percentage or MFI of the DCs. LN, lymph node. N.D., not detected. N.S., not significant. *$P < 0.05$. The result shown is a representative of four similar experiments.

the HP-β-CD-induced DC maturation and its subsequent activation of autologous T cells. These results suggest that HP-β-CD is a promising vaccine adjuvant to potently induce the maturation and activation of DCs.

In the present study, we demonstrated that HP-β-CD has an adjuvanticity. Administration of HP-β-CD efficiently augmented the antigen-specific IgG in the blood. Notably, HP-β-CD predominantly induced IgG1 but not IgG2, indicating preferential enhancement of Th2 responses rather than Th1 responses. Consistent with our findings, intranasal or subcutaneous administration of HP-β-CD increased the immunogenicity of influenza vaccines (7, 23). HP-β-CD seems to be appropriate as a mucosal adjuvant because it induces antigen-specific IgA and IgG in the airway mucosal tissues as well as in the blood (23).

Dendritic cell maturation is a prerequisite for the induction of adaptive immune response. Many previous studies have demonstrated that current vaccine adjuvants, such as aluminum salt (alum), cholera toxin (CT), and monophosphoryl lipid A (MPLA), can efficiently induce DC maturation (26–28). Here, we observed that HP-β-CD increased DCs in the draining lymph nodes as well as the upregulation of DC costimulatory molecules when coadministered with antigen. Additionally, HP-β-CD induced a marked increase in the expression of CD80, CD83, CD86, and MHC proteins on BM-DCs and human monocyte-derived DCs. Furthermore, stimulation with HP-β-CD resulted in weak but significant production of TNF-α, IL-6, and IL-10 in DCs. Therefore, like other adjuvants, HP-β-CD appears to be capable of stimulating DC maturation. However, in contrast to our

observation, a previous study found that HP-β-CD did not induce a maturation marker of $CD11c^+$ DCs in the draining lymph node, even though it potentiated DC antigen uptake (7). Given the differences in a route of injection and doses of HP-β-CD, functional mode of DC activation in the tissues might be different.

Here, we found that HP-β-CD-sensitized DCs markedly induced the proliferation and activation of T lymphocytes, especially $CD4^+$ cells, implying that HP-β-CD can potentially enhance Th-dependent immune responses. Notably, a study of mice immunized with an influenza vaccine suggested that HP-β-CD is a competent adjuvant capable of eliciting follicular Th cells and antibody production (7). Furthermore, other cyclodextrins, such as sulfolipo-cyclodextrin and dimethyl-β-cyclodextrin, have been found to enhance antibody responses in a T lymphocyte-dependent manner (29, 30). Given the fact that enhanced T cell responses have previously been observed with many existing adjuvants, including MPLA, saponin, and CT (31, 32), this enhancement appeared to be mediated through DC activation. These adjuvants influence the types of adaptive immune responses that occur by modulating T lymphocyte differentiation. For instance, alum is an established potentiator of Th2-mediated humoral immunity (33). In addition, MPLA has been shown to preferentially induce Th1-skewed immune responses (34), whereas CT provokes Th1, Th17, and Th2 responses (35). Considering the previous finding that HP-β-CD administration markedly induces IL-13 and IL-5 production, HP-β-CD has been proposed to elicit Th2 responses (7).

In the present study, we observed that HP-β-CD-induced lipid raft formation and contributed to DC maturation. Specifically, filipin-mediated inhibition of lipid rafts abrogated HP-β-CD-mediated phenotypic changes and functional activation of DCs. In line with this observation, many previous studies have demonstrated lipid raft involvement in the activation of various immune cells. Dispersion of lipid rafts has been shown to impair CD1a-mediated antigen presentation by DCs and subsequent activation of T lymphocytes (36). Furthermore, alum adjuvanticity has been proposed to have a critical relationship with the formation of signaling platforms *via* lipid sorting on DCs (26).

2-Hydroxypropyl-β-cyclodextrin can interact with cellular cholesterol (21); however, the precise effects of HP-β-CD on cholesterol seem to depend on its concentration. Low doses of HP-β-CD (below 1 mM) induce efflux or intermembrane transport of cholesterol, while high concentrations (10–100 mM) deplete the lipid molecules from the cell membrane (37, 38). In the present study, DCs were treated with up to 1 mg/ml HP-β-CD, which is equivalent to 1.4 mM. This concentration is relatively low and is likely to mediate cholesterol accumulation and lipid raft formation on the cell membrane rather than to deplete cholesterol. In light of the observation that cholesterol accumulation in the membrane of human monocytes promotes the association of immuno-stimulatory receptor complexes, cholesterol loading in the plasma membrane is believed to be crucial for eliciting inflammatory immune responses (39). Since HP-β-CD can transport free cholesterol to the plasma membrane (40), HP-β-CD is likely to play a role in the formation of cholesterol-rich lipid rafts in DCs, thereby activating signals required for their phenotypic and functional maturation.

Recently, potential issues in relation to the efficacy and safety of vaccine adjuvant have been raised (41–44). Thus, demand has increased for new adjuvants with minimal adverse effects and enhanced capacity to stimulate antigen-specific adaptive immune responses. HP-β-CD is GRAS in many Asian and European countries and is already widely utilized in various commercial products, including food and drugs. Our findings potentially inform the future application and improvement of vaccine adjuvants with HP-β-CD.

AUTHOR CONTRIBUTIONS

SH conceived the idea. SH and SK designed the experiments. SK and SH performed the experiments and/or interpreted the data. C-HY provided critical comments. All authors contributed to discussion of the results followed by writing and reviewing the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2016.00435

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ω3 Polyunsaturated Fatty Acids as Immunomodulators in Colorectal Cancer: New Potential Role in Adjuvant Therapies

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Diet composition may affect the onset and progression of chronic degenerative diseases, including cancer, whose pathogenesis relies on inflammatory processes. Growing evidence indicates that diet and its components critically contribute to human health, affecting the immune system, secretion of adipokines, and metabolic pathways. Colorectal cancer (CRC) is one of the leading causes of death worldwide. Antineoplastic drugs are widely used for CRC treatment, but drug resistance and/or off-target toxicity limit their efficacy. Dietary ω3 polyunsaturated fatty acids (PUFA) have been gaining great interest in recent years as possible anti-inflammatory and anticancer agents, especially in areas such as the large bowel, where the pro-inflammatory context promotes virtually all steps of colon carcinogenesis. Growing epidemiological, experimental, and clinical evidence suggests that ω3 PUFA may play a role in several stages of CRC management exhibiting antineoplastic activity against human CRC cells, improving the efficacy of radiation and chemotherapy, ameliorating cancer-associated secondary complications, and preventing CRC recurrence. These effects are most likely related to the immunomodulatory activities of ω3 PUFA that are able to influence several aspects of the inflammatory process ranging from inflammasome activation, leukocyte recruitment, production of immune mediators to differentiation, and activation of immune cells. In this review, we will focus on the potential use of ω3 PUFA as adjuvant agents together with chemo/radiotherapy, highlighting the immunomodulatory effects most likely responsible for their beneficial effects in different stages of CRC management.

Keywords: ω3 polyunsaturated fatty acids, immunomodulation, colorectal cancer, adjuvant therapy, inflammation

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INTRODUCTION

It has become increasingly clear that dietary habits may affect the risk/progression of chronic diseases with a pathogenic inflammatory component, such as colorectal cancer (CRC) (1). CRC is the third most common cancer and the fourth leading cause of cancer death worldwide and represents a clinical model of cancer showing a close relationship with inflammation and environmental factors (2). Inflammation is nowadays considered an hallmark of cancer (3), and exacerbated inflammatory processes has been reported to favor the insurgence of several types of neoplastic

diseases, their progression, as well as a worse prognosis for the patient with cancer (World Cancer Report, 2014, IARC). Several types of inflammation differing by cause, mechanism, outcome, and intensity can promote cancer development and progression. Interestingly, rising CRC incidence trends are mainly regarded as a part of the change in lifestyle, including calorically excessive high-fat/low-fiber diet, lack of physical activity, and obesity (4). Epidemiological and clinical investigations have consistently evidenced a significant relationship between obesity-driven inflammation and most steps of CRC development, including initiation, promotion, progression, and metastasis (1). Furthermore, cancer therapy as well as surgery for cancer resection can trigger an inflammatory response by causing trauma, necrosis, and tissue injury that stimulate tumor re-emergence and resistance to therapy (5). In spite of the progress made in recent years in cancer treatment, the clinical relevance of the conventional therapeutic modalities alone in solid tumors, including CRC, still pose major questions due to the high costs of drugs and unsatisfactory long-term clinical results (6). Thus, due to the impact of inflammation as a risk factor for CRC development as well as in the course of the disease, new concepts are emerging on the benefits that may derive from the combination of conventional CRC treatments with intervention strategies targeted at inhibiting/attenuating chronic inflammation. Notably, dietary components influence inflammatory processes including intestinal inflammation, and dietary habits have been suggested to play a major role in CRC risk (7). Considerable attention has therefore been directed toward the ability of nutritional agents to target key molecular pathways involved in cancer-related inflammation. It has only recently gained acceptance that fatty acids (FAs) are major determinants in inflammation and may influence the risk of related pathologies. Given their precursor status to signaling lipid mediators, FAs act as ligands for key immune receptors, and their activation can initiate and perpetuate an innate immune response (8).

DIETARY POLYUNSATURATED FATTY ACIDS

ω3 and ω6 FAs are polyunsaturated fatty acids (PUFA) whose definition derives from counting the carbon atoms after the last double bond in the end of the fatty acid chain (9). The type of response they induce strongly depends on their biochemical properties, mainly related to number and position of double bonds (10). In this regard, ω3 and ω6 PUFA exhibit different behaviors with respect to the inflammatory process, exerting anti-inflammatory or pro-inflammatory activity, respectively. Owing to the proposed competitive role of ω3 and ω6 PUFA, their dietary composition has been suggested to be a biologically plausible target for CRC management. ω3 and ω6 FAs represent naturally occurring substances required for biological process with key roles in phospholipid membrane structure and function, cellular signaling, and lipid metabolism (11, 12). In humans, the ω3 α-linolenic acid (ALA, 18:3ω3) as well as the ω6 linoleic acid (LA, 18:2ω6), precursors of several PUFA, are essential FA that cannot be synthesized by humans and must therefore be assumed from dietary sources including fish, flaxseeds, walnuts, and algae (9). Several kind of food, rich in ω3 PUFA, are typical of the so-called Mediterranean diet (MD). Several epidemiological studies have evaluated the effects of this dietary pattern as protective against several diseases associated with chronic low-grade inflammation such as cancer, diabetes, obesity, atherosclerosis, metabolic syndrome, and cognition disorders (13). Adherence to traditional MD was found to be associated with markedly and significantly reduced incidence of cardiovascular diseases and overall cancer (14, 15). Due to the poor endogenous conversion of ALA to its longer and more highly desaturated FAs, there is considerable debate as to whether the longer versions such as eicosapentaenoic acid (EPA, 20:5ω3) and docosahexaenoic acid (DHA, 22:6ω3) may be considered semi-essential (16). These PUFA are key components of cell membrane, lipoproteins, and adipose tissue (AT) whose composition closely reflects the dietary intake (17). For several years, ω3 PUFA have been studied extensively for the prevention of cardiovascular diseases (18, 19). More recently, their capacity to exert anticancer activity has been recognized. A plethora of studies pointed to ω3 PUFA and their oxidative metabolites as potential anti-inflammatory and anticancer agents, especially in areas such as the large bowel, where the influence of orally introduced substances is high and tumors show deranged PUFA patterns. In particular, modified ω3 and ω6 PUFA profiles have been found in serum and cancer tissue of CRC patients, suggesting possible alteration of PUFA metabolism that may play a role in inflammation-driven colorectal carcinogenesis (20, 21). Overall, these studies suggested that the metabolism of PUFA plays a role in inflammation-driven colorectal carcinogenesis and is likely influenced by the tumor size. In this regard, it is of relevance that ω3/ω6 PUFA ratio of the industrialized diets is imbalanced, and this condition results in a well-known pro-inflammatory effect (22). Noteworthy, a decreased ω3/ω6 PUFA ratio of visceral AT correlates with AT inflammatory status in CRC patients suggesting that qualitative, more than quantitative, PUFA changes in AT may influence tissue dysfunctions potentially linked to inflammatory conditions (23). Likewise, the sum of some PUFA [i.e., gamma linolenic acid (GLA), Di-homo-gamma linolenic acid (DGLA), and arachidonic acid (AA)] in both visceral and subcutaneous AT was found statistically higher in cancer patients with respect to healthy controls (24). Overall, these observations support the concept that PUFA derangement occurs not only in tumor tissue but also at microenvironment and systemic levels most likely contributing to tumor-associated inflammation.

There is now sufficient evidence from prospective studies indicating that fish consumption or fish oil ω3 PUFA intake is inversely related to CRC incidence in the general population (25). However, the results achieved in a recent meta-analysis carried out to evaluate the effects of ω3 PUFA on inflammatory markers in CRC patients highlighted that the beneficial effects of ω3 PUFA rely on supplementation protocols (e.g., dose, duration, and route of administration), the concomitant anticancer regimen adopted, as well as on the confounding effects due to other nutrients that might be present in the supplement (26). In this regard, it should be taken into account that the observational studies carried out to evaluate the potential anticancer effects of

ω3 PUFA might have been limited by the insufficient homogeneity of the observations. An explanation for such heterogeneity relies likely on the inherent difficulties associated with epidemiology, including the confounding and dietary pattern context, measurement error, level of intake, and genetic polymorphisms (26, 27).

Despite the majority of studies has proven that ω3 PUFA administration is not toxic or carcinogenic, it is hard to imagine that these compounds could be exploited alone in the treatment of cancer. However, growing epidemiological, experimental, and clinical evidence showing the beneficial effects of ω3 PUFA in several stages of CRC management represent a solid rationale for their exploitation in therapeutic combinatory strategies (28). The antineoplastic effects of ω3 PUFA have been studied for many years, and multiple biological mechanisms have been suggested to explain their inhibitory effects on cancer cell growth (29, 30). However, in recent years, growing attention has been given to the antineoplastic activity of ω3 PUFA related to the action of these bioactive molecules on inflammatory process, due to the pathogenic role of inflammation in the development of many kinds of tumors. ω3 PUFA, particularly EPA and DHA, have emerged as nutrients capable of modulating both metabolic and immune processes. As summarized in **Table 1**, these PUFA are able to modulate a number of aspects of the inflammatory process ranging from inflammasome activation, leukocyte recruitment, and production of immune mediators, to differentiation and activation of immune cells. ω3 PUFA effects are mediated by different mechanisms including both extracellular (e.g., GPR120) (31) and intracellular (e.g., PPARγ) receptors that control inflammatory cell signaling and gene expression (32). In spite of many efforts, it remains to be fully defined how these compounds succeed in influencing such a variety of molecular pathways and cell/tissue functions with pleiotropic beneficial effects, and how these mechanisms are interconnected. Some of the ω3 PUFA-mediated events appear to rely, at least in part, on changes in FA composition of membrane phospholipids, relevant for the production of lipid mediators, and formation of lipid rafts in response to inflammatory stimuli (33).

PROTECTIVE ANTI-INFLAMMATORY EFFECTS OF ω3 PUFA IN COLORECTAL CANCER

Considerable debate still exists on the mechanisms underlying the protective effects exerted by the ω3 PUFA on the establishment/progression of inflammation and cancer (29). Furthermore, the precise mechanism that operates at specific body district, such as the intestine, remains to be defined. Several mechanisms have

TABLE 1 | Immunomodulatory effects of ω3 PUFA in monocyte/macrophages and T lymphocytes.

ω3 PUFA exposure	Experimental/animal model	Observed effect	Suggested mechanism	Reference
DHA[a], EPA[a]	MU and HU monocytic cell lines; MU and HU primary monocyte/Mφ, C57BL/6 mice	↓ inflammasome activation	GPR120-β arrestin-2-mediated NF-kB inhibition Enhanced autophagy Impaired TLR2/TLR1 dimerization	(34–36)
Isocaloric HFD-containing 27% menhaden fish oil (16% EPA, 9% DHA); isocaloric ω3 PUFA-enriched diet (3% menhaden fish-oil + 7% safflower oil)	C57BL/6 mice	↓ leukocyte chemotaxis	GPR120, β arrestin-2 NF-kB and STAT3 inhibition	(37, 38)
DHA[a], EPA[a]	MU monocytic cell lines and primary Mφ	↑ efferocytic activity of Mφ	PPARγ and AKT activation	(39, 40)
DHA[a], EPA[a], isocaloric HFD-containing 27% menhaden fish oil (16% EPA, 9% DHA); HFD + DHA[a]	MU monocytic cell lines and primary Mφ C57BL/6 mice	↑ M2 Mφ polarization	GPR120, β arrestin-2	(37, 40–42)
DHA[a], EPA[a], ω3 PUFA-enriched diet[b], DHA-enriched diet (omegavie DHA90 TG)	BALB/c and C57BL/6 mice MU spleen and bone marrow DC	↓ T cell activation ↑ Th2 lymphocyte polarization ↓ Th17 lymphocyte polarization ↑ expression and generation of T_{reg} ↓ T_{reg} suppressive and migratory function ↓ DC activation ↓ DC-mediated T cell activation ↑ DC-mediated T_{reg} expansion	Interference with STAT3 signaling pathway *via* enhancement of PPARγ-induced SOCS3 M2 Mφ mediated	(43–51)

The table shows a summary of the main publications on the immunomodulatory effects of ω3 PUFA in monocyte/macrophages and T lymphocytes, including both in vitro studies and in vivo animal models.

MU, murine; HU, human; Mφ, macrophage; DC, dendritic cell; HFD, high fat diet; GPR120, G protein-coupled receptor 120.

[a]*Chemically synthesized.*

[b]*ω3 PUFA source: perilla seed oil, menhaden fish oil, and EPAX oil (EPAX-7010) containing ~85% ω3 PUFA (70% EPA, 12% DHA).*

been proposed to explain the capacity of ω3 PUFA to interfere with important steps of CRC carcinogenesis including alterations of the cellular redox state and modulation of membrane dynamics, and surface receptor function which regulate cell proliferation and apoptosis. Due to the close association of CRC with inflammation, the capacity of ω3 PUFA to modulate some aspects of inflammation and immune response is becoming increasingly important. In this regard, it is of interest that ω3 PUFA inhibit agonist-induced activation of pattern recognition receptors, thus dampening inflammation (52). However, only few studies focused on the role of PUFA-mediated immunomodulatory effects in the therapy of CRC.

Due to the abundance of double bonds, EPA and DHA are susceptible to oxidation and can undergo spontaneous non-enzymatic peroxidation or enzymatic oxidation giving rise to a variety of bioactive lipids that induce inflammation, tumorigenesis, and thrombosis, as well as to mediators with antitumorigenic, pro-resolution properties (53, 54). One of the main mechanisms by which ω3 PUFA exert their anti-inflammatory activity involves enzymatic pathways that are hyper-activated in CRC (55), such as cyclooxygenase (COX) and lipoxygenase (LOX), and leads to prostaglandin E_2 (PGE_2) production, a potent pro-inflammatory and procarcinogenic agent. EPA and DHA have been shown to inhibit COX-2 expression in CRC cells (56), and this effect has been attributed to ω3 PUFA capacity to displace ω6 PUFA, in particular AA, from membranes. This metabolic competition is due to structural similarity of EPA and AA, which compete with each other for the same enzymes, COX, and LOX, a very important event in tumor development (57). Furthermore, supplementation with ω3 PUFA significantly decreases AA-derived eicosanoids, while increasing EPA-derived eicosanoids, shifting the balance toward a reduced level of inflammation (58). Interestingly, aspirin, a non-steroidal anti-inflammatory drug, possesses both COX and LOX inhibitory action, and enhances the production of the anti-inflammatory lipid mediator lipoxin A4. Likewise, anti-inflammatory compounds are formed from EPA and DHA by the action of aspirin termed as resolvins (from EPA and DHA) and protectins and maresins from DHA (59, 60). Of note, regular aspirin use substantially reduces the risk of CRC (61). Pro-inflammatory cytokines and tumor-infiltrating myeloid and immune cells play critical roles in almost every developmental stages of inflammation-related cancers, from initiation, promotion, and progression to malignant metastasis (1). A recently published meta-analysis of the ω3 PUFA effects on inflammatory markers highlighted a decreased level of some inflammatory mediators (cytokines or acute phase proteins) in CRC patients who received ω3 PUFA supplementation (26). Likewise, a recent *in vitro* study described a new anti-inflammatory and anticancer properties of DHA. Fluckiger and colleagues reported that inhibition of colon cancer growth and activation of apoptosis by DHA involve autocrine production of TNFα *via* microRNA (miR)-21 (62).

Another mechanism involved in anti-inflammatory and potentially antineoplastic effects of ω3 PUFA regards the possibility that these compounds may modulate epigenetically (through DNA methylation, acetylation modifications, and miR gene regulation) the expression of crucial genes involved in cellular processes associated with colorectal carcinogenesis (63–65). More recently, the epigenetic regulation of gene expression and the polarization of macrophages toward pro-resolving M2 phenotype have emerged as novel mechanisms explaining the antineoplastic effects of ω3 PUFA at the colon level, although additional studies are needed for a deeper comprehension of this issue (30). Finally, the capacity of ω3 PUFA to modulate inflammatory pathways and to generate lipid mediators critical for the resolution of inflammation (e.g., resolvins, protectins, and maresins) has gained attention as a main mechanism involved in the beneficial effects of ω3 PUFA against CRC (66).

ω3 PUFA AS POTENTIAL ADJUVANTS IN THE THERAPY OF CRC

Fatty acids may influence carcinogenesis through various mechanisms. EPA and DHA are incorporated into cellular membranes and through the production of lipid mediators and may exert anticancer properties by affecting gene expression or activating signal transduction molecules involved in the control of cell proliferation, differentiation, apoptosis, and metastasis. These properties of ω3 PUFA suggest that they will have important therapeutic potential in cancer management.

Patients undergoing surgery are at risk of developing complications in the postoperative period partly caused by changes in the immune response following surgery (67). Thus, initially, a hyper-inflammatory response followed by a phase of relative immune incompetence occurs in relation to major surgery (68). Patients who undergo surgery for CRC have a 30% risk of infectious complications, and anastomotic leakages are seen in as many as 15% of patients (69).

Initial studies indicated that administration of PUFA-enriched diets leads to increased incorporation of EPA and DHA not only in liver and gut mucosa tissue but also in tumor tissue in patients with solid tumors of the upper gastrointestinal tract (70). This observation suggested that preoperative administration of oral PUFA-enriched diets could have an impact on the postoperative inflammatory response after major abdominal surgery. Subsequently, in a prospective randomized, double-blind, single-center, placebo-controlled study, it was demonstrated a higher total marine ω3 PUFA content in the colonic mucosa, but not in the muscular layer, after 7 days of oral EPA + DHA supplementation to patients admitted for elective CRC surgery (71). Furthermore, providing ω3 PUFA daily for 7 days before surgery resulted in a significant decrease in the formation of the pro-inflammatory leukotriene B4 (LTB4) from neutrophils with a simultaneous increased production of LTB5. However, the clinical consequences of these changes are still unknown as no correlations between values of LTB4 or LTB4/LTB5, and postoperative complication rates were found (72). Likewise, the higher incorporation of EPA, DHA, and docosapentaenoic acid in granulocytes of the ω3 PUFA-enriched supplement group of CRC patients was not associated with improved postoperative outcomes (69). Thus, the effects of ω3 PUFA on preoperative complications need to

be investigated in larger trials and for a longer period (months) of ω3 PUFA intake to clearly establish whether LTB formation from activated neutrophils is/is not an important determinant of surgical complications.

Several studies highlighted the beneficial effect of ω3 PUFA as chemopreventive and chemotherapeutic agents in the treatment of several chronic pathologies including cancer providing evidence for a potential use of these compounds to enhance chemo/radiotherapy efficacy and reduce the risk of tumor recurrence (73). Furthermore, several studies demonstrated that EPA and DHA, having immunomodulatory capacity, act to reduce inflammation, even when associated with chemo/radiotherapy immune suppression in lung (74, 75), and in other cancers (73).

In particular, Xue and colleagues evaluated whether ω3 PUFA supplementation could affect the efficacy of a cyclical combination treatment with Irinotecan (CPT-11)/5-fluorouracil (5-FU) in a rat CRC model. Administration of a diet enriched with ω3 PUFA prior to initiating chemotherapy, inhibited tumor growth, while, during chemotherapy, ω3 PUFA-enriched diet enhanced tumor chemosensitivity and reduced body weight loss with respect to control diet fed rats (76). Other studies addressed the issue of whether supplementation with ω3 PUFA to CRC patients undergoing chemotherapy could induce changes in inflammation markers, such as the ratio of C reactive protein (CRP) to albumin, considered a relevant clinical/inflammatory marker. In this respect, some clinical trials demonstrated that EPA and DHA supplementation during chemotherapeutic treatments improved CRP values, CRP/albumin status, and positively modulated the nutritional status of CRC patients (77, 78). Likewise, evaluation of clinical outcomes during and after chemotherapy in CRC patients supplemented with fish oil containing EPA and DHA during the first 9 weeks of treatment unraveled that the time to tumor progression was significantly longer in the supplemented with respect to the control group (79). Interestingly, a recent phase II double-blind randomized, placebo-controlled trial of EPA administration to advanced CRC patients undergoing liver resection surgery demonstrated that EPA is incorporated into secondary CRC tissue and has systemic anti-inflammatory activity. In particular, a clear-cut decrease of the urinary metabolite PGE-M, reflecting systemic PGE_2 levels, as well as a reversible reduction in NF-kB binding in peripheral blood leukocytes was observed in EPA-treated patients with respect to the placebo group (80). Overall, these observations that supplementation may represent an innovative combination therapy strategy contributing to delay tumor progression and likely enhancing the antineoplastic activity of chemotherapeutic drugs. The design and main hallmarks of clinical trials performed on CRC patients supplemented with ω3 PUFA, described above, are summarized in **Table 2**.

In the last years, many studies have highlighted the presence of cancer stem cells (CSC) in most tumors, including CRC.

TABLE 2 | Clinical effects of ω3 PUFA in CRC patients.

ω3 PUFA daily treatment	Surgery	*n* Subjects	Timing/ duration	Chemotherapy	Observed effect	Outcome measures	Reference
1 g DHA + 2 g EPA orally	Yes	148	7 days pre-surgery	No	Potential beneficial effect on local immune function	ω3 PUFA content in the colonic mucosa and muscular layer	(71)
1 g DHA + 2 g EPA orally	Yes	148	7 days pre-surgery	No	Anti-inflammatory effects 5-HEPE ↑ LTB4/%-HETE ↓	Inflammatory markers	(72)
1 g DHA + 2 g EPA orally	Yes	148	7 days pre- and 7 days post-surgery	No	No significant difference between group in infectious or non-infectious post-operative complications	Levels of ω3 PUFA into granulocytes	(69)
0.6 g EPA/DHA (fish oil) orally	No	23	63 days	5-FU + irinotecan + folinic acid	Positive modulation of nutritional status. CRP/albumin ↓	Evaluation of nutritional status and inflammatory markers	(77)
0.6 g EPA/DHA (fish oil) orally	No	11	63 days	CAPE + OXA + 5-FU + leucovorin	Improved CRP values, CRP/albumin status, plasma fatty acid profile, and potentially prevented weight loss during treatment	Evaluation of inflammatory markers and nutritional status	(78)
0.6 g EPA/DHA (fish oil) orally	No	30	63 days	Standard chemotherapy	Delayed tumor progression time by enhancing the antineoplastic action of chemotherapy	Evaluation of tumor progression time and CEA values after chemotherapy	(79)
2 g EPA-FFA[a] orally	Yes	88	Median 30 days pre-operative	No	Preoperative treatment may have prolonged benefit on post-operative overall and disease-free survival	Ki67, CD31, and PGE2 levels. Survival statistical analysis (overall and disease-free survival)	(80)

[a]*ALFA (SLA Pharma AG, Liestel, Switzerland).*

5-HEPE, 5-hydroxyeicosatetraenoic acid; LTB4, leukotriene B4; 5-HETE, 5-hydroxyeicosatetraenoic acid; 5-FU, 5-fluorouracil; CAPE, capecitabine (Xeloda); OXA, oxaloplatin; CRP, C reactive protein; CEA, carcinoembryonic antigen; PGE2, prostaglandin E.2.

These cells are considered responsible for tumor relapse and resistance to conventional therapies (81). Thus, novel anticancer strategies have been designed to selectively target CSC, and to this purpose, natural compounds, including ω3 PUFA, might have a relevant role. In colon cancer stem-like cells (CSLC), De Carlo et al. showed that EPA has a pre-differentiating effect and this finding could, at least in part, explain the increased cellular sensitivity to 5-FU (82). In keeping with these observations, Yang and colleagues demonstrated that EPA and DHA, in a single or combined treatment, enhanced the chemotherapeutic sensitivity effect of 5-FU and mitomycin C by inducing apoptotic cell death of CSLC (83).

CONCLUSION

Although the use of ω3 PUFA as potential chemopreventive and chemotherapeutic agents has been frequently proposed in cancer treatment, the evidence achieved points to these compounds as important agents for combined strategies against cancer. In particular, despite some contradictory results, several experimental and clinical data strongly support a role of ω3 PUFA as adjuvants able to counteract the occurrence of inflammatory processes during CRC treatment as well as to increase chemotherapy efficacy or to decrease its toxicity. However, none of the studies has taken into consideration the initial status of the patients prior to PUFA supplementation or dietary advice, a relevant issue to better exploit the preventive/therapeutic/adjuvant potential of these compounds. Further and deepened studies are thus needed to characterize the real ω3 PUFA effectiveness and mechanisms of actions.

AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct, and intellectual contribution to the work and approved it for publication.

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Discovery of Innovative Therapies for Rare Immune-Mediated Inflammatory Diseases via Off-Label Prescription of Biologics: *The Case of IL-6 Receptor Blockade in Castleman's Disease*

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Biologics have revolutionized the field of clinical immunology and proven to be both effective and safe in common immune-mediated inflammatory diseases (IMIDs) such as rheumatoid arthritis, inflammatory bowel diseases, and various hematological disorders. However, in patients with rare, severe IMIDs failing on standard therapies, it is virtually impossible to conduct randomized controlled trials. Therefore, biologics are usually prescribed off-label in these often severely ill patients. Unfortunately, off-label prescription is sometimes hampered in these diseases due to a lack of reimbursement that is often based on a presumed lack of evidence for effectiveness. In the present article, we will discuss that off-label prescription of biologics can be a good way to discover new treatments for rare diseases. This will be illustrated using a case of multicentric Castleman's disease, an immune-mediated lymphoproliferative disorder, in which off-label tocilizumab (humanized anti-IL-6 receptor blocking antibody) treatment resulted in remarkable clinical improvement. Furthermore, we will give recommendations for monitoring efficacy and safety of biologic treatment in rare IMIDs, including the use of registries. In conclusion, we put forward that innovative treatments for rare IMIDs can be discovered via off-label prescription of biologicals, provided that this is based on rational arguments including knowledge of the pathophysiology of the disease.

Keywords: giant lymph node hyperplasia, multicentric Castleman's disease, off-label use, interleukin-6, tocilizumab, biological products, registries

INTRODUCTION

In patients with rare, severe immune-mediated inflammatory diseases (IMIDs), biologics are often the last treatment option when standard therapy with classical immunosuppressive drugs fails. However, clear guidelines on biologic prescription in these diseases are often lacking. A major reason is that proper randomized controlled trials (RCTs) are difficult to design due to the low prevalence,

heterogeneity of patients, and the severe medical condition of patients. Since RCTs are usually required for registration of therapy for a specific disease, physicians often prescribe biologics off-label in these diseases (1). Off-label prescription can be defined as the prescription of a drug for conditions other than current registered indications. This means that the drug is approved by either FDA or EMA for a certain disease, yet there is no definitive proof for the efficacy of the specific drug for other conditions. The physician's decision to prescribe off-label in such cases is often based on the pathophysiology of the disease or shared symptoms with other diseases in which the biologic has proven to be effective. This is called rational prescription and may lead to innovative treatment options, especially in rare diseases. We will illustrate this in the present article by discussing the case of Castleman's disease (CD) and interleukin (IL)-6 receptor blockade, including a case report of a patient who responded very well to this treatment strategy. In addition, we provide an overview of current new developments toward responsible off-label use of biologics in rare, severe, and therapy-refractory IMIDs.

CASTLEMAN'S DISEASE: CLINICAL SYMPTOMS AND THE ROLE OF IL-6

Castleman's disease is a rare and relatively unknown lymphoproliferative disorder. It is characterized by polyclonal B-cell proliferation, usually associated with autoimmune and connective tissue symptoms, and often goes together with a pre-existing autoimmune disorder, such as rheumatoid arthritis (RA), Sjögren's syndrome, and systemic lupus erythematodes (SLE) (2).

Castleman's disease can be divided into unicentric Castleman's disease (UCD) and multicentric Castleman's disease (MCD). In UCD, a single lymphoid region is involved, in contrary to the latter in which multiple lymphoid regions are involved (3). UCD usually causes complaints resulting from enlargement of one or more lymph nodes and mostly lacks systemic symptoms (2). Removal of the lymph node cures 90% of the patients without further complications (4). MCD, on the other hand, can cause "B symptoms" and signs such as anorexia, anemia, and low white blood cell counts (3). MCD has been associated with HIV infection, which is similar to MCD observed in non-HIV-infected patients, except for the high prevalence of pulmonary symptoms and strong association with Kaposi's sarcoma. Interestingly, also HHV-8, a virus causing Kaposi's sarcoma, is also associated with MCD (5). Both UCD and MCD are characterized by hypersecretion of IL-6, most likely by germinal center B-cells in hyperplastic lymph nodes. Furthermore, a correlation between serum IL-6 levels and clinical symptoms of patients with CD was shown (6). IL-6 regulates T-cell function, acute phase reaction, and terminal B-cell differentiation (2). Overproduction of IL-6 can cause various symptoms, including fever and lymphadenopathy, and has been associated with autoimmune disorders, such as RA and juvenile idiopathic arthritis (JIA), as well as lymphoid malignancies (7). The prognosis for MCD is usually better when diagnosed early. Yet, early diagnosis can be very challenging because of the common manifestation of lymph node enlargement in patients with various autoimmune disorders. Moreover, diagnosing MCD can sometimes be hard, because symptoms are often not very specific. Nonetheless, CD should be suspected when a patient's primary diagnosed disease, such as another lymphoproliferative disease or autoimmune disease is unusually hard to treat (8).

TREATMENT OF CASTLEMAN'S DISEASE

Corticosteroids are given as standard therapy in CD, usually resulting in improvement of symptoms, normalization of laboratory parameters, and regression of lymphadenopathy (2, 4). However, relapses are often seen after tapering the dose. Other treatment options are combination chemotherapy and the use of lenalidomide/thalidomine (4). Biologics have also been used in the treatment of CD. Rituximab (chimeric anti-CD20 monoclonal antibody) may be effective, especially in CD patients with pre-existent autoimmune disease (9). Interestingly, long-term remission has also been reported after rituximab administration (2). Clinical improvement after rituximab treatment has been associated with reduced serum levels of pro-inflammatory cytokines, including IL-6 (10). The following case report illustrates the challenges in the treatment of MCD and the potential benefit of rational off-label prescription of biologics in rare IMIDs. In accordance with Dutch laws, the patient gave written informed consent to both the off-label treatment with tocilizumab and the use of his case for this manuscript.

CASE REPORT

A 63-year-old male patient was referred with lymphadenopathy, limb pain, weight loss, and night sweats. Infectious causes and lymphoma were excluded. Eventually, he was diagnosed with MCD, based on clinical manifestations in combination with results of lymph node and bone marrow biopsies demonstrating polyclonal B-cell proliferation. HHV-8 and HIV tests were negative. After failing to respond to high-dose prednisolone treatment, rituximab was administered, dosed in a cycle of 1000 mg intravenously twice every 6 months, which eventually led to a stable condition for 2 years. However, the disease recurred with extensive lymphadenopathy and strongly elevated erythrocyte sedimentation rate (ESR) levels. In addition, he experienced severe weight loss (10 kg), nausea, loss of appetite, increased muscle pain, and extreme fatigue, resulting in disability. Based on the important role of IL-6 in the biology of B-cells, elevated IL-6 levels in patients with CD, and case reports, tocilizumab (anti-IL-6 receptor antibody) treatment was initiated (600 mg biweekly intravenously) combined with methotrexate (7.5 mg weekly). This resulted in a marked decrease in C-reactive protein (CRP) and ESR levels, normalization of hemoglobin (Hb) (**Figure 1**) and albumin levels and remarkable clinical improvement (weight gain and improvement in overall physical condition). In addition, there was a significant reduction of the lymphadenopathy. Lowering the frequency of tocilizumab administration was not possible as this resulted in immediate flaring of the disease. Subsequent increased dosing induced remission again.

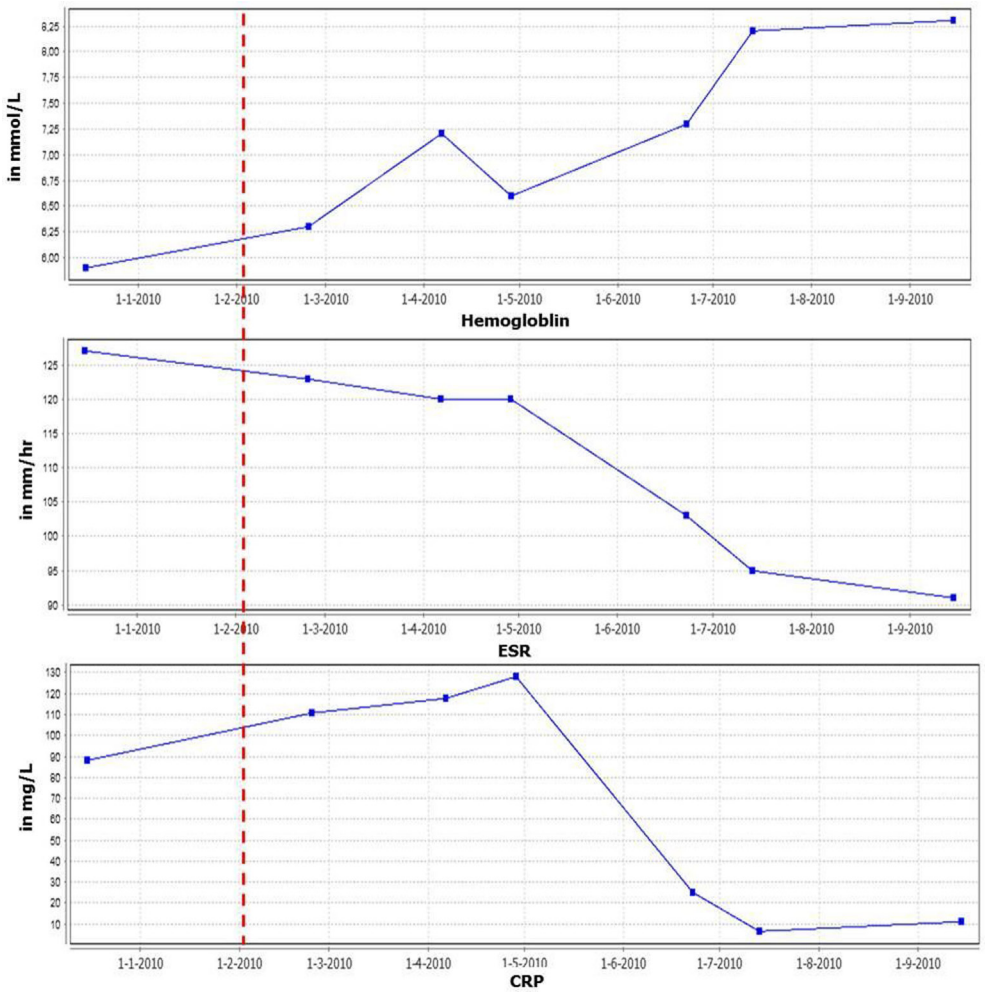

FIGURE 1 | Inflammatory parameters and hemoglobin levels in time. Red line indicates start tocilizumab therapy.

IL-6 BLOCKADE IN CASTLEMAN'S DISEASE

As already mentioned, IL-6 overproduction is associated with the pathogenesis of CD and IL-6 signaling can be blocked by tocilizumab, a humanized monoclonal antibody directed against the human IL-6 receptor. Tocilizumab is used in the treatment of chronic inflammatory diseases in which IL-6 has been implicated in the pathogenesis and exerts its effects by neutralizing the pro-inflammatory effects of IL-6/IL-6 receptor signaling. In addition, tocilizumab treatment may increase the proportion and function of regulatory T cells in the peripheral blood, as has been described in RA (11, 12).

Like most other biologics, the use of tocilizumab is associated with a moderately increased risk of infection. Furthermore, patients sometimes exhibit a transient hypercholesterolemia. Tocilizumab is an effective and safe officially approved treatment for RA and JIA. We describe the successful treatment of a patient with CD with tocilizumab. Over the past few years, several other reports have also described as a good outcome of IL-6 receptor blockade in patients with CD. Nishimoto et al. report an open-label study of 28 patients with MCD. Patients were treated biweekly with 8 mg/kg of tocilizumab for 16 weeks, resulting in improvement of symptoms, reduced acute phase reactants, and decreased need for corticosteroids (13). Interestingly, a recent paper reported persistent improvement of anemia in CD after the administration of tocilizumab. Twelve months after tocilizumab administration, iron-related parameters normalized and symptoms improved in all nine patients (14). This can be explained by the role of IL-6 as inducer of hepcidin, a principal regulator of iron homeostasis (15). Tocilizumab blocks the IL-6 receptor and indirectly downregulates hepcidin. This results in a decrease of iron storage and therefore an increase of serum iron available for Hb synthesis and erythrocyte production, leading to a normalization of the anemia in MCD (2, 14).

As described here, tocilizumab can be very effective in patients with this condition who do not respond to conventional treatment (16). In Japan, these findings have led to the approval

and registration of tocilizumab for the treatment of CD (2, 16). However, in many other countries, including the Netherlands and the USA, tocilizumab has not been approved for the treatment of CD and is therefore often not reimbursed.

RATIONAL PRESCRIPTION OF BIOLOGICS AND MONITORING OFF-LABEL USE

This case report illustrates that off-label prescription is sometimes the only option left after failure of all other possible treatments and can be very effective. Importantly, off-label prescription of biologics should preferably be "rational," i.e., prescription of an approved drug proven to be safe and effective in a certain disease for another disease based on shared signs and symptoms or knowledge on the pathophysiology of the disease. The present case is a good example of rational prescription, as the initiation of tocilizumab treatment was mainly based on the important role of IL-6 in the pathophysiology of CD. Other examples of this are the use of anti-TNF biologics in TNF-driven diseases or the prescription of IL-1 receptor antagonists in autoinflammatory diseases that are characterized by derailed IL-1-dependent intracellular processes (1).

Obviously, it is crucial to carefully monitor efficacy and safety of biologics that are prescribed off-label (1). This is not only important for individual patients but also for generating more evidence to eventually support reimbursement of novel off-label treatments for rare IMIDs; especially since healthcare authorities often decide that off-label prescription of these expensive drugs is not reimbursed due to "lack of evidence" for efficacy in rare IMIDs, which may prevent physicians from prescribing biologics in these patients. We therefore advocate improved monitoring and reporting off-label use of biologics through registries. In several countries, registries have been set up that have provided important information on safety and efficacy in a variety of conditions (17). For example, the RUBRIC registry (acronym for Rational Use of Biologics in Refractory Immune-mediated inflammatory diseases Consortium) is a web-based registry in the Netherlands aiming to identify and monitor all patients suffering from rare therapy-refractory IMIDs who are treated off-label with biologics (18). The information that is generated will be used to develop treatment protocols, which may guide physicians on off-label prescription of biologics and may help payers to make informed decisions about reimbursement.

CONCLUSION

Off-label prescription of drugs is both legal and common and may give early access to new valuable treatments for patients, thereby adding to the innovation of clinical practice. This is especially the case in "orphan diseases" such as rare IMIDs, in which it is very difficult to conduct RCTs, but it also holds true for more common diseases as exemplified by infliximab (anti-TNF) treatment for refractory sarcoidosis. However, it is crucially important to consequently monitor efficacy and safety. We therefore advocate the use of registries, which will result in the collection of unbiased data that can be used to develop treatment guidelines for off-label prescription of biologics in individual rare IMIDs. Ultimately, this will lead to a more evidence-based and rational use of biologics in these diseases.

AUTHOR CONTRIBUTIONS

AM and AA performed the literature search and wrote the manuscript. AM and AA contributed equally to this paper. DG and PT treated the patient, reviewed, and edited the manuscript. ST wrote and edited the paper.

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HIV Latency-Reversing Agents Have Diverse Effects on Natural Killer Cell Function

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In an effort to clear persistent HIV infection and achieve a durable therapy-free remission of HIV disease, extensive pre-clinical studies and early pilot clinical trials are underway to develop and test agents that can reverse latent HIV infection and present viral antigen to the immune system for clearance. It is, therefore, critical to understand the impact of latency-reversing agents (LRAs) on the function of immune effectors needed to clear infected cells. We assessed the impact of LRAs on the function of natural killer (NK) cells, the main effector cells of the innate immune system. We studied the effects of three histone deacetylase inhibitors [SAHA or vorinostat (VOR), romidepsin, and panobinostat (PNB)] and two protein kinase C agonists [prostratin (PROST) and ingenol] on the antiviral activity, cytotoxicity, cytokine secretion, phenotype, and viability of primary NK cells. We found that *ex vivo* exposure to VOR had minimal impact on all parameters assessed, while PNB caused a decrease in NK cell viability, antiviral activity, and cytotoxicity. PROST caused non-specific NK cell activation and, interestingly, improved antiviral activity. Overall, we found that LRAs can alter the function and fate of NK cells, and these effects must be carefully considered as strategies are developed to clear persistent HIV infection.

Keywords: HIV, natural killer cells, latency reversing agents, immune function

INTRODUCTION

The recent description of an HIV-1 infected individual who experienced a sterilizing cure (1), without evidence of replication-competent virus *in vivo*, and others in whom early antiretroviral therapy (ART) resulted in undetectable viremia and maintenance of immune competence despite the cessation of ART (a functional cure) (2), has given rise to a variety of experimental approaches to induce cure or drug-free remission of HIV-1 infection. The most intensively studied eradication strategy [known as "shock and kill" (3)] rests on inducing viral expression within latently infected

Abbreviations: IFN-γ, interferon-gamma; ING, ingenol; LRA, latency reversing agents; PNB, panobinostat; PROST, prostratin; RMD, romidepsin; VOR, vorinostat.

$CD4^+$ T cells, with the goal of reducing the reservoir size through viral cytopathic effects (CPE) or immune-mediated clearance. However, recent *in vitro* experiments demonstrated that proviral reactivation alone did not result in viral CPE, and the autologous HIV-1 specific $CD8^+$ T cells of patients were unable to clear reactivated cells (4). Clearly, the capacity of the host immune system to recognize and kill infected cells upon reactivation requires closer evaluation.

Histone deacetylase (HDAC) inhibitors and protein kinase C (PKC) agonists are two promising classes of latency-reversing agents (LRAs) that are undergoing extensive testing in *in vitro* models and in initial pilot clinical trials to reactivate latent HIV-1 infection. HDAC inhibitors were developed as anticancer drugs as HDACs play important roles in epigenetic and non-epigenetic transcriptional regulation, inducing apoptosis and cell cycle arrest (5). In the context of HIV-1 reactivation, HDAC inhibitors induce transcription at the HIV-1 long terminal repeat (LTR) (6–9). PKC agonists induce latent viral expression though NF-κB signaling (10). Members of these two LRA classes have demonstrated efficacy in inducing HIV-1 expression in cells from patients on ART *in vivo* and *in vitro* (9, 11–16). However, as both histone deacetylation and signaling through NF-κB may impact the function of diverse cell populations, the effect of LRAs beyond latently infected cells must be carefully evaluated.

The influence of LRAs on cytotoxic T-lymphocytes (CTL) has recently been assessed. In one *in vitro* study, selected HDAC inhibitors caused a negative impact on CTL effector function (17), although in both this study and in another study that focused on vorinostat (VOR) (18), little effect of a pharmacologically relevant exposure to VOR was seen. $CD8^+$ T cells are a well-studied and crucial effector cell population contributing to target cell clearance after viral reactivation. However, other effector subsets may also play an important role, including cells from the innate immune system. Natural killer (NK) cells are the main effectors of the innate immune response. NK effector function is elicited immediately upon recognition of activating ligands without prior exposure to the infected cell or to viral antigens, resulting in direct lysis of target cells and/or promotion of antibody-dependent cellular cytotoxicity (ADCC) (19). In addition, NK activity has been associated with HIV post-treatment control of viremia after treatment interruption (20), ADCC has been correlated with protection in a recent HIV-1 vaccine trial (21) and innate immune cell responses were correlated with HIV-1 DNA decline during panobinostat (PNB) treatment *in vivo* (22). Thus, multiple lines of evidence suggest the relevance of NK cells in the clearance of persistent HIV-1 infection.

In the present study, we aim to better understand the impact of LRAs on the innate immune system, and specifically on NK cells. LRAs might impact the capacity of NK cell to clear infected cells in at least two ways: (i) through a direct impact on immune effector cells, causing activation, toxicity, or modifying receptor expression and cytotoxicity capacity or (ii) affecting the expression of ligands in the target population modifying effector recognition and subsequent clearance. Herein, we analyze both the direct impact of candidate compounds from two promising LRA classes on NK cells, and the effects on ligand expression on target cells *ex vivo*, as a means of informing HIV-1 eradication strategies making use of these agents in future pilot clinical trials.

MATERIALS AND METHODS

Cell Samples

Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll gradient from buffy coats of HIV-1 negative healthy donors, under approval of the UNC Biomedical Institutional Review Board. NK and $CD4^+$ T cells were magnetically isolated from the PBMCs by negative selection (StemCell Technologies, Vancouver, BC, Canada). The NK cell enrichment antibody cocktail included monoclonal antibodies against CD3, CD4, CD14, CD19, CD20, CD36, CD66b, CD123, HLA-DR, and glycophorin A. The $CD4^+$ T cell enrichment antibody cocktail included specific antibodies against CD8, CD14, CD16, CD19, CD20, CD36, CD56, CD66b, CD123, TCR-γ/δ, and glycophorin A. After isolation, NK cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% heat inactivated bovine serum and 5% penicillin plus streptomycin (cIMDM), including or not the different LRAs at the appropriate concentrations for 24 h. Then, cells were washed and functional assays performed.

Ethics Statement

Study participants provided written informed consent under a protocol that was approved by the UNC Biomedical Institutional Review Board.

Latency-Reversing Agents

The compounds comprising our LRA panel were provided, and stocks prepared, by the CARE Pharmacology Core of the University of North Carolina. VOR was donated by Merck, and romidepsin (RMD) and PNB obtained from Selleckhem. For all three, a 10 mM stock was prepared in DMSO, and further diluted with IMDM to a working stock concentration of 25 μM (VOR) or 5 μM (RMD and PNB). Prostratin (PROST) was purchased from Cayman Chemical (Ann Arbor, MI, USA) in an ethanol solution, which was lyophilized and reconstituted with DMSO to a concentration of 5 mM, and further diluted in plain IMDM to a working stock solution of 25 μM. Ingenol (ING) 3-20 dibenzoate was obtained from Santa Cruz Biotechnology; stock solution was prepared in DMSO at a concentration of 1 mM and diluted to working stock concentration of 25 μM with non-supplemented IMDM. All stocks and working solutions were stored at −20°C and used avoiding repeated freezing–thawing cycles. LRA concentrations used in the experiments were selected based on pre-clinical data to reflect the potential physiological concentration (23–25). VOR was used at a concentration of 335 nM, RMD at 10 nM, PNB at 20 nM, PROST at 1000 nM, and ING at 100 nM. If physiologic (*in vivo*) data were not available for selecting a concentration, concentrations were chosen to reflect dosing previously shown to be effective in HIV-1 reactivation in *ex vivo* studies (11, 26, 27). In addition, a lower and a higher dose of the one considered physiological

were tested in some experiments to determine if there was a dose-dependent relationship.

Viral Inhibition Assays

$CD4^+$ T cells were isolated by negative selection in parallel to NK cells from each donor. Isolated $CD4^+$ T cells were activated during 24 h with 2 μg/mL PHA (Sigma Aldrich, St Louis, MO, USA) and 60 U/mL IL-2 (Peprotech, Rocky Hill, CT, USA). Cells were then infected with the JR-CSF viral strain by spinoculation for 90 min at 2500 rpm. After spinoculation, cells were extensively washed to remove free virions and 50,000 $CD4^+$ T cells were plated in triplicate for each condition in a 96-well plate. NK cells, previously exposed to LRAs or not (reference control), were added to the wells in an effector:target (E:T) ratio of 1:1, and left in culture for 7 days in cIMDM with 5 U/mL IL-2, with a media change at day 4. Viral production was assessed in the supernatant by p24 ELISA (ABL_{inc}, Rockville, MA, USA), and percentage of viral inhibition of the different conditions was compared to inhibition from untreated NK cells. To assess the impact of LRAs on cell population proportion, we performed FACS analysis of the cells at the end of the viral inhibition assay. Cells were harvested and surface stained with CD3-PerCP, CD4-FITC, and CD56-PE (BD). The proportion of $CD4^+$ T cells was evaluated in the $CD3^+$ population, while the proportion of NK cells was evaluated in the whole sample. For the blocking experiments, NKG2D blockade was performed incubating PROST-treated NK cells with pure NKG2D (Miltenty Biotec) during 30 min at room temperature before starting the viral inhibition culture. Success of blockade was checked by flow cytometry.

Toxicity Assay

Natural killer cells were cultured in cIMDM in the presence or absence of the individual LRAs from our panel for 24 h. After washing, cells were re-suspended in Annexin binding buffer and stained with Annexin V-FITC and 7-AAD (Biolegend, San Diego, CA, USA) following manufacturers' protocol. Samples were analyzed on the Attune Focusing Cytometer (Applied Biosystems), and the percentage of double-positive cells for both Annexin V and 7-AAD was considered as the non-viable population.

Cytotoxicity, IFN-γ Production, and Non-Specific Activation Assays

Natural killer cytotoxicity and IFN-γ production were analyzed in co-cultures of primary NK cells and K562 cells (an NK-sensitive target cell line that lacks MHC-I molecules) with and without previous exposure of the NK cells to individual LRAs from our panel. Cytotoxicity was assessed by analyzing the expression of the degranulation marker CD107a, a reliable marker of NK cell cytotoxic activity (28). A total of 100,000 NK cells were co-cultured with the same number of K562 target cells in 96-well plates for 4–6 h in the presence of PE/Cy7-CD107a antibody, clone H4A3 (BD), adding 1 μL of GolgiStop (BD) after the first hour of culture. Cells were then harvested, washed, and surface-stained with CD56-FITC, clone NCAM 16 (BD) in staining buffer for 20 min on ice in the dark. Cells were then fixed with Fixation buffer (Biolegend, San Diego, CA, USA) during 20 min at room temperature in the dark, washed with Perm/Wash buffer twice and intracellularly stained with IFNγ-PE (Biolegend, San Diego, CA, USA) for 20 min. After washing, cells were re-suspended in staining buffer and analyzed in the Attune Focusing Cytometer (Applied Biosystems). To analyze whether NK cells were non-specifically activated by LRA, NK cells were also incubated in the absence of target cells, and CD69 (CD69-PE, clone FN50, from BD) and CD107a expression was analyzed as described.

Expression of Activating Receptors in NK Cells

A panel of NK cell activating receptors was analyzed by flow cytometry comparing untreated NK cells and cells exposed to individual LRAs from our panel. The following surface monoclonal antibodies were used: CD56-APC/Cy7 (clone HCD56), CD16-Pacific Blue (clone 3G8), NKG2D-Brilliant Violet 510 (clone 1D11), NKp30-PE (clone P30-15), NKp44-AlexaFluor 647 (clone P44-8), NKp46-PE/Cy7 (clone 9E2), and DNAM-1-FITC (clone 11A8) (all from Biolegend, San Diego, CA, USA). Samples were analyzed on LSR Fortessa (Becton Dickinson) cytometer. The expression of each of the receptors was analyzed on the $CD56^+$ population using FlowJo X software (Ashland, OR, USA). To set the gates, fluorescence minus one (FMO) controls were used for each individual experiment.

Expression of NK Ligands on Resting $CD4^+$ T Cells

Primary resting $CD4^+$ T cells were isolated from healthy HIV-uninfected donors and cultured *in vitro* with individual LRAs from our panel. After 24 h in culture with the LRAs, cells were washed and stained with antibodies against seven different NK ligands. Antibodies used included NTB-A (clone NT-7, Biolegend), HLA-E (clone 3D12, Biolegend), Bw4 (clone REA274, Miltenyi Biotec), CD155 (clone TX24, Biolegend), ULBP-1 (clone 170818, R&R Systems), ULBPB-2 (clone 16590, R&R Systems), and CD48 (clone TU145, BD Pharmingen). Flow cytometry was performed to analyze for changes in cell surface expression. Median intensity of fluorescence was compared to medium/carrier solvent alone (negative control, dimethyl sulfoxide).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 6.07 (GraphPad Software, La Jolla, CA, USA). Data are presented either as raw values or fold change (FC) normalized to untreated NK cells for each of the experiments. Statistical significance was determined with a Wilcoxon matched-pairs signed rank test.

RESULTS

NK Cell Antiviral Activity Improves after Prostratin Exposure but It Is Impaired after Panobinostat and Ingenol Treatment

Viral inhibition capacity of NK cells was tested in autologous cell systems, using NK and $CD4^+$ T cells from the same donor. The average purity of NK cells after isolation – measured as

the proportion of $CD3^{-}CD56^{+}$ – was of 91.26% (SEM = 1.5), and the purity of CD4 cells – measured as the proportion of $CD3^{+}CD4^{+}$ – was 93.22 (SEM = 1.6). Results obtained from LRA-treated NK cell conditions were normalized to viral inhibition observed in the untreated NK cell condition. Exposure of NK cells to VOR did not impact the capacity of NK cells to reduce viral replication. However, NK treatment with RMD, PNB, and ING reduced the percentage of viral inhibition (RMD: 79.22%, $p = 0.176$; PNB: 58.6%, $p = 0.016$; ING: 67.69%, $p = 0.001$). Interestingly, PROST exposure improved antiviral activity of NK cells (159.5%, $p = 0.002$) (**Figure 1**, Figure S1 in Supplementary Material).

To elucidate the mechanisms by which some drugs altered NK cell antiviral activity, we investigated the direct impact of exposure to such drugs on NK cytotoxicity, IFN-γ production, viability, activation, and receptor expression. In addition, at the end of the viral inhibition assay, the cells present in the cultures were stained for NK cell markers (CD56) and T cell markers (CD3 and CD4). Interestingly, we found that proportion of NK cells increased following PROST exposure (153%, SEM = 14.0, $p = 0.03$), while the frequency of CD4 cells within the $CD3^{+}$ population was diminished in the PROST-treated NK group compared to the untreated and all the other conditions (88.1%, SEM 2.7, $p = 0.03$). The increased frequency of NK cells in the culture with PROST was primarily due to an increase in the $CD56^{bright}$ population (216.7%, SEM 28.1, $p = 0.03$). For three donors, we also analyzed absolute numbers of cells, finding that PROST treatment increased cell number, specifically NK number (Figure S2 in Supplementary Material). Finally, we performed a three viral inhibition assays blocking the activating receptor NKG2D, showing that blockade of NKG2D in PROST-treated NK cells caused a decrease in viral inhibition capacity (average of 17.43% viral inhibition after blocking of NKG2D compared to 36.12% without blocking, Figure S3 in Supplementary Material).

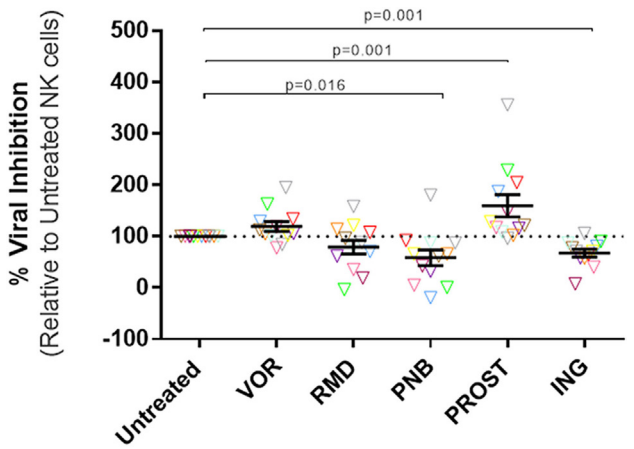

FIGURE 1 | **Antiviral activity of NK cells**. Percentage HIV replication inhibition after 7 days of culture, normalized to untreated NK cells. $CD4^{+}$ T cells were isolated, stimulated, and infected with JR-CSF. Infected targets were cultured with autologous NK cells in triplicate at a ratio 1:1. Each color represents cells from a different donor. At physiologically relevant doses, VOR and RMD did not have significant impact in the antiviral activity of the NK cells, while exposure to PNB and ING impaired NK cell viral inhibition capacity. On the contrary, NK treatment with PROST improves antiviral activity of NK cells. *p*-values were calculated using a Wilcoxon matched-pairs signed rank test. *N* = 12. VOR, vorinostat; RMD, romidepsin; PNB, panobinostat; PROST, prostratin; ING, ingenol.

Romidepsin and Panobinostat Are Toxic to NK Cells

Isolated NK cells were exposed to LRAs for 24 h, washed and stained with Annexin V-FITC and 7-AAD. Dead cells were identified as double-positive cells for Annexin V and 7-AAD (**Figure 2A**). We observed a general trend toward a decrease in viability after exposure to all drugs ($p = 0.02$) except for ING ($p = 0.53$), but the impact of RMD and PNB was more pronounced, with a mean FC of 2 and 2.5 for RMD and PNB, respectively, compared to VOR and PROST (mean FC = 1.3 and 1.2, respectively) (**Figure 2B**). In addition, exposure to increasing concentrations of RMD and PNB decreased cell viability in a dose-dependent manner, while viability of NK cells was not reduced following exposure to higher concentrations of VOR, PROST, or ING (Figure S4 in Supplementary Material). As the observed dead cell numbers were high, we compared the viability of untreated NK cells right after isolation and after 24 h in culture. We observed an increase of the apoptotic population (measured as Annexin V+) from 14.8 to 32%. This high rate of cellular death may be due to the lack of IL-15 or IL-2 in the culture.

LRA Impact on NK Cell-Mediated Cytotoxicity

Natural killer cytotoxicity was measured by analyzing the expression of CD107a after co-culture with K562 cells (**Figure 3A**). Exposure to a physiologically relevant concentration of RMD, PNB, and ING impaired the cytotoxic capacity of NK cells, as shown by a significant decrease in the proportion of $CD56^{+}CD107a^{+}$ cells ($p < 0.0001$). However, VOR and PROST did not have a significant impact on NK cytotoxic function (**Figure 3B**). The observed impairment in NK cell cytotoxic function caused by RMD, PNB, and ING was not due to a direct effect on the viability of NK cells, as we checked in cytotoxic function assays, including a viability stain (Annexin V-FITC and 7-AAD, $n = 4$ donors; data not shown).

We further analyzed whether the impairment in cytotoxic activity was dose dependent. NK cell cytotoxicity showed a dose-dependent reduction after exposure to RMD and PNB, and to some extent to VOR. Interestingly, a higher dose (1000 nM) of ING showed a slight improvement in NK cytotoxic function compared to 100 nM (Figure S5 in Supplementary Material).

Panobinostat and Prostratin Impair IFN-γ Secretion

Interferon-gamma secretion is an antiviral mechanism employed by NK cells and leads to recruitment and modulation of the activity of other effector cells, including $CD8^{+}$ T cells (29). We analyzed IFN-γ production after a 4–6 h culture with K562 target cells (**Figure 4A**). FACS analysis after intracellular IFN-γ staining

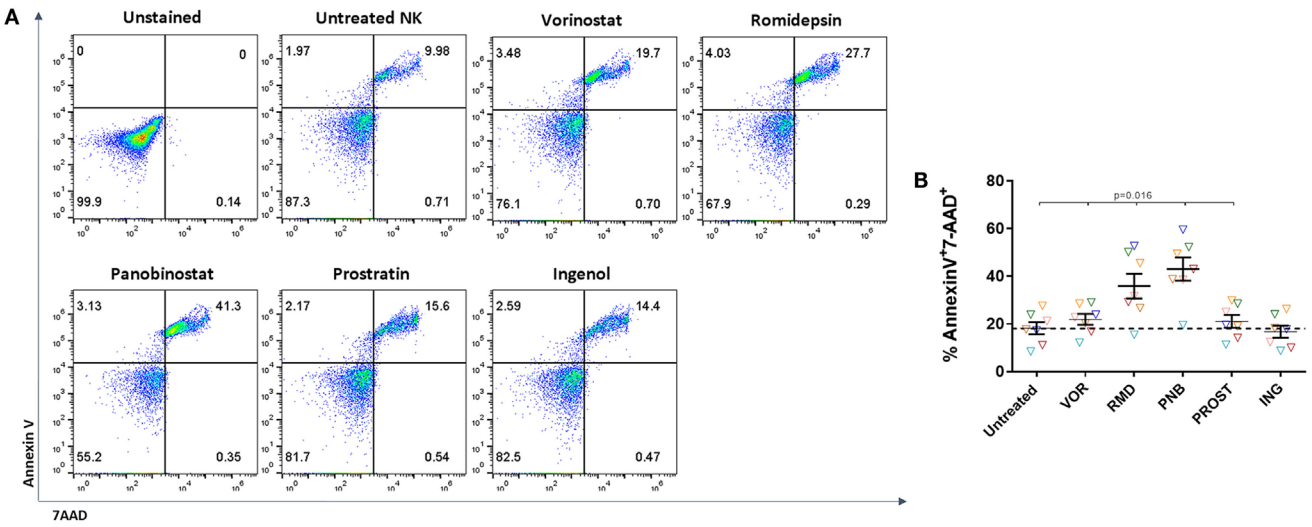

FIGURE 2 | **Toxicity of LRAs on NK cells**. Proportion of dead NK cells after 24-h exposure to the different LRAs. **(A)** Representative FACS analysis showing the gating strategy to identify dead cells according to Annexin V and 7-AAD staining. Cell death was considered when cell were double positive for Annexin V and 7-AAD. **(B)** Proportion of dead NK cells after exposure to the different LRAs, expressed as fold change relative to the untreated condition. Each color represents cells from a different donor. There was a general trend toward a decrease in viability after exposure with all drugs except for ING, but RMD and PNB impact was more pronounced and significantly higher than VOR, PROST, or ING ($p < 0.02$). $N = 7$.

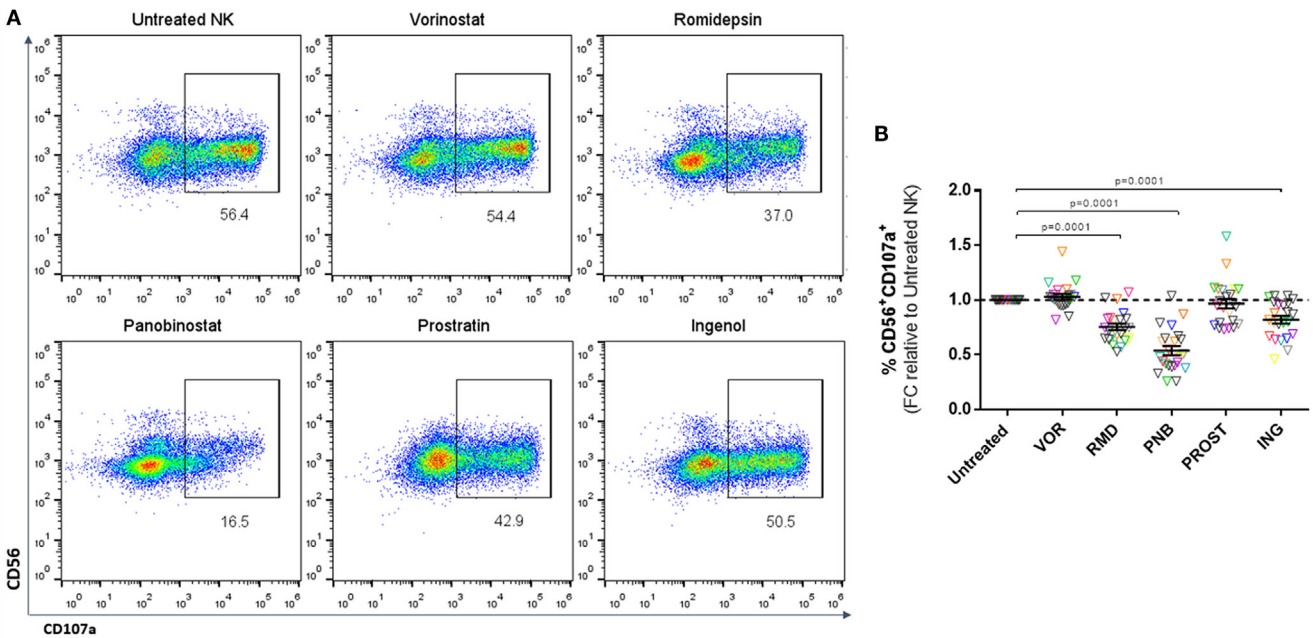

FIGURE 3 | **Cytotoxicity of NK cells**. Proportion of $CD56^+CD107a^+$ cells after 4–6 h culture with K562 cells, expressed as fold change (FC) relative to untreated NK cells. **(A)** Representative FACS analysis plots showing the gating strategy. **(B)** Proportion of $CD56^+$ cells expressing the degranulation marker CD107a. Each color represents cells from a different donor. Exposure to PNB, RMD and ING significantly impaired NK degranulation. $N = 23$.

demonstrated that treatment with VOR modestly increased the percentage of NK cells that produced IFN-γ compared to untreated NK cells; however, this did not reach statistical significance. On the contrary, treatment with PNB or PROST resulted in a significant reduction in the number of IFN-γ^+ NK cells ($p = 0.05$ and $p = 0.01$, respectively) (**Figure 4B**).

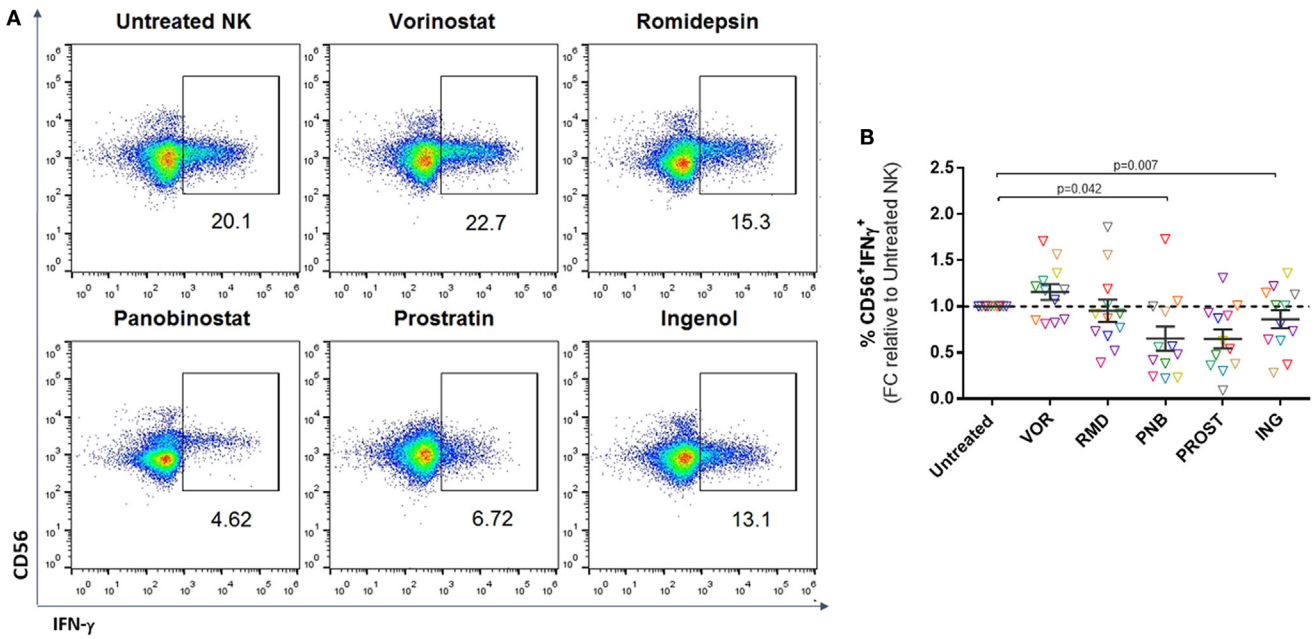

FIGURE 4 | **Cytokine production**. Proportion of CD56⁺IFN-γ⁺ cells after 4–6 h culture with K562 cells. **(A)** Representative FACS analysis showing gating strategy after intracellular staining with IFN-γ. **(B)** Proportion of CD56⁺ cells positive for IFN-γ expressed as fold change relative to the untreated NK condition. Each color represents cells from a different donor. VOR, RMD, or ING do not have a significant effect on IFN-γ production, while PNB and PROST impaired cytokine production. $N = 12$.

PKC Agonists Cause Non-Specific NK Cell Activation

To measure non-specific NK cell activation caused by exposure to LRAs, expression of CD69, CD107a, and IFN-γ was measured in the absence of target cells or any other stimulus. Exposure to all drugs caused non-specific activation as measured by CD69 expression ($p = 0.01$), although activation was much greater after treatment with PROST and ING (27- and 14-fold, respectively). Among the HDACi tested, PNB led to an increase in CD69 expression of nearly 10-fold, while VOR and RMD only produced 1.5- and 2.2-fold increase. In addition, exposure to the PKC agonist PROST caused marked increase in CD107a, while the increase caused by ING was more variable ($p = 0.01$ and $p = 0.06$, respectively; **Figure 5**). IFN-γ production in the absence of target cells was also assessed, and no change was observed after exposure to any of the LRA (data not shown). Figure S6 in Supplementary Material show these results as raw data.

Expression of Activating Receptors on NK Cells Is Downregulated by Exposure to PNB

Natural killer cells express a wide variety of receptors that enable them to differentiate infected or tumor cells from healthy cells. These include inhibitory, activating, adhesion, and cytokine receptors. The balance of these signals determines whether the NK cell becomes activated or not (30). For this study, we analyzed the expression of activating receptors that have been identified to be important for antiviral activity of NK cells. These included CD16, NKG2D, DNAM-1, NKp30, NKp44, and NKp46 (31). Their expression was measured by flow cytometry after gating on the CD56⁺ population (**Figure 6**). PNB exposure caused a significant decrease in the expression of all receptors except from NKp44: CD16 ($p = 0.05$), NKG2D ($p = 0.05$), DNAM-1 ($p = 0.01$), NKp30 ($p = 0.01$), and NKp46 ($p = 0.01$). On the contrary, exposure to ING increased the expression of most of the receptors: CD16 ($p = 0.05$), NKG2D ($p = 0.005$), NKp30 ($p = 0.005$), and NKp46 ($p = 0.01$). PROST also caused a significant increase in NKG2D expression ($p = 0.005$), but a decrease in CD16 ($p = 0.005$). Figure S7 in Supplementary Material shows a representative example of the expression of the activating receptors in the presence of these agents.

Impact of LRAs on NK Ligand Expression in Resting CD4⁺ T Cells

We analyzed by flow cytometry the expression of some ligands on target cells that either trigger NK activation or initiate inhibitory NK signaling by binding to NK inhibitory receptors. The activating ligands included NTB-A, which binds NTB-A on the NK cell surface and leads to activation and secretion of interferon-γ, CD48, which binds 2B4, ULBP-1 and -2, ligands that bind NKG2D and that have been demonstrated to be upregulated in the setting of HIV-1 infection by the viral protein *vpr* (32), and CD155, a NK ligand that binds DNAM-1 on NK cells and induces activation (33). The inhibitory included HLA-E

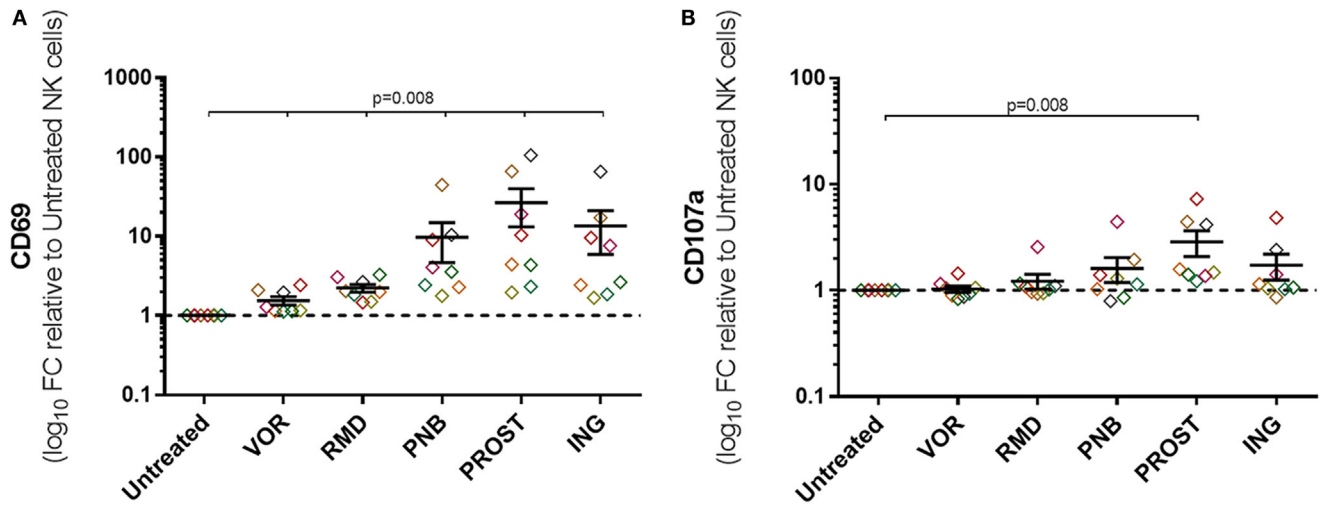

FIGURE 5 | **Non-specific activation of NK cells**. Expression of activation markers on NK cells in the absence of target cells or any other stimuli. Graphs represent $\log_{10}$ of the fold change relative to untreated NKs and each color represents cells from a different donor. **(A)** Expression of CD69. **(B)** Expression of CD107a. Exposure to all drugs caused some unspecific activation as measured by CD69 expression, but exposure to the PKC agonists were the most remarkable. PROST also caused a significant increase in CD107a expression. $N = 8$.

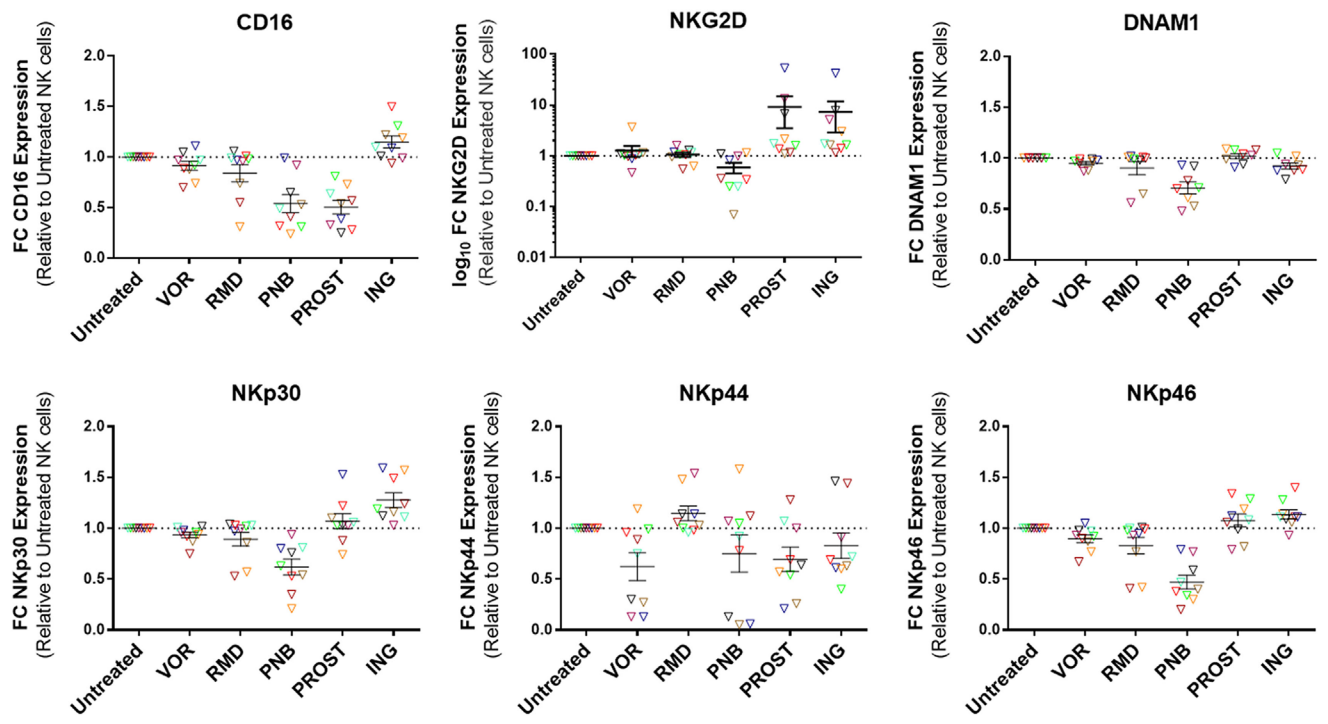

FIGURE 6 | **Expression of activating receptors on NK cells**. Results are expressed as fold change relative to untreated NK cells and each color represents cells from a different donor. PNB caused a general decrease in the expression of activating receptors, as opposed to ING, which caused a significant increase in the expression of all receptors except DNAM1 and NKp44. Of interest, PROST produced a significant increase in the expression of NKG2D. $N = 9$.

and HLA-Bw4, NK ligands that initiate inhibitory NK signaling by binding to NK receptors NKG2A and KIR3DL1, respectively. The expression of most activating ligands on resting $CD4^+$ T cells were unaffected by the LRAs tested, with the exception of CD155, which increased its expression in the presence of both PKC agonists [2.3-fold increase with PROST ($p = 0.01$) and 2.1-fold increase with ING ($p = 0.02$)], as well as PNB (1.7-fold increase, $p = 0.02$). Regarding inhibitory ligands, PKC agonists

significantly upregulated expression of HLA-E (ING: twofold, $p = 0.005$; PROST: 2.3-fold, $p = 0.01$) and Bw4 (ING: 2.6-fold, $p = 0.01$, PROST: 2.8-fold, $p = 0.01$). None of the HDACi significantly affected HLA-E expression, although modest trends for decreased HLA-Bw4 expression were observed with all HDACi, reaching statistical significance for VOR ($p = 0.05$). In sum, exposure to PKC agonists PROST and ING significantly increased two inhibitory NK ligands (HLA-E and HLA-Bw4) and one activating ligand (CD155) on the cell surface of primary $CD4^+$ T cells, while HDACi exposure had little effect on NK ligand expression on resting $CD4^+$ T cells (**Figure 7**).

DISCUSSION

For successful implementation of the HIV eradication strategy of latency reversal and clearance, LRAs doses and administration regimens must be selected so that they induce viral antigen expression in latently infected cells but do not interfere with the clearance function of immune effector cells. In this study, we have evaluated the impact of five LRAs on NK cell function. Overall, we observed a heterogeneous effect of the different LRAs evaluated, differing even within each drug class. VOR did not have any significant effect on any of the parameters of NK cell function or viability, while exposure to the other HDACi tested, RMD and PNB, had deleterious effects. Particularly, PNB caused a significant decrease in cytotoxicity, antiviral activity, activating receptor expression, and cytokine secretion of NK cells, along with a decrease in viability. A similar trend was observed for RMD, but milder that the observed with PNB. PKC agonists induced expression of some markers of activation in NK cells and, remarkably, PROST exposure lead to an improvement in antiviral activity.

To our knowledge, no studies of the impact of PNB or PKC agonists on NK cell function have been performed. A previous study reported VOR suppression of NK cell cytolytic activity by impairing granule exocytosis and decreasing expression of activating receptors (34). However, a 96-h incubation with the drug was carried out in those experiments, which exceeds the *in vivo* pharmacokinetic exposure to VOR, cleared in less than 6 h (35). On the contrary, we did not observe any negative impact of VOR on NK cell function. Clinical treatment with RMD in patients with cutaneous T-cell lymphoma induced a decrease of NK cell cytolytic activity, similar to our *ex vivo* results, although interestingly the activity was restored after stimulation with a toll-like receptor agonist (36).

Here, we observed that PNB exposure caused a decrease in the antiviral activity of NK cells. This impairment in viral inhibition was likely due to the reduction in cytotoxicity and IFN-γ production that we also observed, as well as the down-modulation of important activating receptors, such as NKG2D, on the surface of the NK cells. In addition, PNB decreased NK cell viability, so at least part of the decreased antiviral activity observed in the viral inhibition experiments could be due to an actual reduction of number of NK cells in culture, with a consequent reduction in E:T cell ratio. In fact, using our whole dataset, we found that both cytotoxicity and cell viability correlated significantly with a decrease in antiviral activity (Figure S8 in Supplementary Material). PNB has been shown in a study to be the most potent of all HDAC inhibitors (37), and could represent a very promising option for HIV reactivation. However, our *in vitro* results point out the potential deleterious effects that PNB can have on NK cell function, and this should be monitored in future clinical studies. Interestingly, the recent *in vivo* pilot clinical trial with PNB observed that patients with more pronounced proviral DNA

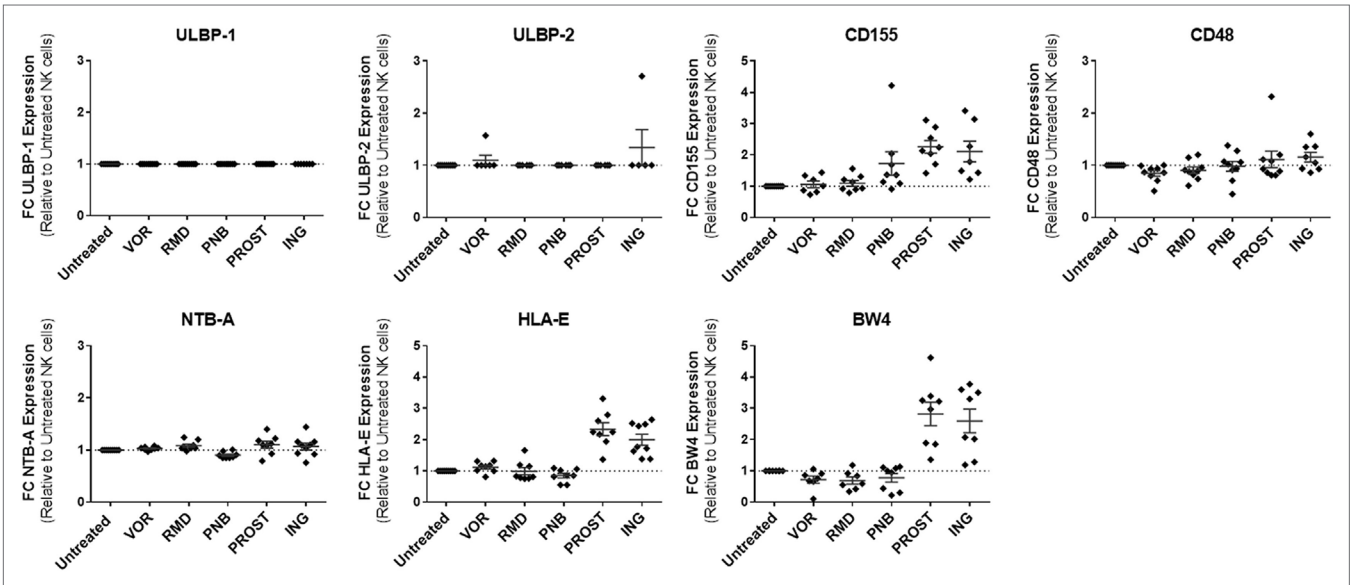

FIGURE 7 | NK ligand expression on resting $CD4^+$ T cells in the presence of LRAs. LRAs had little effect on NK ligand expression with the exception of PKC agonists, which caused increased expression of the inhibitory NK ligands HLA-E and HLA-Bw4 as well as activating ligand CD155. Median fluorescence intensity was compared to the medium/carrier solvent alone condition (negative control, dimethyl sulfoxide). $N = 8$.

decline during PNB treatment had a higher frequency of NK cells (22), suggesting that the function of the innate immune system should be monitored during latency reversal and clearance studies.

On the other hand, we observed an improvement in NK antiviral activity after exposure to PROST. This observation could be due to the increase in NK activation upon culture with the drug; however, it is also possible that PROST remained attached to cellular membranes despite washing after drug exposure as has previously been suggested (38), leading to blunted HIV infection in target cells. When we analyzed the cell composition of the cultures after a 7-day viral inhibition assay, we consistently observed that in the cultures where NK cells were exposed to PROST, the $CD3^{-}CD56^{+}$ subset was increased compared to all the other conditions, with an especially marked increase in the $CD56^{bright}$ population, while the proportion of cells expressing CD4 within the $CD3^{+}$ population was decreased. Moreover, exposure to PROST upregulated the expression of NKG2D, which is a NK cell activating receptor known for its importance in NK cell antiviral activity (39, 40). Thus, the increase in antiviral activity observed after PROST exposure was most likely due to a combination of several effects, including changes in both the target population and in the effector population. This may have interesting implications for *in vivo* HIV-1 eradication strategies in which PKC agonists might simultaneously induce several desirable effects: reactivation of latently infected cells, inhibition of viral replication, and enhancement of the antiviral effect of NK cells. ING, the other PKC agonist tested in our experiments, did not have such a marked effect in any of the functional characteristics analyzed, but we did observe upregulation of NKG2D and some activation measured by CD69. Beyond the specific characteristics of each of these components of the PKC agonist family, a possible reason for the difference in the magnitude of the effect observed with PROST and ING could be the dose of each drug used in our experiments. In fact, when a higher dose (1 μM) of ING was used for the viral inhibition experiments, an improvement in NK cell antiviral activity was observed (mean of twofold compared to untreated NK cells, Figure S9 in Supplementary Material). However, we also observed a decrease in IFN-γ production when NK cells were exposed to PKC agonists. This conflicts with our observations of the increase of the $CD56^{bright}$ population, given that generally $CD56^{dim}$ subpopulations are more cytotoxic, while $CD56^{bright}$ are able to produce IFN-γ (41). We also observed a decrease in CD16 expression on NK cells treated with PROST, which in this case would correlate with the increase in the $CD56^{bright}$ subpopulation, as CD16 is expressed largely in $CD56^{dim}$ subsets. Down-modulation of CD16 is concerning given that ADCC is mediated by antibody engagement to this receptor, and further study is required to determine whether this decrease in expression has functional consequences.

Our experiments were performed using unstimulated NK cells, with the aim of recapitulating *in vivo* exposure *to* LRAs. However, for immunotherapeutic purposes, an alternative strategy involves *ex vivo* stimulation and expansion of NK cells, with the intention of improving their cytotoxic potential. Susceptibility of these expanded cells to LRAs may differ from what we have observed in non-stimulated cells, as it has been already shown for impact on CTL (17). In fact, we observed a reduction in K562 target cell lysis when expanded NK cells were exposed to PROST (data not shown). On the contrary, and although we did not observe relevant impact of VOR on NK cell function, Schmudde et al. reported an impairment of NK cell degranulation by VOR exposure when cells were not stimulated, but no impact if NK cells were previously stimulated with IL-2 (42).

In addition to investigating the direct impact of LRAs on NK cells, we analyzed the impact of exposure to these agents on target cells relevant for HIV infection (resting $CD4^{+}$ T cells) to assess expression of surface ligands that would render them more susceptible to NK cell recognition and clearance. Overall, we did not observe a striking effect on ligand expression on $CD4^{+}$ T cells, with the exception of a modest increase in HLA-E and BW4 (inhibitory ligands), and CD155 (an activating ligand) when cells were exposed to PKC agonists. Different observations have been reported from oncology studies, where it has been consistently reported that HDACi upregulate the expression of NKG2D ligands on tumor cells (43–46), helping their recognition by innate immune system. On the other hand, it has also been seen that HDACi can down-modulate the expression of NKp30 ligands on tumor cells, reducing NKp30-dependent effector functions of NK cells (47).

To achieve clinically significant reversal of HIV-1 latency, several studies suggest that combinations of mechanistically different LRA will be needed (48–50). Our results add an additional factor to consider when designing an adequate LRA combination, as not only reactivation potency should be taken into account but also the impact that each of the drugs have on immune effector function. Thus, if a certain LRA is selected to be used because of its potency for reactivating the reservoir but it has shown to cause an impair in immune function, the second component of the LRA combination ideally should have proven to cause an improvement to some extent in the effector activity. The optimum situation for cure strategies would be finding compounds that simultaneously can disrupt latency and boost the immune response, and some agents with these capabilities are beginning to be described (51).

In summary, we have evaluated *ex vivo* the impact of five different latency-reversing agents on the effector function, phenotype, and viability of NK cells. This is of clinical relevance given the necessity of a potent immune response after reactivation of the latent HIV reservoir in order to achieve viral eradication. We have found a heterogeneous effect of the different agents studied, highlighting the lack of impact of VOR, the negative effects of PNB and RMD, and the potential beneficial impact of PKC agonists. Impact of LRA on immune function should be considered when designing LRA combinations to reactivate the latent HIV-1 reservoir. This is the first study to address the impact of LRAs on innate immune functions in the context of HIV-1 eradication, and demonstrate the importance of further evaluation of NK cell function. However, *in vitro* results might differ from *in vivo* effects. Our analysis of the effect of LRAs on NK cell function is, of necessity, only a preliminary one. In clinical trials, multiple doses of LRAs will be given over time, a phenomenon that is more difficult to model. Thus, innate immune function should be evaluated in HIV-1 positive patients undergoing latency reversing therapy.

AUTHOR CONTRIBUTIONS

CG and DM conceived the study. AS performed the experiments for ligand expression on target cells, MC performed target lysis experiments, and CG conducted all other experimental work. NS-S, AS, JK, EB, and VP contributed with ideas and experimental design of the study. CG wrote the manuscript, with edits from DM, NS-S, AS, MC, and VP. All authors revised the contents of the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2016.00356

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Glatiramer Acetate, Dimethyl Fumarate, and Monomethyl Fumarate Upregulate the Expression of CCR10 on the Surface of Natural Killer Cells and Enhance Their Chemotaxis and Cytotoxicity

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In vitro harnessing of immune cells is the most important advance in the field of cancer immunotherapy. Results shown in the current paper may be used to harness natural killer (NK) cells *in vitro*. It is observed that drugs used to treat multiple sclerosis such as glatiramer acetate, dimethyl fumarate, and monomethyl fumarate upregulate the expression of chemokines receptor 10 (CCR10) on the surface of human IL-2-activated NK cells. This is corroborated with increased chemotaxis of these cells toward the concentration gradients of the ligands for CCR10, namely CCL27 and CCL28. It is also demonstrated that these three drugs enhance NK cell cytotoxicity against tumor target cells, an activity that is abrogated by prior incubation of the cells with anti-CCR10 antibody. Because CCL27 and CCL28 are secreted by selective tumor types such as malignant melanoma, squamous cell carcinomas, and colorectal cancer, respectively, it is hypothesized that activated NK cells may be harnessed *in vitro* with any of these drugs before utilizing them as a therapeutic modality for cancer.

Keywords: NK cells, glatiramer acetate, dimethyl fumarate, monomethyl fumarate, cancer, chemotaxis, cytotoxicity

INTRODUCTION

Natural killer (NK) cells perform several important functions; among them, the regulation of the adaptive immune response by secreting cytokines such as IFN-γ, shaping the innate immune system by interacting with dendritic cells, defending against viral infection, and lysing and destroying tumor cells (1–5). Resting NK cells respond to dangers occurring at sites of injury. Evidence gathered from a mouse xenograft tumor model testing functionally deficient NK cells or antibody-mediated NK cell depletion supports that NK cells can eradicate tumor cells. An 11-year follow-up study in patients indicated that low NK-like cytotoxicity was associated with increased cancer risk (6). High levels of tumor-infiltrating NK cells are associated with a favorable tumor outcome in patients with colorectal carcinoma, gastric carcinoma, and squamous cell lung cancer, suggesting that NK cell infiltration into tumor tissues represents a positive prognostic marker (7).

Chemokines are molecules that play essential roles in linking the innate and adaptive immune responses (8) and are crucial in health and diseases (9). They have low molecular weights and are divided into four subfamilies based on the position of the cysteine (C) residue in the amino terminal end of the molecules; these are known as CXC, CC, C, and CX3C. They play important roles in NK cell biology maintaining them in the bone marrow, guiding them into the circulation, and aiding their accumulation at sites of injury. The field examining the expression of chemokine receptors in NK cells, and their ability to induce their migration, started in early 1990s (10, 11). There are overwhelming results describing the effects of chemokines on various biological activities of NK cells [reviewed in Ref. (12)].

Glatiramer acetate (GA; commercial name Copaxone) is a synthetic compound made up of the four amino acids Glu, Ala, Lys, and Tyr that are most common in myelin basic protein (13). GA is a first-line immunomodulatory therapy in relapsing-remitting multiple sclerosis (RRMS) patients (14). Although the drug is not as effective as second-line therapies such as Natalizumab and Fingolimod, GA is widely used due to few serious side effects. This drug showed promise in maintenance therapy, when used after more intensive immunosuppression (15). It was also reported that GA enhanced the *in vitro* killing of autologous and allogeneic human immature and mature monocyte-derived dendritic cells (DCs) by activated human NK cells (16). Further, administration of GA into mice ameliorated the EAE clinical scores, and this was associated with high killing of dendritic cells by NK cells isolated from the same mice (17).

Dimethyl fumarate (DMF), also known as Tecfidera (Biogen, Cambridge, MA, USA), has been approved by the FDA as an oral therapy for multiple sclerosis (MS) patients due to its efficacy. The mechanism of action of DMF is not completely understood. However, it was suggested that DMF may be hydrolyzed by esterases to monomethyl fumarate (MMF), although it is not yet clear whether MMF might mediate the *in situ* effects of DMF (18). It has also been demonstrated that DMF inhibits the proliferation of A375 or M24met cell lines and reduces melanoma growth and metastasis in experimental melanoma mouse models (19).

We recently reported that MMF increased primary human $CD56^+$ NK cell lysis of K562 and RAJI tumor cells (20). Moreover, MMF upregulated the expression of NKp46 on the surface of NK cells, which was correlated with upregulation of CD107a (lysosomal-associated membrane protein-1 "LAMP-1") on the surface of $CD56^+$ NK cells, and the release of Granzyme B from CD56 NK cells (20). Moreover, MMF inhibited the EAE clinical score in SJL mice correlated with enhanced NK cell lysis of dendritic cells (21).

In the present report, we describe a novel effect of GA, MMF, and DMF. We observed that these drugs upregulate the expression of CCR10 on the surface of IL-2-activated NK cells, corroborated with increased cytotoxicity, and induced chemotaxis toward the ligands for CCR10, namely CCL27 and CCL28. These observations may have implications for utilizing the highly antitumor effector NK cells in the therapy of cancer, particularly for those patients where tumor cells secrete the ligands for CCR10.

MATERIALS AND METHODS

Reagents

FITC-conjugated mouse antihuman CCR3, CCR4, CCR5, CCR6, CCR7, CCR9, CXCR1, CXCR3, CXCR4, and CXCR5 or unconjugated monoclonal mouse-antihuman CCR1, CCR2, and CXCR6, as well as PE-conjugated rat antihuman CCR8, PE-conjugated rat antihuman CCR10, and PE-conjugated rat IgG2b, were obtained from R&D Systems Europe Ltd. (Abingdon, UK). FITC-conjugated mouse antihuman CX_3CR1 was purchased from Medical and Biological Laboratories Co. Ltd. (Nagoya, Japan). FITC-conjugated monoclonal mouse antihuman CD3, PE-conjugated monoclonal mouse antihuman CD56, and FITC-conjugated goat anti-mouse were purchased from Becton-Dickinson (San Diego, CA, USA). FITC-conjugated mouse IgG, PE-conjugated mouse IgG, unconjugated mouse IgG, and unconjugated rat IgG were obtained from either Becton-Dickinson or from R&D Systems. Pertussis toxin (PTX), MMF, and DMF were obtained from Sigma-Aldrich (Saint Louis, MO, USA). CCL1, CCL27, CCL28, and CXCL10 were purchased from PeproTech (London, UK).

Preparation and Culture of NK Cells

Buffy coats from normal human volunteers were obtained from the blood bank (Ulleval Hospital, Oslo). Human IL-2-activated NK cells were prepared using Histopaque-1077 (Sigma-Aldrich) and RosetteSep human NK cell enrichment cocktail (Stemcell Technologies, SARL, Grenoble, France). NK cells were negatively selected by removing cells expressing CD3, CD4, CD19, CD36, CD66b, CD123, and glycophorin A. More than 95% of these cells expressed the CD56 molecule but lacked the CD3 molecule as determined by flow cytometric analysis (Figure S1 in Supplementary Material). Purified NK cells were then placed in flasks at 1×10^6/mL and 200 U/mL IL-2 (PeproTech, Rocky Hill, NJ, USA), and then incubated at 37°C in a 5% CO_2 incubator for 5–7 days.

NK Cell Cytotoxicity Assay

The human myeloid leukemia cell line K562 cells (CCL-243 obtained from American type culture collection "ATCC," Manassas, VA, USA) or RAJI human lymphoma cells (CCL-214, ATCC), were used as target cells. Target cells were incubated at 1×10^6 cells/mL with 5 μg/mL Calcein AM (Sigma-Aldrich) for 45 min. The cells were pelleted by centrifugation and resuspended in RPMI. To obtain total lysis, these cells were incubated in 96-well plates with 0.05% Triton X, whereas they were incubated with medium alone to obtain total viability. In other cultures, Calcein-AM-labeled cells were incubated at 37°C in a 5% CO_2 incubator with activated NK cells at different NK target cell ratios for 4 h. The plates were centrifuged, supernatants were removed, and replaced with PBS. Fluorescence units (FUs) were measured in Cytofluor plate reader. The percentage of cytotoxicity was calculated according to the following formula: % viability = FU of targets incubated with IL-2-activated NK cells (experimental) minus FU of targets incubated with Triton X, divided by FU of targets incubated in medium only (total viability), minus FU of

targets incubated with Triton X (total lysis). In the figures, the 10:1 effector:target (E:T) cell ratio is shown; however, similar results were obtained using other E:T cell ratios (2.5:1 and 5:1). Of note, the media of incubation contains RPMI plus 10% FCS. For anti-CCR10 treatment, the cells were washed and then treated with 10 μg/mL anti-CCR10 or 10 μg/mL isotype control antibody for 45 min at 4°C, washed and examined for viability. Only more than 95% viable cells were then added to Calcein-AM-labeled target cells and incubated for 4 h in the NK cytotoxicity assay.

In Vitro Chemotaxis Assay

Nucleopore blind well chemotaxis chambers with a lower well volume of 200 μL were used. A maximum volume of 200 μL medium containing RPMI plus 2% FCS was placed in the lower wells in the presence or absence of various chemokines. Cells (2×10^5) were placed in the upper compartments and incubated for 2 h at 37°C in a 5% CO_2 incubator separated by polycarbonate filters (Nucleopore Polycarbonate 13 mm size 8 UM, Whatman International Ltd., Kent, UK). Filters were removed, dehydrated, stained with 15% modified Giemsa stain for 7 min, and then mounted on glass slides. Cells in 10 high power fields were counted and averaged for each sample. Migration index (MI) was calculated as the number of cells migrating toward the concentration gradients of chemokines divided by the number of cells migrating toward medium only as previously described (22). For pretreatment with PTX, NK cells (1×10^6/mL) were either left intact or were treated for 2 h at 37°C with 100 ng/mL-activated PTX, as previously described (22). Only more than 95% viable cells were examined.

Flow Cytometric Analysis

IL-2-activated NK cells were either left intact or incubated with various concentrations of GA, MMF, or DMF for 24 h. The cells were washed and incubated in a 96-well plate (v-bottom, 2×10^5 cells per well), washed again, and resuspended in PBS buffer containing 0.1% sodium azide and 2% fetal bovine serum. Cells were first checked for viability with Trypan blue exclusion test, and only more than 95% viable cells were used in the assay. These viable cells were labeled with antibodies for 45 min in the dark at 4°C, washed twice, and examined in the flow cytometer (FACSC II, Becton-Dickinson Biosciences, San Jose, CA, USA). Markers were set according to the isotype control FITC- or PE-conjugated mouse IgG. More than 95% viable NK cells were also incubated at 4°C in the dark with FITC-conjugated anti-CD107a or FITC-conjugated IgG1 isotype control (Becton-Dickinson Pharmingen, San Diego, CA, USA). They were washed twice and examined in the flow cytometry. Gating was performed according to the isotype control. Analysis was done by FlowJo (Flow cytometry analysis software, Ashland, OR, USA).

Cell Lysis and Immunoblotting

More than 95% viable NK cells treated with 100 ng, 1 μg, or 10 μg GA for 24 h were lysed with ice-cold Non-idet P-40 lysis buffer containing 1% Non-idet P-40, 30 mM Tris–HCl, pH 7.4, 150 mM NaCl, 10 mM NaF, 1 mM EDTA, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethyl-sulfonyl fluoride (PMSF), and a protease inhibitor cocktail (Sigma-Aldrich). The solutions were centrifuged at 13,000 × *g* for 15 min at 4°C to separate the cell lysates from the supernatants. Total protein concentration in the samples was determined using Bio-Rad Protein Assay (Bio-Rad, Uppsala, Sweden), and samples with 20-μg protein were used for each well in the gel. The samples were run on SDS-PAGE criterion gels and then electro transferred to PVDF-membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% skim milk in TBS for 1 h, and then incubated overnight at 4°C with 1:250 dilution of polyclonal rabbit antihuman CCR10 or 1:8000 dilution of polyclonal rabbit anti-GADPH (Both from Abcam, Cambridge, UK). The membranes were washed four times in TBS-T-buffer, before incubation with HRP-conjugated secondary antibody (1:2500) (Bio-Rad) for 1 h at room temperature. Binding of antibodies to the target proteins was detected by secondary HRP-labeled antibodies and Super Signal West Pico Stable Peroxide Solution (Pierce, Rockford, IL, USA), using chemiluminescence film and CURIX 60 (Agfa HealthCare, Mortsel, Belgium).

Detection of CCL27 (CTACK) and CCL28 (MEC) by ELISA

Natural killer cells (1×10^6/mL) were incubated with 100 ng, 1 μg, or 10 μg of GA, or with culture medium as a control for 24 h at 37°C in 5% CO_2 incubator. After incubation, the cells were harvested, and the cell suspensions were centrifuged at 1000 *g* for 8 min. Supernatants were collected and stored at −80°C until further analysis. Levels of chemokine were measured using ELISA kits from RayBiotech Inc. (Norcross, GA, USA). Color intensity was measured at 450 nm in a BioTek PowerWave XS plate reader. The standard curves and concentrations were calculated using Gen5 Data Analysis Software (BioTek Instruments, Winooski, VT, USA).

Detection of Granzyme B by Flow Cytometric Analysis

IL-2-activated NK cells (1×10^6/mL) were left intact for 24 h. Supernatants were collected from these cells and were kept at −80°C until use. To detect the expression of Granzyme B (GrB), the cells (1×10^6/mL) were incubated with either media alone or with 10 μg/mL of GA overnight. In addition, the cells treated as above (with or without GA) were preincubated with supernatants collected from activated NK cells either alone or with 1 μg/mL mouse antihuman CCL27 or mouse antihuman CCL28 (R&D systems), and as a control mouse IgG1.

To stain the intracellular GrB, cells were incubated with 10 μg/mL Brefeldin A (Sigma-Aldrich) for 4 h. They were then fixed with 4% paraformaldehyde for 15 min at 4°C and then washed twice with SAP buffer (PBS with 0.1% Saponin and 0.05% NaN3) before staining intracellularly with either PE-conjugated mouse antihuman Granzyme B or isotype control PE-conjugated mouse IgG antibody (both from ImmunoTools, Friesoythe, Germany) in the dark at 4°C for 45 min. Cells were washed with flow cytometric medium and resuspended with PBS in 5-mL tubes to perform flow cytometric analysis. Gating was done according to the PE-conjugated isotype control antibody. Analysis was done by FlowJo (flow cytometry analysis software, Ashland, OR, USA).

Statistical Analysis

Significant values were generated using several tests. We used one-way ANOVA, two-way ANOVA with Bonferroni *post hoc* correction, or the Student's *t*-test. A P value <0.05 was considered to be statistically significant.

RESULTS

GA Upregulates the Expression of CCR10 on the Surface of IL-2-Activated NK Cells and Induces Their Chemotaxis Toward Its Ligands

Natural killer cells migrate into inflammatory sites aided by sets of chemokine receptors, which direct them toward the chemokines present at tumor growth sites. To this end, we investigated the expression of CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR8, CCR9, CCR10, CXCR1, CXCR2, CXCR3, CXCR4, CXCR5, CXCR6, CXCR7, and CX_3CR1 on the surface of IL-2-activated NK cells after 24 h incubation with 1 or 10 μg/mL of GA. Only more than 90% viable cells as determined by Trypan blue exclusion test were used in this and subsequent assays. The reasons for choosing activated NK cells are due to the fact that at, inflammatory sites such as the tumor microenvironment, inflammatory molecules including cytokines and chemokines are released, which activate NK cells. In addition, activated NK cells migrate toward sites of inflammation much more efficiently than non-activated cells [reviewed in Ref. (12)].

From all the chemokine receptors examined, GA upregulated the expression of CCR1 and CCR10 on the surface of activated NK cells (**Figure 1A**). Also shown are histograms where CCR10 is expressed on activated NK cells after incubating overnight with 10 μg/mL of GA (**Figure 1B**). We focused the rest of this work on the expression of CCR10, since another drug used for treating MS patients, namely DMF also upregulates similar expression (also see **Figure 4**). To confirm the upregulation of CCR10 after GA stimulation, we performed immunoblot analysis. Results in **Figure 1C** demonstrate that overnight incubation with 0.1, 1, or 10 μg/mL of GA induced the expression of this receptor on activated NK cells, despite the fact that flow cytometric analysis did not show increase in expression of this receptor after incubation with 1 μg/mL of GA (**Figure 1A**).

Consequently, we examined the chemotaxis of activated NK cells treated for 24 h with 10 μg/mL GA toward various concentrations (ranging from 1 to 1000 ng/mL) of CCL27 and CCL28, the ligands for CCR10. Cells untreated with GA migrated with low intensity toward high concentrations of CCL27 (100 and 1000 ng/mL) and CCL28 (10 and 100 ng/mL), which could be due to the low number of CCR10 positive NK cells. However, cells treated with GA for 24 h migrated toward much lower concentrations of CCL27 and CCL28 (**Figure 1D**). When compared to untreated cells, the migration of cells toward the lower concentrations of these chemokines was enhanced by twofold to fivefold after treatment with GA, an enhancement that was statistically significant (**Figure 1D**). As a control, we also observed that these cells migrated toward the concentration gradients of CCL3, one of the ligands for CCR1 after treatment with GA (**Figure 1D**), which is corroborated with increased CCR1 expression on the surface of these cells after similar treatment.

Pertussis Toxin Inhibits the Chemotaxis of NK Cells

We also investigated whether G proteins might be involved in mediating the chemotactic response. Consequently, activated NK cells were either left intact or were pretreated with PTX for 2 h, which intoxicates and inhibits the function of G_i/G_o (22). As shown in **Figure 2A**, NK cells migrated toward 10 ng/mL CCL27 after preincubating for 24 h with 10 μg/mL GA ($P < 0.001$, as compared to the control). This effect was inhibited after preincubating NK cells with PTX ($P < 0.04$). Similarly, 1 ng/mL of CCL28 induced the chemotaxis of NK cells pretreated overnight with GA ($P < 0.04$, as compared to the control), and this activity was abrogated upon pretreating the cells with PTX ($P < 0.05$, **Figure 2B**).

GA Enhances NK Cell Lysis of Tumor Cells: Inhibition by Anti-CCR10 Antibody

To evaluate whether GA might induce activities other than chemotaxis in NK cells, we performed the cytotoxicity assay. Results shown in **Figure 3** demonstrate that incubating NK cells overnight with 10 μg/mL of this drug increased activated NK cell lysis of K562 cells (**Figure 3A**), or RAJI cells (**Figure 3B**). Because GA upregulates the expression of CCR10 on the surface of NK cells, we sought to demonstrate whether such expression might influence the cytolytic activity of these cells. NK cells either untreated or incubated with 10 μg/mL anti-CCR10, or as a control with isotype control IgG antibody, were examined for their ability to lyse tumor target cells in the NK cytotoxicity assay. We observed that anti-CCR10, and not the isotype control IgG, tended to inhibit NK cell lysis induced by GA of K562 (**Figure 3A**), but significantly inhibited lysis of RAJI cells ($P < 0.05$, comparing killing in the presence of anti-CCR10 to isotype control IgG, **Figure 3B**). Of note, cells treated overnight with GA and then incubated with isotype control IgG antibody also lysed K562 cells (**Figure 2A**) or RAJI cells (**Figure 2B**), but this did not reach statistical significance when compared to untreated cells incubated with the isotype control antibody.

IL-2-Activated NK Cells Secrete CCL27 and CCL28

Because anti-CCR10 inhibited GA-induced NK cell cytotoxicity, we entertained various possibilities to explain such activity. First, we sought to determine whether GA might induce the release of CCL27 or CCL28 from activated NK cells, and that these chemokines might facilitate NK cell cytolytic activity by binding CCR10. Our results demonstrate that IL-2-activated NK cells released an average of 280 pg/mL of CCL27 (Figure S2A in Supplementary Material), or 500 pg/mL CCL28 (Figure S2B in Supplementary Material). However, addition of various concentrations ranging from 0.1 to 10 μg/mL of GA to IL-2-activated NK cells did not significantly affect the levels of these chemokines,

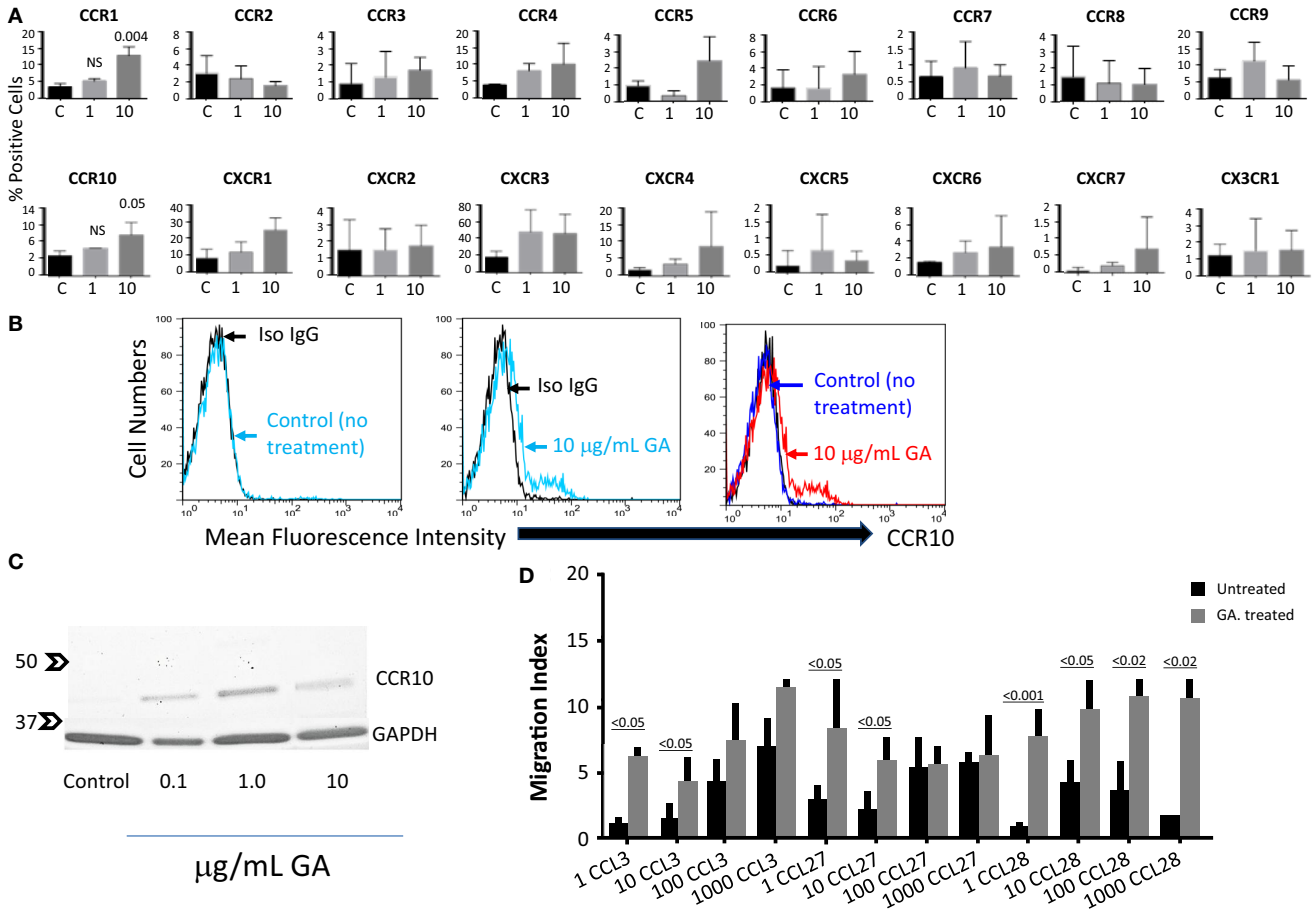

FIGURE 1 | **GA upregulates the expression of CCR10 on the surface of NK cells**. **(A)** NK cells (1×10^6/mL) were preincubated overnight with either media (C = control) or with 1 or 10 µg/mL of GA. The cells were washed and the expression of CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR8, CCR9, CCR10, CXCR2, CXCR2, CXCR3, CXCR4, CXCR5, CXCR6, CXCR7, and CX3CR1 was determined by flow cytometric analysis. Mean ± SEM of three experiments. *P* values were determined using Student's *t*-test. **(B)** Representative experiment showing the expression of CCR10 on the surface of IL-2-activated NK cells after incubating with media alone (control) or 10 µg/mL of GA. Gating was done according to the PE-conjugated isotype control antibody. The right panel shows an overlay combined results of the first two panels. **(C)** Immunoblot analysis showing the expression of CCR10 after 24-h pretreatment with media (control) or with 0.1, 1, or 10 µg/mL of GA. The expression of housekeeping GAPDH protein is also shown. Numbers to the left represent the molecular weights. **(D)** NK cells migrate toward the concentration gradients of CCR10 or CCR1 ligands. Various concentrations ranging between 1 and 1000 ng/mL of CCL3, CCL27, or CCL28 were placed in the lower wells of Boyden chambers, whereas 1×10^5 NK cells either untreated (black columns), or pretreated for 24 h with 10 µg/mL of GA (gray columns), were placed in the upper wells. Two hours later, the filters were collected, the cells fixed, and then stained with modified Giemsa stain. Migration index was calculated as the number of cells migrating in the presence of the chemokine divided by the number of cells migrating in its absence (C = control). A comparison of migration toward CCL3, CCL27, and CCL28 among untreated NK cells (black columns) and GA-treated cells (gray columns) was performed using two-way ANOVA test of six experiments.

although a trend in increased release of CCL28 was noted after pretreatment with 10 µg/mL GA (Figure S2 in Supplementary Material).

Next, we examined whether GA or a combination of GA with CCL27 or CCL28 might induce the expression of CD107a on the surface of activated NK cells. Histograms of flow cytometric analysis showed a trend of increased CD107a expression after incubating the cells with GA, CCL27, or CCL28 (Figure S2C in Supplementary Material, left). Combining GA with CCL27 or CCL28 did not augment such expression when compared to cells incubated with GA alone (Figure S2C in Supplementary Material, right). When three or more experiments were performed, the trend of increased expression of CD107a was also noticed after incubating the cells with GA, CCL27, or CCL28. However, such enhancement did not reach significant levels after treating the cells with GA, any of the two chemokines, or GA plus the chemokines (Figure S2D in Supplementary Material).

GA or Supernatants Collected from IL-2-Activated NK Cells Induce the Expression of Granzyme B in NK Cells: Inhibition by Anti-CCL28

To further investigate the plausible mechanisms of CCR10/chemokines axis, we measured the expression of Granzyme B in activated NK cells. For this, NK cells were either incubated with

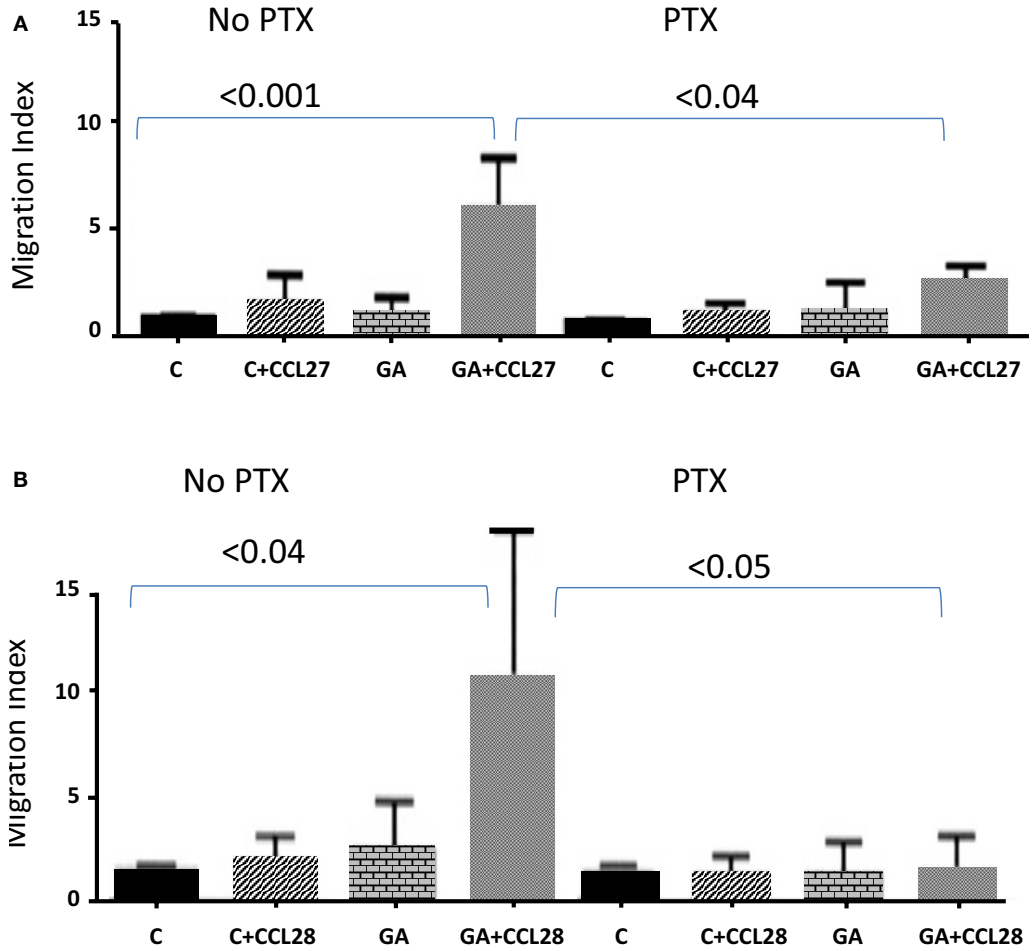

FIGURE 2 | **Pretreatment with pertussis toxin (PTX) inhibits GA activity**. **(A)** Migration of cells either left untreated (C = control) or pretreated overnight with 10 μg/mL of GA toward 10 ng/mL CCL27. Before the assay, these cells were either left intact (left columns) or intoxicated with 100 ng/mL of PTX for 2 h (right columns). **(B)** Migration of cells either untreated (C = control) or pretreated overnight with 10 μg/mL of GA toward 1 ng/mL CCL28. Before the assay, these cells were either left intact (left columns) or intoxicated with 100 ng/mL of PTX for 2 h (right columns). *P* values comparing the migration of cells toward chemokines, and its inhibition by pretreatment with PTX, are determined by two-way ANOVA test of six different experiments. C = control. C+ chemokine = untreated cells migrating toward the chemokine. GA = cells treated with GA overnight and migrated toward media only (no chemokine). GA+ chemokine = cells treated with GA overnight and then migrated toward the chemokine.

media alone or with 10 μg/mL of GA overnight, and the expression of Granzyme (GrB) was detected by flow cytometric analysis. Such treatment increased the percentages of cells expressing GrB from 6 to about 30% after pretreatment with the drug (Figure S3 in Supplementary Material). Supernatants collected from activated NK cells, when added to NK cells, also increased such expression to about 12%. The enhancement of GrB expression by the supernatants was lowered by pretreatment with anti-CCL28 to about 6%, but not with anti-CCL27, whereas the isotype control for the anti-CCL27 and anti-CCL28 also did not affect the activity of the supernatants (Figure S3 in Supplementary Material, upper panels). Addition of the supernatants to GA did not result in any synergy among their activities. However, anti-CCL28, and to a lesser extent anti-CCL27, reduced the effect of GA on GrB expression (Figure S3 in Supplementary Material, lower panels).

DMF or MMF Also Upregulates the Expression of CCR10 on the Surface of IL-2-Activated NK Cells and Induces Their Chemotaxis

To determine whether other drugs or metabolites used to treat or have potential for treating MS or cancer patients might affect the expression of CCR10 on NK cells similar to GA, we investigated the effects of various drugs. We observed that none of the concentrations of vitamin D_3, its metabolite Calcipotriol, or FTY720 upregulated the expression of CCR10 on the surface of activated NK cells (not shown). In contrast, two different concentrations of MMF (1 and 100 μM) or the 100 μM concentration of DMF significantly upregulated the expression of CCR10 on the surfaces of NK cells (**Figure 4A**). MMF and the drug DMF also upregulated the expression of CXCR3, but not any other

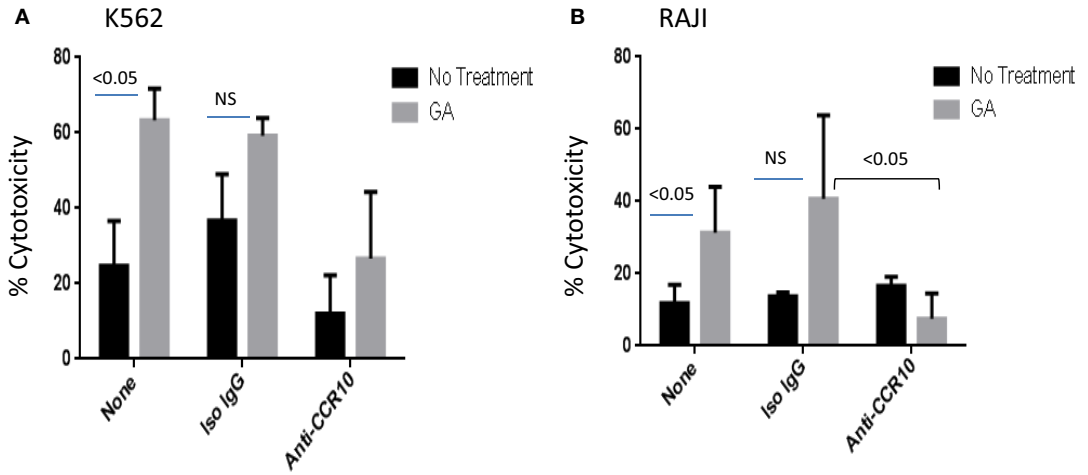

FIGURE 3 | Anti-CCR10 inhibits NK cell lysis of tumor target cells. **(A)** NK cells were either left untreated (black columns) or incubated with 10 μg/mL of GA overnight (gray columns). Before the assay the cells were incubated for 45 min with isotype control IgG antibody or with 10 μg/mL of anti-CCR10, washed, and then mixed with Calcein-AM-labeled K562 cells in the 4-h NK cytotoxicity assay. **(B)** This is similar to **(A)** except that RAJI tumor cells were used as targets instead of K562 cells. Mean ± SEM of four separate experiments.

chemokine receptor examined (**Figure 4A**). Histograms of one representative experiment showed that 1 and 100 μM of MMF or 100 μM of DMF upregulated the expression of CCR10 on the surface of human IL-2-activated NK cells when compared to cells unstimulated with the drugs (**Figure 4B**). To corroborate the expression of CCR10 with functional activity, we performed the chemotaxis assay. Treatment of NK cells with MMF for 24 h enhanced their chemotaxis toward CCL27, CCL28, and CXCL10, the ligand for CXCR3 (**Figure 4C**). These results correlated well with increased expression of CCR10 and CXCR3 on NK cells after incubation with 1 or 100 μM MMF. Surprisingly, pretreatment of IL-2-activated NK cells with DMF did not increase their chemotaxis toward CCL27. However, their chemotaxis was increased toward 1 μg/mL of CCL28 or CXCL10 after overnight incubation with 100 μM DMF (**Figure 4D**).

Anti-CCR10 Inhibits MMF or DMF-Induced NK Cell Lysis of Tumor Target Cells

We recently reported that MMF enhanced freshly isolated NK cell lysis of tumor cells (20). Because of the differences in the expression of NK cytotoxicity receptors among naive vs. activated NK cells, we sought to investigate whether MMF or DMF might enhance activated NK cell lysis of tumor cells. Pretreatment with either MMF or DMF enhanced IL-2-activated NK cell killing of K562 tumor cells (**Figure 5A**). However, only pretreatment with MMF, and not DMF, augmented activated NK cell lysis of the B cell lymphoma RAJI cells (**Figure 5B**). These results mirrored our recent findings showing that MMF, and not DMF, increased lysis of naive NK cells against RAJI cells (20).

Next, we asked whether CCR10 might play a role in MMF or DMF augmentation of NK cell cytotoxicity. Similar to its effect on GA-enhanced NK cell cytotoxicity, incubating activated NK cells with anti-CCR10 for 45 min abrogated the cytotoxicity induced by MMF or DMF against K562 cells (**Figure 5A**) or MMF-enhanced activated NK cell cytotoxicity against RAJI tumor cells (**Figure 5B**). This effect is not related to a general toxic effect of anti-CCR10 antibody as more than 95% of the cells were viable after treatment for 45 min with the antibodies.

DISCUSSION

Natural killer cells stand at the cross road among treatment of autoimmune diseases and immunodeficient diseases. In autoimmune diseases, the best protocol is to suppress the immune system, which is achieved by immunosuppressive drugs. On the other hand, in immunodeficient diseases, such as cancer or AIDS, the immune system must be activated to fight cancer cells or virally infected cells, respectively. From a first look, it appears that these methods of treatment are contradictory to each other, i.e., immunosuppression vs. immunostimulation. However, NK cells can be used as a therapeutic modality for both autoimmune diseases and cancer (23). Although there are more than 600 NCI approved clinical trials using NK cells (http://www.cancer.gov/search/results) to treat various forms of cancer, investigators are faced with the problems of targeting these highly antitumor effectors toward the sites of tumor growth.

Given the fact that NK cells can kill tumors, several strategies for the therapeutic use of NK cells have been proposed and tried in a clinical context. Cytokines have been used in the treatment of some human cancers and, in some instances, the mechanisms of action are through direct or indirect activation of NK cells (24). NK cell differentiation and activation is affected by cytokines such as IL-2, IL-12, IL-15, IL-18, IL-21, and IFNs. Several clinical trials have assessed the effects of IL-2 administration on activation and expansion of NK cells in patients with cancer (25). Apart from specific cytokines and/or growth factors, broad activators of immune function that are used in cancer treatment may also act

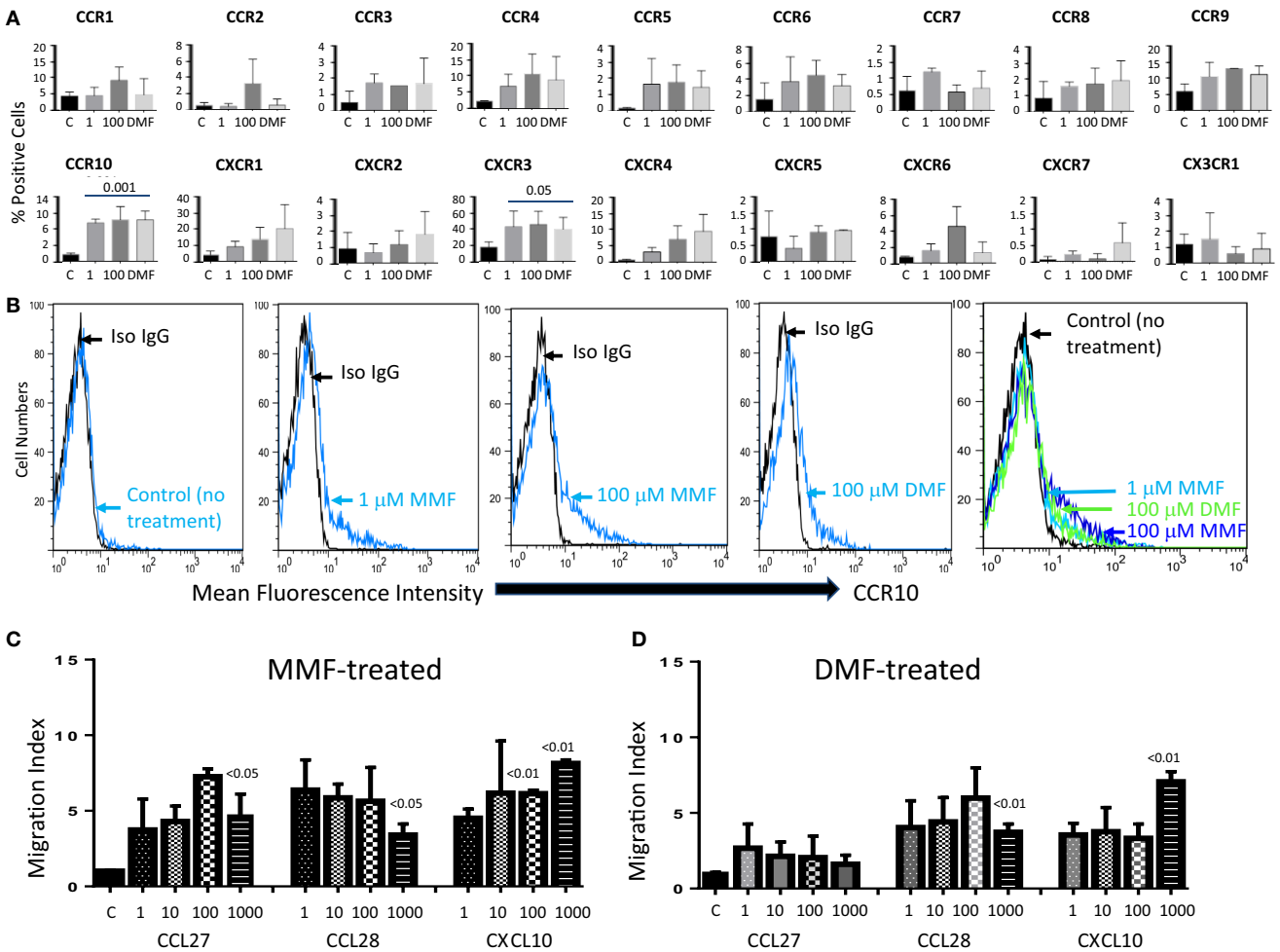

FIGURE 4 | MMF or DMF upregulates the expression of CCR10 and CXCR3 on NK cells and induces their chemotaxis toward the corresponding chemokines. **(A)** NK cells (1×10^6/mL) were preincubated overnight with either media (C = control) or with two different concentrations of MMF (1 and 100 μM) or with 100 μM DMF. The cells were washed, and the expression of CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR8, CCR9, CCR10, CXCR2, CXCR2, CXCR3, CXCR4, CXCR5, CXCR6, CXCR7, and CX3CR1 was determined by flow cytometric analysis. *P* values were determined using Student's *t*-test of three experiments comparing the percentages of $CCR10^+$ or $CXCR3^+$ NK cells after treatment with MMF or DMF to the controls. **(B)** Representative experiment showing the expression of CCR10 on the surface of IL-2-activated NK cells after incubating with media alone (control), 1 or 100 μM MMF, or 100 μM DMF. Gating was done according to the PE-conjugated isotype control antibody. The right panel shows an overlay combined results of the first four panels. **(C)** NK cells migrate toward the concentration gradients of CCR10 or CXCR3 ligands. Various concentrations ranging between 1 and 1000 ng/mL of CCL27, CCL28, or CXCL10 were placed in the lower wells of Boyden chambers, whereas 1×10^5 NK cells either untreated (C = control) or pretreated with 100 μM MMF were placed in the upper chambers. Two hours later, the filters were collected, the cells fixed, and then stained with modified Giemsa stain. Migration index was calculated as the number of cells migrating in the presence of the chemokine divided by the number of cells migrating in its absence (C = control). A comparison of migration toward CCL27, CCL28, or CCL3 among untreated NK cells (black columns) and MMF-treated cells was performed using two-way ANOVA test of six experiments. **(D)** This is similar to **(C)**, except that 100 μM DMF was used instead of MMF.

on NK cells (25). Similarly, NK cells may have a role in the clinical efficacy of *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) treatment of bladder cancer (26), indicating that mediators, which activate endogenous NK cells, can induce immune-mediated control of cancer. The potential efficacy of NK cells in treating various forms of cancer has been extensively reviewed (27–33).

CCL27 (CTACK) is expressed in epidermal keratinocytes and is responsible for attracting $CCR10^+$ T cells into the skin (34, 35). On the other hand, CCL28 is expressed by epithelial cells of mucosal sites (36–38) and is upregulated in inflamed tissues such as colon, duodenal mucosa, lungs, and liver ducts (39–41). Further, treatment of Crohn's disease patients resulted in reduced expression of CCR10 corroborated with reduced inflammation in those patients [reviewed in Ref. (42)].

Intratumoral administration of CCR10 as well as other chemokine-expressing adenoviral vector into tumor-bearing animals resulted in the recruitment and activation of T cells at sites of tumor growth (43). Similarly, transfecting CCL27 into ovarian carcinoma cells resulted in reduced tumor growth by antitumor immune responses (44). Human keratinocyte-derived skin tumors can evade T cells antitumor activity by downregulating the expression of CCR10. Further, *in vivo* work demonstrated that

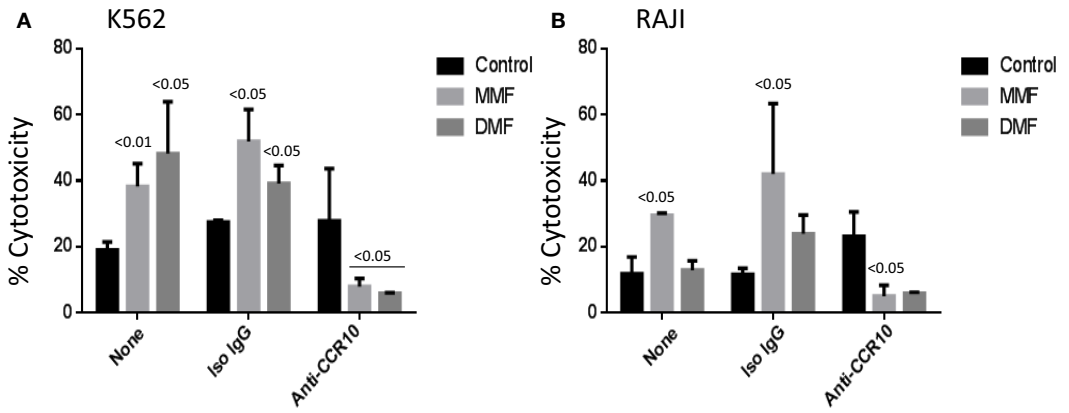

FIGURE 5 | **MMF or DMF augments activated NK cell cytotoxicity against tumor cells: inhibition by anti-CCR10 antibody**. **(A)** NK cells were either left intact (black columns) or incubated with 100 μM MMF or DMF overnight (gray columns), washed, and then incubated with K562 cells. **(B)** is similar to **(A)** except that RAJI cells were used in the NK cytotoxicity assay. For treatment with anti-CCR10, the cells were washed and incubated with either 10 μg/mL of anti-CCR10 or isotype control IgG antibody for 45 min before incubating with the target cells. Percent cytotoxicity was then measured, and significant values as compared to the control in each treatment are shown on top of the columns. Significance of cytotoxicity in the presence of anti-CCR10 was compared to the cytotoxicity in the presence of isotype control IgG antibody. Mean ± SEM of four experiments.

neutralization of CCL27 decreased leukocyte recruitment toward cutaneous tumor sites, resulting in enhanced tumor growth (45).

In contrast, it was observed that overexpression of CCR10 and CCR7 resulted in severe outcome of human cutaneous melanoma growth, as determined by high risk of relapse and death of patients (46). Along these lines, Kai et al. (47) observed that CCR10 and CCL27 were strongly expressed in human cutaneous squamous cell carcinoma, which is advantageous for tumor cell survival and proliferation. Further, hypoxic tumor cells released CCL28 recruiting $CCR10^+$ Treg cells, which promote tumor growth and angiogenesis, an activity that was abrogated by anti-CCL28 (48).

To this end, we observed that GA upregulates the expression of CCR10 on the surface of human NK cells. This activity corroborated with enhanced migration of these cells toward the concentration gradients of CCL27 and CCL28, the ligands for CCR10. PTX inhibited the chemotactic effects induced by chemokines, suggesting that G protein-coupled receptors (GPCRs), and in particular $G_{i/o}$, are involved. PTX also inhibited the cytolytic activity of NK cells induced by GA against tumor cells. Because chemokines bind GPCRs, we entertained the possibility that CCR10 could be involved in mediating NK cell cytotoxicity. We observed that anti-CCR10 reduced NK cell lysis of tumor target cells. Although the reasons behind such enigmatic effect are not clear, it is plausible that a complex interaction takes place resulting in activating the lytic potential of NK cells. It is conceivable that CCR10 upregulated by GA might interact with the CCR10 ligands such as CCL27 and CCL28 secreted by activated NK cells. Such binding may result in inducing intracellular signaling molecules that may mediate the cytolytic activity of NK cells. Hence, anti-CCR10 or PTX might interfere with such interaction resulting in inhibiting NK cells mediating lysis of tumor cells. Our results did not detect any increases in the levels of CCL27 or CCL28 secreted by NK cells after stimulation with GA. Neither there was a synergy among GA and these chemokines in the upregulation of CD107a on the surface of these cells. This molecule is considered an activation marker for NK cell-mediated lysis of tumor cells, and its expression is increased after incubating resting NK cells with MMF (20). Further analysis showed that supernatants collected from activated NK cells increased the percentages of Granzyme B positive cells, and that such activity was inhibited by pretreatment with anti-CCL28 indicating that CCL28 secreted by activated NK cells might bind CCR10 and consequently, upregulate the expression of lytic molecules on higher numbers of NK cells. GA also increased the percentages of Granzyme B positive NK cells, and this effect was reduced by anti-CCL28. Albeit not examined, it is plausible that GA might also utilize CCL28, perhaps to increase the expression of GrB. Although we have not seen a significant increase of CCL28 release after pretreatment with GA, we noted a trend of such enhancement.

Similar to GA, MMF and DMF also upregulated the expression of CCR10 on the surface of activated NK cells. Further, these chemicals enhanced activated NK cell lysis of tumor target cells. Similar to GA effect, such enhancement of cytotoxicity was abrogated by pretreatment with anti-CCR10. More work is needed to explore in details such an exciting possibility involving chemokines/chemokine receptors axis in mediating not only the migration of NK cells but also other activities such as cytotoxicity.

We consider the most important aspect of this work is the finding that drugs for MS such as GA and DMF as well as MMF upregulate the expression of CCR10 on the surface of IL-2-activated NK cells. Such finding might have potential relevance for treating cancer, particularly those tumors that secrete chemokines, which bind CCR10, including malignant melanomas and squamous cell carcinomas, which secrete CCL27 (47, 49), or colorectal cancer cells that secrete CCL28 (50). This is particularly relevant when one considers that there is an estimated $2–5 \times 10^9$ NK cells in 5 L of blood. A 1% of this NK cell number represents a subset of NK cells that expresses CCR10 (an estimated number of $20–50 \times 10^8$/5 L of blood). A 10% increase after treatment with the drugs described in this manuscript suggests that the number of $CCR10^+$ NK cells

might be increased to about 200–500 × 10^8/5 L of blood; a substantial number of killer cells that may potentially inhibit tumor growth. Our next step is to examine in tumor-bearing animals whether these $CCR10^+$-activated killer cells may migrate into the sites of colorectal cancers or melanomas after pretreatment with GA, MMF, or DMF. A number of cancers are, at present, incurable. For others, chemotherapy is only partially effective, and a significant proportion of patients relapse following treatment. Some hematological malignancies are treatable by hematopoietic stem cell transplantation (HSCT), but fewer than 30% of patients requiring HSCT have a suitable donor. NK cells possess the ability to spontaneously lyse certain target cells, including tumor cells. The major issue that faced investigators in this field is the inability of NK cells to migrate toward sites of tumor growth. This, despite the existence of more than 600 approved clinical trials using NK cells to treat various cancers. Our present work may provide a novel approach of harnessing activated NK cells *in vitro* for the purpose of administering into cancer patients. We propose that these cells can be harnessed *in vitro* with drugs such as GA, DMF (or MMF). These drugs perform two important functions for these cells; first, they increased their cytotoxicity against tumor cells, and second, they upregulate the expression of CCR10 on their surfaces. Consequently, NK cells can be targeted toward the growth of tumor cells that secrete CCL27 and CCL28.

CONCLUSION

We previously described the effects of GA on chemokine receptor expression in an MS patient receiving this drug (51). However, this is the first demonstration that GA, DMF, and MMF upregulate the expression of an important chemokine receptor, i.e., CCR10, on the surface of activated NK cells. Such activity might have important implications in NK cell immunotherapy. Because immunotherapy using NK cells suffers from the inability of these cells to migrate into tumor stroma, it is now feasible to direct these cells toward sites of tumor growth, particularly those secreting CCL27 and CCL28. Hence, we suggest the feasibility of using such approach to treat cutaneous squamous cell carcinomas and squamous melanoma, as well as colorectal cancer.

AUTHOR CONTRIBUTIONS

AAM designed the experiments, performed the chemotaxis assays, analyzed the data, and wrote the paper. KS and ZA-J performed the flow cytometric experiments and other assays.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2016.00437

FIGURE S1 | Expression of CD56 molecule on the surface of IL-2-activated NK cells. (A) Activated NK cells were labeled with either mouse FITC-conjugated isotype IgG or mouse FITC-conjugated anti-CD3 antibody. **(B)** Activated NK cells labeled with mouse PE-conjugated isotype IgG or mouse PE-conjugated anti-CD56 antibody. **(C)** Activated NK cells labeled with both FITC-conjugated anti-CD3 antibody and PE-conjugated anti-CD56 and examined in the flow cytometry. One of the four representative experiments was performed.

FIGURE S2 | IL-2-activated NK cells release CCL27 and CCL28 and express CD107a. (A) NK cells were either left intact or were incubated for 24 h with 0.1, 1 or 10 μg/mL GA. Supernatants were collected, and the levels of CCL27 were measured. **(B)** CCL28 levels were evaluated using ELISA assay. **(C)** A representative experiment showing histograms for the expression of CD107a after incubating overnight with 10 μg/mL GA, 10 ng/mL CCL27, or 10 ng/mL CCL28 (left panel), or 10 μg/mL GA either alone or in combination with 10 ng/mL CCL27 or 10 ng/mL CCL28 (right panel). **(D)** Activated NK cells (1 × 10^6/mL) were either untreated (–) or incubated overnight with 10 μg/mL GA (GA), 100 ng/mL CCL27, 100 ng/mL CCL28, or a combination of 10 μg/mL GA with 100 ng/mL CCL27 (GA + CCL27), or 100 ng/mL CCL28 (GA + CCL28). The cells were washed, and the expression of CD107a was evaluated by flow cytometric analysis. Mean ± SEM of values from three different donors. The binding of isotype control antibody is also shown.

FIGURE S3 | GA or supernatants collected from IL-2-activated NK cells increase the percentages of NK cells expressing Granzyme B. IL-2-activated NK cells were incubated for 24 h with either media alone or with 10 μg/mL GA. In both treatments, the cells were incubated with supernatants collected from IL-2-activated NK cells. In other cultures, the cells were pretreated with the supernatants in the presence of 1 μg/mL mouse IgG isotype control for anti-CCL27 and anti-CCL28, or with 1 μg/mL of neutralizing mouse anti-CCL27 or mouse anti-CCL28. Upper panels show expression of Granzyme B in the absence of GA, whereas lower panels show expression of the same molecule in the presence of 10 μg/mL GA. One of the two representative experiments was performed. Percentages of positive cells are shown between brackets.

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Utilization of Dimethyl Fumarate and Related Molecules for Treatment of Multiple Sclerosis, Cancer, and Other Diseases

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Several drugs have been approved for treatment of multiple sclerosis (MS). Dimethyl fumarate (DMF) is utilized as an oral drug to treat this disease and is proven to be potent with less side effects than several other drugs. On the other hand, monomethyl fumarate (MMF), a related compound, has not been examined in greater details although it has the potential as a therapeutic drug for MS and other diseases. The mechanism of action of DMF or MMF is related to their ability to enhance the antioxidant pathways and to inhibit reactive oxygen species. However, other mechanisms have also been described, which include effects on monocytes, dendritic cells, T cells, and natural killer cells. It is also reported that DMF might be useful for treating psoriasis, asthma, aggressive breast cancers, hematopoeitic tumors, inflammatory bowel disease, intracerebral hemorrhage, osteoarthritis, chronic pancreatitis, and retinal ischemia. In this article, we will touch on some of these diseases with an emphasis on the effects of DMF and MMF on various immune cells.

Keywords: NK cells, dimethyl fumarate, cancer, multiple sclerosis, monomethyl fumarate

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INTRODUCTION

Twenty years ago, there was no treatment for multiple sclerosis (MS). Today, there are wide varieties of immunomodulatory drugs, which have been licensed to treat MS patients. For relapsing-remitting MS (RRMS) patients, several drugs have been approved. During the 1990s, beta interferon and glatiramer acetate were the only drugs available. Other drugs, such as natalizumab, teriflunomide, and fingolimod, were later approved. These drugs modify the immune system to slow disease progression, decrease attacks, and reduce the development of new brain lesions.

Dimethyl fumarate (DMF) has been lately approved by the US Food and Drug Administration (FDA) as an oral drug for MS patients. This drug was first used to treat inflammatory skin diseases, such as psoriasis. The beneficial effects of this medication corroborated with regulating $CD4^+$ Th1 cell differentiation. In clinical trials, it showed positive benefits for MS patients by lowering risk of relapse and reducing the number of brain lesions (1–6).

The mechanism of action is not fully known. After oral intake, DMF is completely absorbed in the small intestine, and only small amounts are excreted in the feces and urine (7). DMF possesses a short half-life of ~12 min inside the body (8). After absorption, DMF is rapidly hydrolyzed by esterases to monomethyl fumarate (MMF) (9), which has a short half-life of 36 h. This molecule interacts

with the immune cells in the blood circulation and crosses the blood–brain barrier (BBB) to the central nervous system (CNS) (10). **Figure 1** shows the structures of DMF and MMF.

Dimethyl fumarate is an α, β-unsaturated carboxylic acid ester. It is demonstrated that DMF by activating nuclear factor erythroid 2-related factor (Nrf2), stimulated the production of glutathione (GSH), the cells most important scavenger of reactive oxygen species (ROS) (11), hence, protecting against ROS-induced cytotoxicity. Further studies demonstrate that DMF downregulated nuclear factor kappa B (NF-κB) in cells, inhibited the anti-apoptotic protein Bcl-2, and induced apoptosis. Cells challenged with oxidative stressors increase their antioxidant capacity as a response to increase ROS production and maintain homeostasis. Nrf2 acts as a key control of the redox gene transcription; under oxidative stress, the Nrf2 signaling is activated to enhance the expression of a large number of antioxidants and enzymes that restore redox homeostasis. Nrf2 interacts with the cysteine-rich protein Kelch-like ECH-associated protein 1 (Keap1) and acts as an adaptor protein for the Cul3-dependent E3 (Cul3) ubiquitin ligase complex. In normal conditions, Keap1 promotes ubiquitination and repeatedly eliminates Nrf2 within a half-life of 13–21 min (12, 13). Keap1 possesses many cysteine residues in the amino acid terminal that act as sensors detecting changes in cellular redox state. During cellular stress, Keap1 is less effective at promoting Nrf2 degradation (12, 14).

Under normal conditions, Nrf2 is sequestered in the cytoplasm *via* binding to its inhibitory molecule Keap1. ROS/stress causes dissociation of Nrf2-Keap1 complex, leading to activation of Nrf2 and its translocation into the nucleus. In the nucleus, Nrf2 heterodimerizes with other transcription factors, such as MAF, and consequently, binds the antioxidant responsive elements (ARE) in the target genes. Nrf2 promotes transcriptional activation of antioxidants and detoxifying enzymes. At the same time, phosphorylation of the repressor molecule IκB by ROS/stress causes activation of NF-κB, leading to activating gene transcription encoding inflammatory mediators. Studies have shown that Nrf2 and NF-κB pathways have inhibitory influence on one another.

EFFECTS OF DMF ON THE INNATE IMMUNE SYSTEM

The main components of the innate immune system are epithelial barriers, leukocytes, dendritic cells (DCs), and natural killer (NK) cells. NK cells are large granular lymphocytes that spontaneously lyse target cells and are important for defending against viral infections as well as controlling tumor growth. NK cells have also immunoregulatory role by secretion of cytokines, chemokines, as well as cell-to-cell cross-talk (15). These cells express several activating and inhibitory receptors that detect target cells and control NK cell activity. In human, NK cells are divided by the expression of CD56 molecule into $CD56^{dim}$ and $CD56^{bright}$ subsets (15, 16).

The flow cytometric analysis of peripheral blood immune cells in 41 DMF-treated MS patients shows that these patients had significantly fewer circulating $CD8^+$ T cells, $CD4^+$ T cells, $CD56^{dim}$ NK cells, $CD19^+$ B cells, and plasmacytoid DCs (17). Furthermore, the expression of $CXCR3^+$ (a potential marker for Th1) and $CCR6^+$ (a potential marker for Th17) was reduced, while the number of regulatory T cells (Treg) was unchanged. Interestingly, DMF did not affect circulating $CD56^{bright}$ NK cells, $CD14^+$ monocytes, or myeloid DCs. However, DMF-treated patients had significantly fewer $CD56^{dim}$ NK cells when compared with healthy controls (17). A clinical study of 35 RRMS patients at baseline, 3 months, 6 months, and 12 months after initiation of DMF treatment shows that total leukocyte and lymphocyte counts diminished after 6 months, whereas after 12 months of DMF therapy total T cells counts decreased by 44%, $CD8^+$ T cell

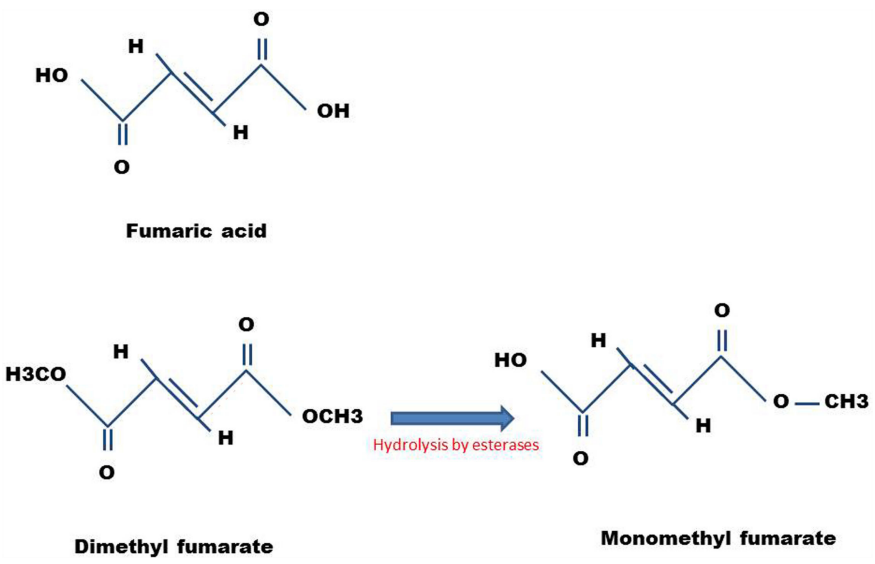

FIGURE 1 | Chemical structures of DMF and MMF. Also shown is the structure of fumaric acid, the precursor molecule.

counts declined by 54.6%, and $CD4^+$ T cell counts decreased by 39% (18). $CD19^+$ B cell counts were also reduced by 37.5%, and eosinophils counts were decreased by 54%, whereas the percentages of neutrophils, monocytes, basophils, and NK cells were not significantly altered (18).

It has been previously demonstrated that MMF is a potent agonist of hydroxycarboxylic acid receptor 2 "(HCA_2), a G protein coupled receptor also known as GPR109A" (19). It is reported that HCA_2 mediates the therapeutic effects of DMF or MMF in experimental autoimmune encephalomyelitis (EAE) mouse model (20). Recently, we observed that MMF enhanced primary non-activated human $CD56^+$ NK cell lysis of leukemic cell line K562 and B-cell lymphoma RAJI cells *in vitro* (21). Furthermore, MMF upregulated NKp46 expression on the surface of $CD56^+$ NK cells, an activity correlated with upregulation of CD107a expression and the release of Granzyme B from $CD56^+$ NK cells (21).

Moreover, MMF delayed EAE clinical score in SJL/J mice and prevented the disease progression in treated mice. These results are linked to enhanced NK cells lysis of DCs isolated from the same mice (22). To correlate these findings with human settings, we recently observed that human NK cells incubated with various concentrations of DMF or MMF robustly lysed immature DCs *in vitro* (manuscript in preparation). These findings suggest that one mechanism of action for these "drugs" is plausibly due to activating NK cells to lyse DCs, and consequently, impeding antigen presentation to autoreactive T cells.

Dendritic cells represent key links between the innate and adaptive immune system (23). T cells and NK cells are stimulated through direct contact with activated DCs (24). DCs play a major role in regulating the immune response by releasing cytokines and expressing co-stimulatory molecules. They are capable of processing both exogenous and endogenous antigens and present them in the context of MHC class I or II molecules. It has been reported that DMF inhibited DCs maturation through a reduction in the release of the inflammatory cytokines IL-6 and IL-12. Furthermore, DMF activated type II DCs, which have anti-inflammatory effects and suppressed type I DCs, which are inflammatory (25, 26). DMF induced type II DCs by regulating GSH depletion, followed by increased heme oxygenase-1 (HO-1) expression and suppressing STAT1 phosphorylation in DCs (26). This, combined with the reduction in the inflammatory cytokines by nuclear translocation of NF-κB, resulted in inhibiting CD1a, CD40, CD80, CD86, and HLA-DR expression (27). Consequently, the capacity of DCs to stimulate allogeneic Th1 and Th17 cells is reduced. It was also determined that increased production of the Th2 cytokines and increased expression of IL-10, instead of IL-12 and IL-23 by DCs, enhanced the development of T regulatory (Treg) cells (28).

We reported that monocyte-derived DCs isolated from EAE mice treated with MMF did not increase the expression of CD80 molecule (22). Interestingly, E-cadherin expression was upregulated in EAE mice, and MMF reversed this upregulation. Increased E-cadherin expression suggests a shifting of the immune system toward inflammatory Th1/Th17 response. These results support previous study showing that inflammatory E-$cadherin^+$ bone marrow-derived DCs isolated from animals with colitis promoted Th17 response (29). The study also demonstrates that E-$cadherin^+$ DCs enhanced Th1 cell responses (29). Furthermore, E-$cadherin^+$ DCs increased the number of IFN-γ^+ $CD4^+$ T cells and decreased the number of IL-4^+ $CD4^+$ T cells (30). MMF by decreasing E-cadherin expression on DCs may decrease inflammatory Th1/Th17 proliferation and may enhance the anti-inflammatory Th2 cells.

Although DMF but not MMF induced apoptosis in iDCs and moderately inhibited the ability of DCs to induce proliferation of allogeneic T cells, it is reported that MMF affected the polarization but not maturation of monocyte-derived DCs, resulting in downregulating Th1 lymphocyte responses (31). *In vitro* study for the effects of MMF on DCs differentiation shows that MMF inhibited monocyte-derived DCs differentiation in response to LPS, resulting in cells that are incapable of appropriately mature to DCs. In addition, MMF did not decrease the capacity of DCs to capture antigens, but MMF/DCs interaction resulted in producing low levels of IL-12, IL-10, and TNF-α, whereas IL-8 production was not altered (32). Consequently, MMF/DCs interaction partially affected IFN-γ production by naive T cells, whereas the production of IL-4 and IL-10 was not influenced by MMF (32). Another study demonstrates that DMF inhibited DCs maturation by reducing the production of the inflammatory cytokines IL-6 and IL-12 as well as the expression of MHC class II, CD80, and CD86 (27). Furthermore, immature DCs activated fewer T cells characterized by low IFN-γ and IL-17 production (27). In contrast, de Jong et al. (33) demonstrate that MMF increased the production of IL-4 and IL-5 without altering the production of IL-2 and interferon-γ in stimulated peripheral blood mononuclear cells challenged with bacterial antigens.

TREATMENT OF PSORIASIS AND OTHER SKIN DISEASES WITH DMF

Psoriasis is a type-1 cytokine-mediated chronic autoimmune skin disease aided by the infiltration of Th1/Th17 cells into the skin (34–36). DMF is utilized to treat psoriasis in European countries for more than 30 years. Fumaric acid was first used for treatment of psoriasis by the German chemist Walter Schweckendiek in 1959. In 1994, DMF was licensed in Germany under the trade name Fumaderm for the treatment of psoriasis. DMF inhibited Janus kinas (JAK) signaling and interfered with intracellular proteins trafficking and consequently, inhibited the release of pro-inflammatory cytokines, such as IL-12, IL-23, and TNF, whereas the release of anti-inflammatory cytokines, such as IL-10, was increased. DMF also inhibited the production of IFN-γ and enhanced the production of IL-10 in the culture of psoriatic keratinocytes (37).

Previous experimental and clinical studies were focused on the mechanism of action for DMF that could affect the immune system. The immunohistochemical studies of psoriatic plaques indicate that DMF has several anti-inflammatory effects *via* a number of pathways, leading to reduction in the levels of several inflammatory T cell subsets (38, 39) and decreased recruitment

of inflammatory cells (40). The ability of DMF or MMF to induce apoptosis of $CD4^+$ and $CD8^+$ T cells and *in vitro* switching the immune system toward a Th2 anti-inflammatory type response in psoriasis patients could be through impaired DCs maturation and induction of apoptosis. In addition, DMF inhibited the formation of new blood vessels, a process that is involved in the formation of psoriatic plaques (41).

Clinical studies demonstrate that DMF reduced $CD4^+$ T cells and $CD8^+$ T cells by inducing apoptotic cell death (42). *In vivo* studies indicate that DMF inhibited T cell mediated organ rejection in a rat model (43). A study of allergic contact dermatitis, a skin disorder in which an exaggerated T cell response occurs, shows that DMF suppressed allergen-induced T cell proliferation, corroborated with modulating cytokines/chemokines expression by reducing the levels of IFN-γ but not IL-5 and downregulating CXCR3 but not CCR4 expression (44).

TREATMENT OF MULTIPLE SCLEROSIS PATIENTS WITH DMF

Multiple sclerosis is a chronic inflammatory autoimmune disease of the CNS in which the insulating myelin sheaths of nerve cell axons in the brain and spinal cord are attacked by the immune system (45). The principal mechanism responsible for this disease is still incompletely understood. The consensus is that activated T cells attack oligodendrocytes, leading to destruction of myelin sheaths (demyelination). Furthermore, the presence of inflammatory T cells in the CNS triggers recruitment of more T cells, B cells, dendritic cells, microglia cells, and NK cells (46). Due to the progressive neurodegenerative nature of MS, therapeutic modalities that exhibit direct neuroprotective effects are needed. A phase 3 clinical trial study of 2667 RRMS patients demonstrates the efficacy and safety of DMF in MS (1). *In vitro* study indicates that DMF increased the frequency of the multipotent neurospheres resulting in the survival of mouse and rat neural stem progenitor cells (NPCs) following oxidative stress with hydrogen peroxide (H_2O_2) treatment (47). Using motor neuron survival assay, DMF significantly promoted survival of motor neurons under oxidative stress. Furthermore, DMF increased the expression of Nrf2 at both RNA and protein levels in the NPC cultures (47).

There is agreement that antioxidants reduce the risk of certain pathological conditions, such as neurodegenerative diseases. *In vivo* animal studies have shown that DMF or MMF inhibited the disease course in the EAE model (48). It is also demonstrated that MMF crossed the BBB, indicating it may have a direct cytoprotective function in the CNS (49). The detoxification capabilities of DMF or MMF reduced the production and release of inflammatory molecules, such as TNF-α, IL-1β, and IL-6 as well as nitric oxide from microglia and astrocytes activated with LPS *in vitro* (50, 51). DMF or MMF increased the production of detoxification enzymes, such as nicotinamide adenine dinucleotide phosphate quinone reductase 1 (NQO-1), HO-1, and cellular glutathione, abolishing NF-kB translocation into the nucleus (52). NQO-1 is also detected in the liver and in the CNS of DMF-treated animals. This results in decreased expression of NF-kB-dependent genes that regulate the expression of inflammatory cytokines, chemokines, and adhesion molecules, and consequently, reduced the damage to CNS cells. Reducing the expression of adhesion molecules in the BBB represents a critical step in the transmigration of immune cells into the CNS. DMF inhibited TNF-α-induced expression of intracellular adhesion molecule-1 (ICAM-1), E selection, and the vascular cell adhesions molecule-1 (VCAM-1) in endothelial cells *in vitro* (53, 54). This is correlated with activating Nrf2 (55–58), which is released from the Keap-1complex *via* the activity of fumarates (see above). This may lead to reducing free radicals, preventing the synthesis of reactive nitrogen species, and thus protecting the CNS from degeneration and axonal loss (59, 60). These immunomodulatory activities of DMF or MMF, which constitute inhibiting cytokine production and nitric oxide synthesis, are important for the protection of oligodendrocytes against ROS-induced cytotoxicity and consequently, oligodendrocytes survival during an oxidative attack is augmented.

Multiple sclerosis animal models, such as EAE, are induced by immunization with different myelin antigens, such as proteolipid peptide ($PLP_{139–151}$) in SJL/J mice, an animal model disease that may represent relapsing-remitting form of MS, or C57BL/6J mice immunized with $MOG_{35–55}$, a model closely resembles chronic progressive MS. These models are characterized by inflammation, demyelination, and axonal lose. Treatment of EAE mice with DMF reduced macrophage-induced inflammation in the spinal cord (48). DMF suppressed Th1 and Th17 cell differentiation as well as expression of pro-inflammatory cytokines IFN-γ, TNF-α, and IL-17 (61). The drug also promoted Th2 cells that produce IL-4, IL-5, and IL-10 (33). In chronic MS, microglia cells are activated and released pro-inflammatory cytokines and stress-associated molecules leading to neurodegeneration and alteration of synaptic transmission (62). Modulation of microglia activation toward an alternatively activated phenotype can modify the outcome of some experimental models of neurological diseases. The study on EAE demonstrates that exposure to MMF switched the molecular and functional phenotype of activated microglia from pro-inflammatory type to neuroprotective effect (49). This switch in activity may occur through activation of HCAR2. MMF binding to HCAR2 triggered a pathway driven by the AMPK/Sirt axis resulting in inhibition of NF-κB and reducing pro-inflammatory cytokine production (49).

EFFECTS OF DMF ON THE CENTRAL NERVOUS SYSTEM

A recent study reports that administration of DMF protected claudin-5 expression in the BBB along with reduced brain edema formation in C57BL/6 mice undergoing experimental ischemia reperfusion injury (63). Using the immortalized murine brain endothelial cell line bEND.3, a preservation of zonula occludens-1 (ZO-1) and VE-cadherin localization in oxygen–glucose deprived cells in the presence of DMF was observed. Reduced transendothelial migration of the human monocyte cell line THP-1 toward CCL2 chemokine in the lower chamber of a transwell system after pretreatment of the bEND.3 cells with DMF was also

noted. Further observations demonstrate decreased ICAM-1, VCAM-1, and E-selectin mRNA expression in bEND.3 cells after treatment with DMF for 6 h (54).

In vitro human umbilical vein endothelium examination indicates that DMF or MMF modulated pro-inflammatory intracellular signaling and T-cell adhesiveness of human brain microvascular endothelial cells (64). Neither DMF nor MMF reduced the basal expression of ICAM-1 under inflammatory condition or blocked NF-κB in human brain microvascular endothelial cells compared to solvent control. Hence, it is suggested that brain endothelial cells do not directly mediate a potential blocking effect of fumaric acid esters on the infiltration of inflammatory T cells into the CNS (64). It is also determined that DMF ameliorated inflammation, reduced BBB permeability and improved neurological outcomes by casein kinase 2 and Nrf2 signaling pathways in experimental intracerebral hemorrhage (ICH) mouse model (65).

Evidence from clinical and animal studies suggests that inflammation and oxidative stress, which occur after hematoma formation, are involved in ICH-induced secondary brain injury and neurological dysfunction (66). VCAM-1 and ICAM-1 are adhesion molecules expressed in the endothelium important during inflammation and after tissue injury. Both are increased upon activation of NF-κB-mediated TNF-α signaling pathway. TNF-α increases early onset endothelial adhesion by protein kinase C-dependent upregulation of ICAM-1 expression, which exacerbates ICH. Investigating the experimental autoimmune neuritis indicates that DMF treatment reduced the neurological deficits by ameliorating inflammatory cell infiltration and demyelination of sciatic nerves. In addition, DMF treatment decreased the level of pro-inflammatory M1 macrophages, while increasing the number of anti-inflammatory M2 macrophages in the spleens and sciatic nerves of EAN rats (67, 68). In RAW 264.7 macrophage cell line, a shift in macrophage polarization from M1 to M2 phenotype was demonstrated to be dependent on DMF application. In sciatic nerves, DMF treatment elevated the level of Nrf2 and its target HO-1, which may facilitate macrophage polarization toward M2 type (68). In addition, by reducing NF-kB in astrocytes, DMF inhibited the degradation of IkBa and reduced the expression of nitric oxide synthase (69). Moreover, DMF improved the inflammatory milieu in the spleens of EAN rats, characterized by downregulating mRNA for IFN-γ, TNF-α, IL-6, and IL-17 and upregulating mRNA level for IL-4 and IL-10 (68).

EFFECTS OF DMF ON TUMOR DEVELOPMENT

It has been reported that fumarase is involved in DNA repair (70). By studying yeast cells, it is observed that cytosolic fumarase plays a role in detecting and repairing DNA damage, particularly double-stranded DNA breaks. According to this theory, if the cells lack the fumarase, they may need to repair damaged DNA and are most likely prone to develop tumors. Further study on the role of redox demonstrates that high levels of ROS are harmful to normal cells and may lead to development of tumor by inducing DNA damage. Malignant transformation also increases cellular stress, leading to high ROS levels. On the other hand, Keap1-Nrf2 system protects cells from the effects of oxidants by regulating the expression of cytoprotective proteins (71). *In vivo* evidence indicates that Nrf2 has a protective role against tumor development in mouse models and in prostate cancer in humans (72). The mechanism by which Nrf2 is protective against tumor development has been attributed to the ability of Nrf2 to reduce the amount of ROS and DNA damages in cells.

It has also been demonstrated that DMF inhibited the proliferation of A375 and M24met cell lines and reduced melanoma growth and metastasis in experimental melanoma mouse models (73). Furthermore, DMF arrested the cell cycle at the G2-M boundary and was pro-apoptotic, inhibiting tumor cell growth. On the other hand, MMF increased primary human $CD56^+$ NK cell lysis of K562 and RAJI tumor cells, suggesting that this molecule may have *in situ* antitumor activity (21).

ROLE OF DMF IN GASTROINTESTINAL ULCERATION

It has been demonstrated that stress can play a pathogenic role for gastrointestinal ulceration, by disrupting gastric mucosal defensive barrier (74). Activators of stress give rise to the release of corticotropin-releasing hormone (CRH). CRH acts on the pituitary gland and stimulates the secretion of ACTH, which promotes glucocorticoids release from the adrenal cortex (75). Glucocorticoids not only interfere with tissue repair, elevate levels of gastric acids, and pepsin but also reduce the secretion of gastric mucus and eventually impair gastric mucosal barrier resulting in peptic ulcer. Low daily oral doses of MMF may prevent the chronic foot-shock stress-induced gastric ulcers and may associate with differential hormonal and oxidative processes (76). MMF suppressed the stress-induced elevation in adrenal gland corticosterone level and modulated the oxidative stress responses. Interestingly, DMF did not inhibit the effect of innate defense against microorganism. Treatment of monocytes and neutrophils with DMF after stimulation with *Staphylococcus aureus*, *Escherichia coli*, or the yeast *Candida albicans* in addition to zymosan particles or the tripeptide fMLP resulted in increased production of superoxide anion, which exerts anti-microbial effects (77).

EFFECTS OF DMF ON COLLAGEN TYPE II DEGRADATION

In vivo study of collagen type II degradation suggests that DMF ameliorated the disease by inhibiting the expression of metalloproteinase (MMP)-1, MMP-3, and MMP-13 that are induced by TNF-α (78). DMF may attenuate MMPs expression by suppressing JAK1 and JAK2/STAT3 pathways and by blocking TNF-α-induced STAT3 phosphorylation and DNA-binding activity (79). *In vivo* mice study on renal fibrosis, where TGF-β plays a key role in the development of the disease, demonstrates that DMF treatment may prevent renal fibrosis *via* Nrf2-mediated suppression of TGF-β signaling (80).

TABLE 1 | Immunoregulatory effects of DMF and/or MMF on various immune cells.

Cell type	Molecule	Cytokine/other molecules involved	Effect(s)	Reference
T cells	DMF/MMF	↓IFN-γ, ↓TNF-α, ↓IL-17, ↑IL-4, ↑IL-5, ↑IL-10, ↓CXCR3, ↓CCR6	↓Bcl-2, ↑Apoptosis, ↓Th1, ↓Th17, ↑Th2, ↓CD4, ↓CD8, ↑Treg	(17, 18, 27, 32, 42, 44, 61)
B cells	DMF	↑Nrf2→↑GSH→↓ROS, ↓NF-kB	↓Bcl-2, ↑Apoptosis, ↓CD19 B cells	(17, 18)
Monocytes	DMF	↑Nrf2, ↓NF-kB	No effect on cell numbers, ↑Antioxidant response	(18, 77)
DCs	DMF/MMF	↓GSH→↑HO-1, ↓NF-kB, ↓IL-6, ↓IL-12, ↑IL-10, ↓TNF-α↓, E-cadherin	↑Apoptosis, ↓plasmacytoid DCs, ↓DC maturation, ↓type I DCs, ↑type II DCs	(17, 22, 25–27, 31, 32)
NK cells	DMF/MMF	↑NKp46, ↑CD107, ↑Granzyme B	↓$CD56^{dim}$ NK cells, No effect on $CD56^{bright}$ numbers, ↑$CD56^{bright}$ NK cells lysis of tumor cells, ↑Lysis of DCS	(17, 18, 21, 22)
Macrophages	DMF	↑Nrf2, ↓mRNA of IFN-γ, ↓mRNA of TNF-α, ↓mRNA of IL-6, ↓mRNA of IL-17, ↑mRNA of IL-4, ↑mRNA of IL-10	↓M1 macrophages, ↑M2 macrophages	(68)
Neutrophils	DMF/MMF	↓HCA2	↓Number of infiltrating neutrophils	(20)
Keratinocytes	DMF	↓IL-12, ↓IL-23, ↓TNF, ↓IFN-γ, ↑IL-10, ↓IL-6, ↓TGF-α	↓Proliferation of keratinocytes	(37)
Endothelial cells	DMF	↓TNF-α, ↓ICAM-1, ↓VCAM-1, ↓E-selection, ↑Nrf2	↓BBB permeability→↓Immune cell migration	(41, 54, 55, 65)
Microglia	DMF/MMF	↓IL-1, ↓IL-6, ↓TNF-α, ↓NO, ↑Nrf2→↑GSH→↓ROS, ↓NF-kB, ↑NQO-1, ↑HO-1, ↑HCAR2	↑Antioxidant response, switching activated microglia from pro-inflammatory to neuroprotective	(49, 50, 52)
Astrocytes	DMF/MMF	↑Nrf2→↑GSH→↓ROS, ↓NF-kB, ↓IL-1, ↓IL-6, ↓TNF-α, ↓NO	↑Antioxidant response	(50, 52, 58)
Neurons	DMF	↑Nrf2→↑GSH→↓ROS	↓Apoptosis, ↑Neurons survival under oxidative stress	(47, 58)
Tumor cells	DMF	Arrest the cell cycle at G2-M, ↓pro-apoptotic	↓Proliferation of melanoma cells, ↓Proliferation of tumor cells, ↑Apoptosis	(73)

↓, *decreased;* ↑, *increased.*

CONCLUSION AND FUTURE DIRECTIONS

Dimethyl fumarate was originally used for treatment of psoriasis. Its success in treating RRMS patients led for its approval as an oral drug to treat MS patients. One mechanism of action that our group pursued is that DMF might enhance natural killer cell lysis of dendritic cells, hence, impeding presenting encephalitogen to autoreactive T cells. Further investigations suggest that DMF may also be used in the oncology field due to its ability to suppress the growth of melanoma cells. On the other hand, it appears that related molecules, such as MMF, may have even more potent antitumor activity than DMF. Although the association among DMF and MMF is at present conjectural, it is documented that MMF has robust antitumor activity by activating natural killer cells to kill tumor cells. This new mechanism of action for MMF should provide imputes for investigating this molecule not only as a therapeutic tool for autoimmune diseases but also for cancer and immunodeficient diseases. **Table 1** shows the current knowledge regarding the effects of DMF and MMF on various immune cells.

AUTHOR CONTRIBUTIONS

Both authors contributed to writing this review article.

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Increased Autophagy-Related 5 Gene Expression Is Associated with Collagen Expression in the Airways of Refractory Asthmatics

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Background: Fibrosis, particularly excessive collagen deposition, presents a challenge for treating asthmatic individuals. At present, no drugs can remove or reduce excessive collagen in asthmatic airways. Hence, the identification of pathways involved in collagen deposition would help to generate therapeutic targets to interfere with the airway remodeling process. Autophagy, a cellular degradation process, has been shown to be dysregulated in various fibrotic diseases, and genetic association studies in independent human populations have identified autophagy-related 5 (ATG5) to be associated with asthma pathogenesis. Hence, the dysregulation of autophagy may contribute to fibrosis in asthmatic airways.

Objective: This study aimed to determine if (1) collagen deposition in asthmatic airways is associated with ATG5 expression and (2) ATG5 protein expression is associated with asthma *per se* and severity.

Methods: Gene expression of transforming growth factor beta 1, various asthma-related collagen types [collagen, type I, alpha 1; collagen, type II, alpha 1; collagen, type III, alpha 1; collagen, type V, alpha 1 (COL5A1) and collagen, type V, alpha 2], and ATG5 were measured using mRNA isolated from bronchial biopsies of refractory asthmatic subjects and assessed for pairwise associations. Protein expression of ATG5 in the airways was measured and associations were assessed for asthma *per se*, severity, and lung function.

Main results: In refractory asthmatic individuals, gene expression of ATG5 was positively associated with COL5A1 in the airways. No association was detected between ATG5 protein expression and asthma *per se*, severity, and lung function.

Conclusion and clinical relevance: Positive correlation between the gene expression patterns of ATG5 and COL5A1 suggests that dysregulated autophagy may contribute to subepithelial fibrosis in the airways of refractory asthmatic individuals. This finding

highlights the therapeutic potential of ATG5 in ameliorating airway remodeling in the difficult-to-treat refractory asthmatic individuals.

Keywords: autophagy, ATG5, asthma, collagen, airway remodeling

INTRODUCTION

The Global Initiative for Asthma estimated that globally there are 300 million people who suffer from asthma and the number is expected to reach 400 million by 2025 (1). Among asthmatic individuals, between 5 and 10% are considered severe who need a combination of oral steroids, inhaled steroids, short-acting bronchodilator, long-acting bronchodilator, and leukotriene modifiers to control their asthma. However, a proportion of these severe asthmatic subjects, despite aggressive treatment schemes continue to have exacerbations, obstructive airways, emergency visits, and even near fatal asthma attacks. The injury and repair associated with severe persistent asthma results in irreversible airflow obstruction due to airway remodeling (2, 3). Fibrosis is an important characteristic of tissue remodeling, and in asthmatic airways, fibrosis is associated with increased collagen deposition in the subepithelium (4–7). Simplistically put, in normal airways, collagen and other extracellular matrix (ECM) proteins are deposited and degraded in a homeostatic fashion; yet in asthmatic airways, such homeostasis is dysregulated, as reviewed elsewhere (8). In asthmatic airways, collagen type I (4), type III (4, 9), and type V (9, 10), among other ECM proteins, were found in greater quantity than in non-asthmatic airways. Furthermore, the presence of fibrosis below the epithelium of airways is associated with asthma severity and lung function decline (6). While commonly used asthma medications are effective in reducing inflammation and dilating constricted airways, they are ineffective in reducing or preventing fibrosis (6, 11, 12).

Autophagy is a cellular degradation process in which the cell hydrolytically removes cytoplasmic contents, such as damaged organelles and protein aggregates, by first engulfing the target within a double membrane vacuole and followed by fusion with lysosomes, as reviewed elsewhere (13, 14). During autophagy, autophagy-related 5 (ATG5) is covalently conjugated with ATG12 and interacts with ATG16 to form the ATG12–ATG5–ATG16 complex (15). This complex enhances the formation of the membrane destined to form an autophagosome and is thus, vital for autophagosome formation (16). Intricate relationships exist between autophagy and other forms of cell death (i.e., apoptosis and necrosis) (17, 18). It has been postulated that autophagy serves as a cell survival mechanism to remove triggers that are threatening cell survival, yet when such threats become overwhelming, cell death processes such as apoptosis and necrosis take over (18, 19). Dysregulation of autophagy has been linked to fibrosis in a number of fibrotic diseases, including cirrhosis (20), idiopathic pulmonary fibrosis (21), and renal fibrosis (22). The upregulation of autophagy during activation of fibrogenic cells, such as hepatic stellate cells from mice as well as hepatitis B-, hepatitis C-virus-infected human liver, and human fibroblasts from idiopathic pulmonary fibrosis, suggests that autophagy is a central pathway in fibrosis (23, 24). The loss of autophagy function, with specific autophagy inhibition by siRNAs against *Atg5*, results in the attenuation of matrix accumulation and fibrogenesis in stellate cells and renal, embryonic and lung fibroblasts (23), further supporting the role of autophagy in the fibrotic process. Recently, elevated autophagic activities have been detected in cells from sputum and blood from severe asthmatic patients as compared to the milder asthmatics and healthy controls (25). Furthermore, two candidate gene association studies detected associations between variations in the gene encoding ATG5 and asthma (26, 27), and elevated ATG5 gene expression was found in the nasal epithelium of children with acute asthma as compared to those with no asthma or stable asthma (27). In addition to asthma *per se*, *ATG5* polymorphism was associated with lung function in asthmatic individuals (26). This genetic association coupled with the histological observation of increased autophagosomes in moderately severe asthmatics provides evidence of autophagy in the pathogenesis of asthma (25, 26). Stemming from these reports, we hypothesize that ATG5 expression is associated with collagen deposition in severe asthmatic patients.

MATERIALS AND METHODS

Sample Collections

Gene expression measurement using microarray was performed using RNA isolated from bronchial biopsy samples of study participants in the Bronchoscopic Exploratory Research Study of Biomarkers in Corticosteroid-refractory Asthma (BOBCAT) study (28). The BOBCAT study was a multicenter study conducted in Canada, United States, and United Kingdom, and patient recruitment has been described previously (28). Briefly, patients with uncontrolled moderate-to-severe asthma accompanied by forced expiratory volume in one second (FEV_1) percent (%) predicted of 40–80%, airway obstruction of >12% and reversibility with a short-acting bronchodilator or methacholine sensitivity (PC_{20}) <8 mg/ml in the past 5 years were recruited. The asthma of the participants must be refractory as defined by at least two exacerbations in the previous year or an asthma control quality (ACQ) score of >1.50 while on high-dose inhaled corticosteroid (ICS) (>1,000 μg of fluticasone or equivalent daily) with or without long-acting β-agonist. Processing of the bronchial biopsy tissues for RNA isolation and gene expression microarray analyses has been described previously (29).

Protein expression was measured in bronchial biopsy tissues obtained from fiberoptic bronchoscopy of asthmatic and non-asthmatic healthy subjects archived at the Tissue Bank of the Respiratory Health Network of the Fonds de Recherche du Québec – Santé (McGill University Health Centre site). Patient recruitment and sample processing have been described previously (6, 30). Asthma severity (mild, moderate, and severe) was determined based on medication usage, frequency of exacerbation, and lung function as previously described (30, 31). Briefly, severe asthma subjects met the criteria proposed by the American Thoracic Society workshop on refractory asthma (32). Moderate

asthmatic subjects were individuals with persistent asthma whose symptoms were under-control with a dosage between 176 and 800 μg/d of fluticasone (or equivalent) with or without add-on controller medication, no more than two steroid bursts in the past 12 months and none in the past 3 months with total days on oral steroids <30 days in the prior 12 months, predicted FEV_1 >70% and >90% of personal best from the past 2 years, and a maximum of one unscheduled visit for asthma in the prior 12 months. Mild asthmatic subjects were individuals with prebronchodilator predicted FEV_1 >80% and treated with either no or low-to-moderate dose of ICS (<880 μg fluticasone or equivalent). In addition to asthmatic subjects, non-asthmatic subjects with no history of asthma diagnosis, predicted FEV_1 >90% and free of respiratory or systemic diseases, were included as control subjects.

All subjects have given their informed consent in accordance with the Declaration of Helsinki, and the study has been approved by the Research Ethics Board of the Research Institute-McGill University Health Centre. The BOBCAT protocol was approved by the Copernicus Group independent review board and respective institutional review boards associated with other participating study centers in the United States, Canada, and UK.

Gene Expression

Gene expression data were available from previously performed microarray analyses (33). Briefly, amplified single-stranded cDNA from homogenized bronchial biopsy tissues was hybridized to Affymetrix (Santa Clara, CA, USA) U133 plus 2.0 arrays, and array images were analyzed with Affymetrix GeneChip Expression Analysis Software. Gene expression data in the airways of various asthma-related collagen types [collagen, type I, alpha 1 (COL1A1); collagen, type II, alpha 1 (COL2A1); collagen, type III, alpha 1 (COL3A1); collagen, type V, alpha 1 (COL5A1) and collagen, type V, alpha 2 (COL5A2)], transforming growth factor beta 1 (TGFB1), and ATG5 were obtained.

Immunocytochemistry

Immunocytochemistry staining of formalin-fixed paraffin-embedded biopsy samples was performed to determine the protein level of ATG5. ATG5 immunoreactivity was detected using an ATG5 specific antibody (Abcam, Cambridge, MA, USA, ab109490) on 5 μm thick tissues as previously described (34). Briefly, heat-activated antigen-retrieval in citrate buffer was performed to expose antigens; endogenous peroxidase activity was blocked with 1% H_2O_2, protein detection and signal amplification were achieved with streptavidin-horseradish peroxidase complex (Dako, Carpinteria, CA, USA), brown color stains were developed by 3,3′-diaminobenzidine (Dako, Carpinteria, CA, USA), and tissues were counter stained with hematoxylin and lithium carbonate. Image analyses were performed using the image processing program, ImageJ (version 1.46). Protein expression of ATG5 in the submucosal area was measured as the proportion of positively stained area.

Statistical Analyses

Microarray data analyses were performed using Bioconductor in the R statistical environment as previously described (35). Pairwise correlation of gene expressions was performed using Spearman's rank order correlation. A Bonferroni corrected p value <0.004 was considered as statistically significant. The association between ATG5 protein expression in the bronchial biopsy samples and asthma *per se*, severity, and lung function were assessed by Wilcoxon, Wilcoxon rank sums, and Pearson's correlation tests, respectively.

RESULTS

Population Characteristics

Bronchial biopsy tissues from 35 refractory asthmatic subjects that participated in the BOBCAT study were used to assess for pairwise correlations between autophagy and different collagen subtypes gene expressions. Patients characteristics have previously been published (28). Briefly, the mean age of the subjects was 46 years (SD = 11) with 62.9% of the subjects being male. The mean FEV_1% predicted was 61% (SD = 12). The mean ACQ score was 2.6 (SD = 0.8). Bronchial biopsy tissues from 42 asthmatics (15 mild, 12 moderate, and 15 severe) and 15 non-asthmatic healthy subjects were used to measure and assess correlation between ATG5 protein expression and asthma *per se* and severity. The mean ages of the four groups (normal, mild, moderate, and severe) were 32.7 years (SD = 14.9), 31.6 years (SD = 9.5), 42.1 years (SD = 10.6), and 40.9 years (SD = 8.1), respectively. The percentages of the samples being female were 60, 63, 50, and 27%, respectively. In terms of lung function, the mean FEV_1% predicted values of the four groups were 109.1% (SD = 14.9), 90.0% (SD = 15.6), 90.4% (SD = 14.5), and 58.3% (SD = 15.4), respectively. The mean FEV_1/forced vital capacity values were 0.83 (SD = 0.07), 0.76 (SD = 0.10), 0.77 (SD = 0.09), and 0.67 (SD = 0.13), respectively.

Gene Expression of TGFB1 in the Airways Is Associated with COL1A1 Gene Expression in Refractory Asthmatic Subjects

Pairwise gene expression comparisons demonstrated a significant correlation between *TGFB1* and *COL1A1* ($\rho = 0.59$, p-value $= 2.4 \times 10^{-4}$) (**Table 1**). The correlation between *TGFB1* and *COL1A2* demonstrated a positive trend but was not significant after correction ($\rho = 0.32$, p-value = 0.06). No significant correlations or trends were observed between *TGFB1* and the other investigated collagen types in this study [*COL3A1* ($\rho = 0.01$, p-value = 0.95), *COL5A1* ($\rho = 0.07$, p-value = 0.71), and *COL5A2* ($\rho = 0.01$, p-value = 0.96)]. Therefore, *COL1A1* gene expression positively correlated with *TGFB1* gene expression in refractory asthmatics.

Gene Expression of ATG5 in the Airways Is Associated with COL5A1 Gene Expression in Refractory Asthmatic Subjects

Pairwise gene expression comparisons demonstrated a significant correlation between *ATG5* and *COL5A1* ($\rho = 0.72$, p-value $= 2.9 \times 10^{-6}$) (**Table 1**). The correlation between *ATG5* and *COL1A1* demonstrated a positive trend but was

TABLE 1 | Pairwise correlations between gene expression of various collagen types and *ATG5* and *TGFB1* in bronchial biopsy tissues of refractory asthmatic individuals.

	COL1A1	*COL1A2*	*COL2A1*	*COL3A1*	*COL5A1*	*COL5A2*	*ATG5*
TGFB1	$\rho = 0.59$, $p^* = 2.4 \times 10^{-4}$	$\rho = 0.32$, $p = 0.06$	Did not analyze	$\rho = 0.01$, $p = 0.95$	$\rho = 0.07$, $p = 0.71$	$\rho = 0.01$, $p = 0.96$	$\rho = 0.22$, $p = 0.21$
ATG5	$\rho = 0.42$, $p = 0.01$	$\rho = 0.25$, $p = 0.14$	$\rho = -0.3$, $p = 0.08$	$\rho = 0.33$, $p = 0.05$	$\rho = 0.72$, $p = 2.9 \times 10^{-6}$	$\rho = 0.30$, $p = 0.08$	

**Bonferroni adjusted p-value threshold for statistical significance (i.e., 12 tests) is 0.004.*

not significant after correction ($\rho = 0.42$, p-value $= 0.01$). No significant correlations or trends were observed between *ATG5* and the other investigated collagen types in this study [COL1A2 ($\rho = 0.25$, p-value $= 0.14$), COL2A1 ($\rho = -0.3$, p-value $= 0.08$), COL3A1 ($\rho = 0.33$, p-value $= 0.05$), and COL5A2 ($\rho = 0.30$, p-value $= 0.08$)]. Therefore, *COL5A1* gene expression positively correlated with *ATG5* gene expression in refractory asthmatics.

Protein Expression of ATG5 in the Airways Is Not Associated with Asthma, Asthma Severity, or Lung Function in Asthmatic Subjects

Since fibrosis is often associated with asthma severity and decline in lung function (6), and *ATG5* gene expression demonstrated positive correlation with *COL5A1* gene expression, the ATG5 protein expression in the asthmatic airways was investigated to determine the association, if any, with asthma *per se*, severity, and lung function. ATG5 proteins were detected in the epithelium, airway smooth muscle cell bundles, and inflammatory cells in all asthmatic and non-asthmatic subjects (**Figure 1**). Pre-absorption of the ATG5 monoclonal antibodies with ATG5 peptides prevented any positive staining of the biopsy tissues (**Figure 1F**). ATG5 protein expression in the submucosal area was measured and expressed as a proportion of positively stained tissue area. No significant difference was detected in the ATG5 expression between non-asthmatic control and asthmatic subjects (Wilcoxon p-value = 0.1) (**Figure 2**). When asthmatic subjects were stratified by severity (mild, moderate, and severe), no significant difference in ATG5 expression was observed among the three asthmatic severity groups and with the non-asthmatic group (p-value = 0.7 Wilcoxon rank sums) (**Figure 2**). In terms of lung function in asthmatic subjects, ATG5 protein expression did not correlate with FEV_1% predicted in asthmatic subjects [Spearman's rank correlation coefficient (ρ) = 0.04, $p = 0.83$] or when stratified by asthma severity ($0.3 <$ Spearman's $\rho < 0.8$, $0.26 < p < 0.83$) (data not shown).

DISCUSSION

Albeit the genetic findings of association between ATG5 polymorphism and various asthma-related traits in a number of independent populations, no association was found between the ATG5 protein level and either the presence of asthma *per se*, severity of asthma, or by lung function in this study. A number of factors may contribute to the lack of association at the protein level despite associations detected at the gene level. For example, genotypes of the bronchial biopsy tissues were not determined due to limited availability of archived tissues. Furthermore, elevated gene expression was observed in nasal mucosal cells of acute asthmatic children as compared to stable asthmatic and control children (27). The biological consequence may only be detectable in the downstream autophagic pathway. The detection of LC3B-II punctae using immunocytochemistry is often used to indicate the presence of autophagy (24); however, the use of archived formalin-fixed paraffin-embedded tissues in this study limited its use. In this study, the investigation was focused on ATG5 proteins in the submucosal area of the airways; hence, the impact of the genetic and gene expression association may not be carried to protein expression in different cell types. Finally, the phenotypes of interest were different from previous studies reporting genetic associations. Although the disease of investigation was asthma, the underlying mechanism for the genetic association with acute asthma in children or with FEV_1% predicted in asthmatic subjects may, and likely be, different from that of severity.

Subepithelial fibrosis is a hallmark of asthmatic airways, and fibrosis can be primarily attributed to the deposition of collagen of types I, III, and V as well as fibronectin (6). The findings of positive association between the gene expression of various collagens (i.e., COL5A1 and less significantly COL1A1) and ATG5 supported the speculation that enhanced autophagy is associated with asthma pathogenesis and in particular collagen deposition. It has been suggested that TGFB1 is a pro-fibrotic element present in asthmatic tissues (6, 36), as evidenced by the gene expression data of the BOBCAT study. TGFB1 gene expression positively correlated with type 1 collagen gene expression in this study. This is in concordance with the observation that TGFB1 simultaneously promotes COL1A2 synthesis and autophagy induction in human atrial myofibroblasts, and ATG5 knockout of mouse embryonic fibroblasts is associated with a parallel decline in the fibrotic effect of TGFB1 when compared to wild-type cells, further stressing the role of autophagy in TGFB1-induced fibrosis (37). Interestingly, in this study TGFB1 gene expression did not correlate with COL5A1 and COL5A2, yet ATG5 gene expression correlated with the type V collagen gene expression. Type V collagen is a minor collagen that is intercalated within fibrils of the major lung collagen, type I collagen (38). Under normal conditions, the epitopes of the type V collagen are masked within the fibrils; yet in conditions with prominent tissue remodeling, the type V epitopes are exposed and have been shown to induce autoimmunity in a murine model of allergic airway disease (10), lung transplant-associated obliterative bronchiolitis (39), and idiopathic pulmonary fibrosis (40). In the context of asthma, an observational study of asthmatic subjects has detected higher levels of type V collagen antibody in the serum of asthmatic subjects

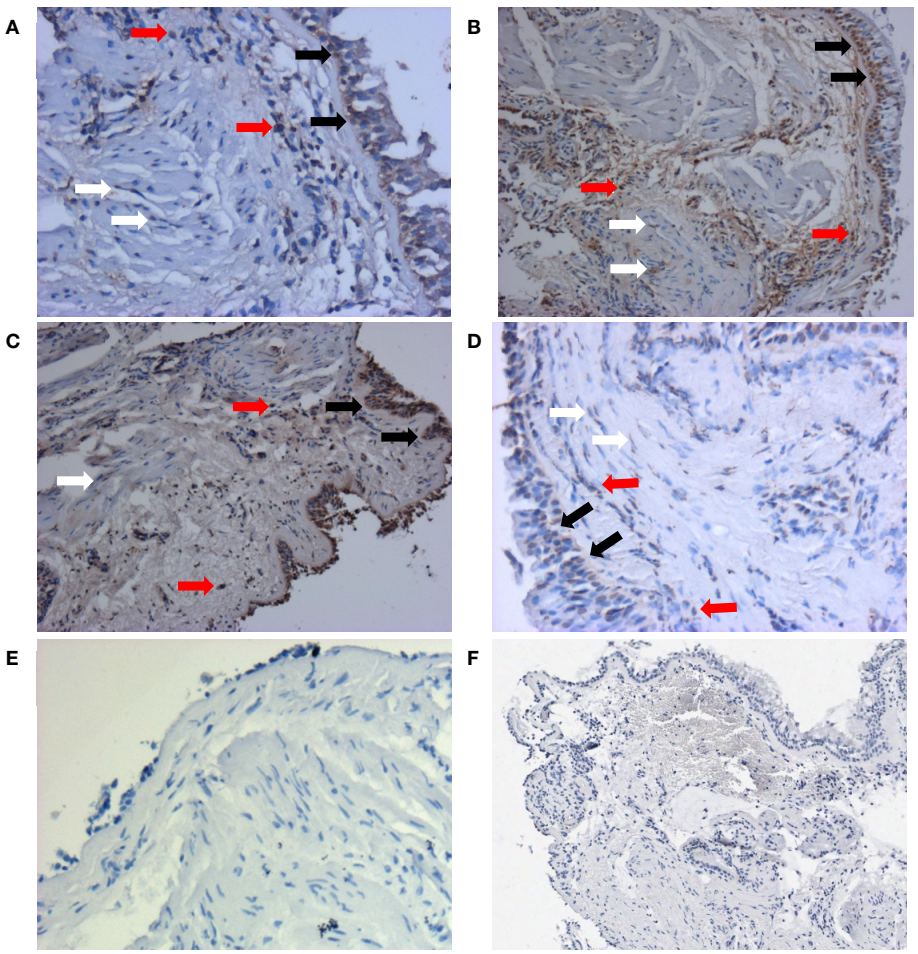

FIGURE 1 | Representative staining patterns of ATG5 protein expression from each group were shown here: non-asthmatic control (A), mild (B), moderate (C), and severe (D). Monoclonal antibody for ATG5 was used as the primary antibody and developed with 3,3′-diaminobenzidine diaminobenzidine (brown). Nuclei were stained with hematoxylin (blue). Negative controls of ATG staining were performed using IgG1 isotype **(E)** and pre-absorption with ATG antigens **(F)**. Pictures were taken at 200× magnification. Positive stainings could be detected in epithelial cells (black arrows), airway smooth muscle cells (white arrows), and inflammatory cells (red arrows). Enlarged staining patterns can be found in Figure S1 in Supplementary Material.

than in non-asthmatic healthy subjects (10). Histopathological examination of a lung biopsy of an individual with fatal asthma also demonstrated greater type V collagen staining than normal lung biopsy (10). The murine model of allergic airway disease further demonstrated the positive association between anti-type V collagen antibody and IgE antibody production, and the protective effect of type V collagen-induced tolerance in airway resistance and airway hyperresponsiveness (10). Given that the BOBCAT study subjects are of moderate-to-severe asthma severity, airway remodeling is likely to be prevalent, and increased synthesis and deposition of type V collagen may be an important contributor to the associated fibrosis in these asthmatics.

Fibrosis in different organs has been associated with both autophagic upregulation as well as downregulation, emphasizing the diversity in the functional role of autophagy in tissue repair (41). On the one hand, it has been shown that in proximal epithelial cells, ATG5-mediated autophagy reduced type I collagen deposition by blocking the G_2/M phase arrest (42), a cell cycle phase whose arrest would initiate DNA repair and synthesis of pro-fibrotic factors (43). Furthermore, bleomycin-induced pulmonary fibrosis in a mouse model led to increased autophagy activation in the lungs as revealed by upregulated ATG5 protein expression levels and increased autophagosome formation (44). However, deficient autophagy in this model enhanced lung fibrosis, which was characterized by upregulation of collagens, COL1A2 and COL3A1. On the other hand, in human oral fibroblasts, suppression of autophagy led to reduction in type I collagen (i.e., COL1A2) gene expression, promotion of apoptosis, and suppression of proliferation (45). Additionally, prolonged starvation of human embryonic lung fibroblasts triggered the simultaneous activation of myofibroblast differentiation, which was accompanied by increased COL1A1 and COL3A1 at mRNA or protein levels, and autophagy (41). Autophagy inhibition was shown to prevent collagen mRNA and protein levels and myofibroblast differentiation (41). These discrepancies clearly demonstrate tissue and cell specificity in the downstream effects of autophagy. Other

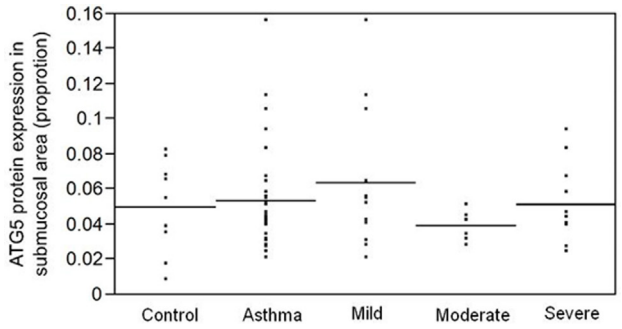

FIGURE 2 | ATG5 protein expression, measured as proportion of positively stained area in the submucosa, is not associated with asthma *per se* ($p = 0.1$) or with asthma severity ($p = 0.7$).

models of fibrosis suggested that autophagy may regulate fibrosis through a large number of pathways including the activation of the unfolded protein response (46), the activation of the IL-17A/STAT3 signaling pathway (47), the suppression of mitochondrial reactive oxidative species–NF-κB-IL1α/β pathways (48), and the degradation of activated caspase-8 (49).

The mechanism behind the observed association between autophagy and type V collagen production is unknown and elusive. Angiotensin may be involved in tissue remodeling, and angiotensin II type I receptor signaling has been shown to induce autophagy in cardiomyocytes (50). Angiotensin II stimulation was observed to activate autophagy in rat cardiac fibroblasts both *in vitro* and *in vivo*, and ATG5 knockdown augmented angiotensin II-mediated accumulation of collagen type I (51). In another study, angiotensin II type I receptor antagonist, valsartan, suppressed types III and V collagen synthesis by modulating TGFB1 expression at the mRNA and protein levels (52), suggesting a plausible role of autophagy in type V collagen deposition *via* angiotensin II type I receptor signaling. In order to draw a mechanistic conclusion of the ATG5–type V collagen association in this study, further investigations involving various cell types such as fibroblasts, epithelial cells, and smooth muscle cells need to be studied separately. However, the findings that type V collagen and autophagy are associated in the lung tissues of moderate-to-severe asthmatic subjects are novel and exciting. Though there have been recent health authority approvals of two drugs to treat idiopathic pulmonary fibrosis, no pharmaceutical agents have yet been shown to directly ameliorate or reverse fibrosis. This finding supports ATG5 as a new target for anti-fibrotic drug development.

AUTHOR CONTRIBUTIONS

AP, DC, SA, AM, JH, JA, CL, and QH conceptualized and designed the study; acquired, analyzed, and interpreted the data generated; drafted, revised, and approved the manuscript; and agreed to be accountable for all aspects of the work. RR and BM edited, revised, and analyzed the data and content in the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00355/full#supplementary-material.

FIGURE S1 | ATG5 positive staining in various cell types in a bronchial biopsy tissue of a severe asthmatic subject. Nuclei were stained with hematoxylin (blue). Positive stainings could be detected in epithelial cells (black arrow), airway smooth muscle cells (white arrow), and inflammatory cells (red arrow).

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Endogenous n-3 Polyunsaturated Fatty Acids Attenuate T Cell-Mediated Hepatitis *via* Autophagy Activation

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Omega-3 polyunsaturated fatty acids (n-3 PUFAs) exert anti-inflammatory effects in several liver disorders, including cirrhosis, acute liver failure, and fatty liver disease. To date, little is known about their role in immune-mediated liver diseases. In this study, we used *fat-1* transgenic mice rich in endogenous n-3 PUFAs to examine the role of n-3 PUFAs in immune-mediated liver injury. Concanavalin A (Con A) was administered intravenously to wild-type (WT) and *fat-1* transgenic mice to induce T cell-mediated hepatitis. Reduced liver damage was shown in Con A-administrated *fat-1* transgenic mice, as evidenced by decreased mortality, attenuated hepatic necrosis, lessened serum alanine aminotransferase activity, and inhibited production of pro-inflammatory cytokines (e.g., TNF-α, IL-6, IL-17A, and IFN-γ). *In vivo* and *in vitro* studies demonstrated that n-3 PUFAs significantly inhibited the activation of hepatic T cells and the differentiation of Th1 cells after Con A challenge. Further studies showed that n-3 PUFAs markedly increased autophagy level in Con A-treated *fat-1* T cells compared with the WT counterparts. Blocking hepatic autophagy activity with chloroquine diminished the differences in T cell activation and liver injury between Con A-injected WT and *fat-1* transgenic mice. We conclude that n-3 PUFAs limit Con A-induced hepatitis *via* an autophagy-dependent mechanism and could be exploited as a new therapeutic approach for autoimmune hepatitis.

Keywords: omega-3 polyunsaturated fatty acids, immune-mediated liver injury, concanavalin A-induced hepatitis, T cell activation, autophagy

INTRODUCTION

Autoimmune hepatitis is a medical problem in which the body's immune system attacks the liver, causing cirrhosis and liver failure (1). Although the etiology is not entirely understood, impaired apoptosis and excessive T cell activation are believed to be associated with the pathophysiology of autoimmune hepatitis (2, 3). The T cell responses involved in autoimmune hepatitis comprise the

Abbreviations: ALT, alanine aminotransferase; ALA, alpha-linolenic acid; BSA, bovine serum albumin; CFSE, 5-(and-6)-carboxy fluorescein diacetate succinimidyl ester; Con A, Concanavalin A; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; H&E, hematoxylin and eosin; MNCs, mononuclear cells; mTOR, mammalian target of rapamycin; n-3 PUFAs, Omega-3 polyunsaturated fatty acids; NF-κB, nuclear factor-κB; PI, propidium iodide; PVDF, polyvinylidene fluoride; WT, wild-type.

predominant IFN-γ-producing Th1 cells over IL-4-producing Th2 cells and the reduced frequency and function of regulatory T cells (Tregs) (4–7). It is of critical importance to identify new immune modulators that can counter-regulate the hepatic immune response, especially the T cell response, in autoimmune hepatitis.

Omega-3 polyunsaturated fatty acids (n-3 PUFAs) are a collection of polyunsaturated fatty acids, including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from fish and alpha-linolenic acid (ALA) in plants (8), which have acknowledged effects on both structural integrity and function of cellular membranes. Emerging evidence showed that n-3 PUFAs could regulate the inflammatory response by decreasing inflammatory cytokines and reactive oxygen production (9). *Fat-1* transgenic mice, which express the *Caenorhabditis elegans fat-1* gene, are capable of synthesizing n-3 PUFAs from the n-6 type, leading to elevated amounts of n-3 PUFAs in their tissues compared with the wild-type (WT) littermates (10). Thus, these mice exhibit more anti-inflammatory derivatives generated from n-3 PUFAs (e.g., resolvin E1, resolvin D3, protectin D1, and maresin 1), resulting in protection against inflammatory disorders in different organs, such as allergic airway inflammation, chemically induced colitis, pancreatitis, and diabetic neuropathy (11–14). Since *fat-1* transgenic mice have significant endogenous amounts of n-3 PUFAs in their liver tissue (15), the function of n-3 PUFAs in liver injury and inflammation has been investigated. *Fat-1* transgenic mice developed less severe D-galactosamine/lipopolysaccharide (D-GalN/LPS)-induced inflammatory liver injury than WT mice, associated with a reduction of pro-inflammatory cytokines (e.g., TNF-α, IL-1β, IL-6, and IFN-γ) (15). Moreover, tissue n-3 PUFAs protected against acute ethanol-induced hepatic steatosis and diet-induced fatty liver disease in *fat-1* transgenic mice, through activation of cholesterol catabolism to bile acid and downregulation of hepatic inflammatory response (16, 17). However, the effect of endogenous n-3 PUFAs on liver immune responses that involve hepatic T lymphocytes remains unclear.

Intravenous injection of mice with T cell mitogen concanavalin A (ConA) induces polyclonal activation of T lymphocytes, resulting in a liver-specific inflammatory response (18). This model is characterized by elevated serum levels of alanine transaminase (ALT) and pro-inflammatory cytokines (e.g., TNF-α, IL-6, and IFN-γ), as well as infiltration of T lymphocytes and necrosis of hepatocytes in the liver tissue (4, 19). Therefore, Con A-induced hepatitis is a well-established murine model that can simulate the pathophysiology of human autoimmune hepatitis and has been extensively employed to elucidate the underlying mechanisms of T cell-mediated autoimmune hepatitis. In this study, we used *fat-1* transgenic mice to explore the protective effect of endogenous n-3 PUFAs on liver injury in the model of autoimmune hepatitis induced by Con A. The results showed that *fat-1* transgenic mice were resistant to Con A-induced hepatitis, which attributed to the suppressed T cell activation and Th1 differentiation in the presence of n-3 PUFAs. We also provided evidences that endogenous n-3 PUFAs enhanced T cell autophagy upon Con A challenge, which may be involved in the inhibition of T cell activation and subsequent liver injury. In summary, our findings revealed that hepatic n-3 PUFAs controlled T cell responses during immune-mediated hepatitis, which may be potentially employed as a new therapeutic strategy for autoimmune hepatitis.

MATERIALS AND METHODS

Mice

Wild-type C57BL/6 mice were purchased from the Laboratory Animal Center of Southern Medical University (Guangzhou, China). *Fat-1* transgenic mice were backcrossed with WT C57BL/6 mice, and the *fat-1* genotypes of each animal were characterized using isolated genomic DNA from mouse tails by PCR analysis as we previously described (20). All animal experiments in this study were approved by the Welfare and Ethical Committee for Experimental Animal Care of Southern Medical University.

Reagents

Con A, chloroquine, and DHA were purchased from Sigma–Aldrich (St. Louis, MO, USA). Antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA), including the antibodies against p62 (Cat# 5114), LC3 (D11), phospho-STAT1 (58D6), STAT1 (D1K9Y), phospho-STAT3 (D3A7), STAT3 (D3Z2G), phospho-NF-κB p65 (93H1), NF-κB p65 (C22B4), and GAPDH (D16H11). Mouse monoclonal antibodies against CD3 (145-2C11), CD4 (RM4-5), IFN-γ (XMG1.2), and CD69 (H1.2F3) were purchased from BD Pharmingen (San Jose, CA, USA). Anti-NK1.1 (PK136), anti-CD16/CD32 (2.4G2), goat anti-rabbit IgG, and 7-aminoactinomycin D (7-AAD) were from MultiSciences (Hangzhou, China). Also, 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) was purchased from Invitrogen (San Diego, CA, USA).

Con A-Induced Hepatitis Model

Con A-induced hepatitis was initiated as described previously with modification (21). Briefly, Con A (C2010, Sigma–Aldrich) was dissolved in PBS at 1 mg/ml, and age-matched mice received an intravenous injection of Con A to induce hepatitis. The high dose (35 mg/kg body weight) of Con A was used to generate survival curves in WT and *fat-1* transgenic mice, while the low dose (15 mg/kg body weight) of Con A permitted assessment of the liver pathology with hematoxylin and eosin (H&E) staining and other *in vitro* assays at indicated time after Con A challenge. To test the benefit of dietary n-3 PUFAs in immune-mediated liver injury, WT mice were fed with an n-3 PUFAs-enriched diet, as we previously described (20), for 3 weeks before Con A administration. For autophagy inhibition, chloroquine was injected intraperitoneally at the dose of 40 mg/kg body weight 1 h before Con A administration.

TUNEL Assay

Formalin-fixed and paraffin-embedded tissue sections of liver were deparaffinized in xylene, then gradually rehydrated in decreasing concentrations of ethanol and distilled water. Subsequently, proteinase K permeabilized sections were subjected to incubation with TdT enzyme and fluorochrome mixture (Promega, Madison, WI, USA) for 1 h at 37°C in the dark. After DAPI (Roche, Mannheim, Germany) staining, the slides were analyzed under a fluorescence microscope.

Serum ALT Assay and Cytokine Assessment

Individual mouse serum was collected at different time points after Con A injection. Serum alanine aminotransferase (ALT) activity was measured with a commercial kit (Jiancheng Biotech, Nanjing, China), according to the manufacturer's instruction. Cytokine levels in the sera and cell culture supernatants were assessed using commercial ELISA kits purchased from eBioscience (San Diego, CA, USA).

Western Blotting Analysis

Protein samples were separated on SDS-polyacrylamide gels and then transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Bovine serum albumin (BSA, 5%) was used to block non-specific sites of the membranes for at least 1 h at room temperature. Subsequently, the membranes were incubated overnight at 4°C with primary antibodies, followed by incubation with the horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Finally, the membranes were washed three times, and detection of the target protein was conducted with enhanced chemiluminescence (Thermo Fisher, Carlsbad, CA, USA).

Preparation of Liver Mononuclear Cells and Purification of Hepatic T Cells

Mouse livers were removed and pressed through a 200-gage stainless steel mesh. The filtrate containing non-parenchymal cell was centrifuged at 50 × *g* for 5 min. Supernatants containing mononuclear cells (MNCs) were collected, followed by washing once with PBS. The cells were resuspended in 30% Percoll (GE Healthcare, Uppsala, Sweden) and gently overlaid onto 70% Percoll. After centrifugation at 1000 × *g* for 20 min, liver MNCs were collected from the interphase, washed twice with PBS, and then resuspended for further proliferation assay and FACS analysis. T cells were purified from liver MNCs using mouse T lymphocyte enrichment set from BD Biosciences (San Jose, CA, USA).

Flow Cytometry

Isolated liver MNCs were resuspended in PBS containing 1% BSA. NKT cells and T cells were determined by staining with anti-CD3, anti-CD4, anti-NK1.1, and anti-CD69 (BD Pharmingen). For detection of IFN-γ and LC3, cells with surface staining were fixed and permeabilized by Cytofix/Cytoperm kit (eBioscience), further stained with anti-IFN-γ or anti-LC3 antibodies. To evaluate nuclear factor-κB (NF-κB) activity in T cells, hepatic MNCs from Con A-injected mice were permeabilized by methanol followed stained with fluorescence-conjugated antibodies against phospho-NF-κB p65 and CD3. T cell apoptosis was monitored by FACS analysis using Annexin V/propidium iodide (PI) staining, according to the manufacturer's instruction (Keygen Biotech, Nanjing, China).

Isolation of RNA and qRT-PCR Analysis

Mice liver or hepatic MNCs' total RNA was extracted using Trizol (Invitrogen) and then transcribed into cDNA using the reverse transcription kit (TaKaRa, Dalian, China), as instructed by the manufacturer. SYBR Green quantitative RT-PCR was performed to determine the gene expression level using a 7900HT fast real-time PCR system (Applied Biosystems, San Francisco, CA, USA), according to the protocols provided with the SYBR Premix EX Taq (TaKaRa). The levels of target gene were normalized with respect to GAPDH gene expression. The primer sequences used in the experiment are shown in **Table 1**.

In vitro T Cell Stimulation

Liver MNCs (4×10^5 per well) were treated with various concentration of Con A in the flat-bottomed 96-well plate for 72 h for proliferation assay. Supernatants were collected at 48 h for cytokine assays using ELISA. Purified hepatic T cells were stimulated with 5 μg/ml of Con A for indicated time followed by immunoblotting and FACS analysis. In some studies, DHA was added 4 h before treatment with Con A.

Statistical Analysis

The experimental data were evaluated by calculating the mean ± SD. One-way ANOVA was used for comparisons among multiple groups. Differences between two groups within experiments were analyzed by Student's *t*-test. Comparison of the survival curves was performed using the log-rank test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

n-3 PUFAs Play a Protective Role in Con A-Induced Fulminant Hepatitis

To define the role of n-3 PUFAs in Con A-induced hepatitis, sex- and age-matched C57BL/6 WT and *fat-1* transgenic mice

TABLE 1 | Primers for inflammatory cytokines and T cell-specific transcript factors.

	Forward primer (5′–3′)	Reverse primer (5′–3′)
IFN-γ	CATCAGCAACAACATAAGCGTCA	CTCCTTTTCCGCTTCCTGA
TNF-α	CTCTTCTGCCTGCTGCACTTTG	ATGGGCTACAGGCTTGTCACTC
IL-6	TACCACTTCACAAGTCGGAGGC	CTGCAAGTGCATCATCGTTGTTC
IL-17A	CAGACTACCTCAACCGTTCCAC	TCCAGCTTTCCCTCCGCATTGA
IL-4	ATCATCGGCATTTTGAACGAGGTC	ACCTTGGAAGCCCTACAGACGA
IL-10	CGGGAAGACAATAACTGCACCC	CGGTTAGCAGTATGTTGTCCAGC
T-bet	CCACCTGTTGTGGTCCAAGTTC	CCACAAACATCCTGTAATGGCTTG
GATA3	CCTCTGGAGGAGGAACGCTAAT	GTTTCGGGTCTGGATGCCTTCT
RORγt	CCGCTGAGAGGGCTTCAC	TGCAGGAGTAGGCCACATTACA
Foxp3	CCTGGTTGTGAGAAGGTCTTCG	TGCTCCAGAGACTGCACCACTT
GAPDH	CATCACTGCCACCCAGAAGACTG	ATGCCAGTGAGCTTCCCGTTCAG

were treated with a high dose of Con A (35 mg/kg body weight), and the survival rate of mice was determined. Strikingly, all of the WT mice died within 60 h. In contrast, 90% of *fat-1* transgenic mice survived the challenge (**Figure 1A**), indicating a protective role of endogenous n-3 PUFAs against Con A-induced liver damage. To evaluate the effect of n-3 PUFAs on liver injury, mice received a low dose of Con A (15 mg/kg body weight), followed by the examination of serum ALT activity and liver pathological changes. Sera collected from *fat-1* transgenic mice showed low levels of ALT activity compared to that from WT mice after

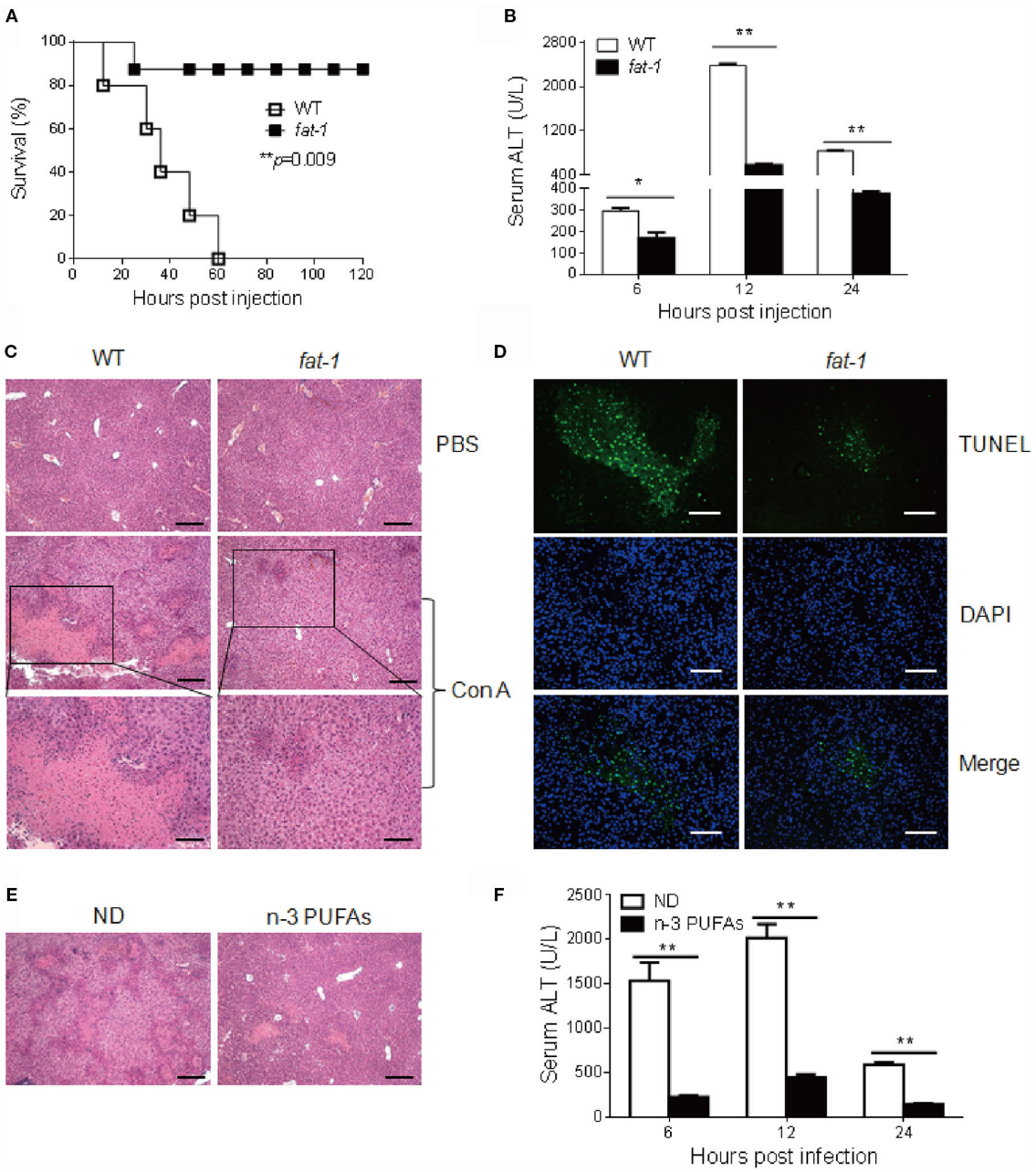

FIGURE 1 | n-3 PUFAs protect mice from Con A-induced hepatitis. (A) WT and *fat-1* transgenic mice ($n = 10$) were injected with Con A at the dose of 35 mg/kg, and overall survival of mice was monitored ($p = 0.009$). **(B–D)** A low dose of Con A (15 mg/kg) was injected into WT and *fat-1* transgenic mice ($n = 5$). Serum ALT activities in different time points were determined **(B)**. Histological analysis of mouse livers was performed with H&E staining. Scale bars = 100 μm. Bottom panels showed the higher-magnification views of the necrotic area. Scale bars = 50 μm. **(C)**. The apoptosis in mice liver tissues was evaluated by fluorescence TUNEL staining. Scale bars = 50 μm. **(D)**. $^{*}p < 0.05$, $^{**}p < 0.01$. Data are representative of three independent experiments with similar results. **(E,F)** WT mice were injected with Con A (15 mg/kg) after consuming normal diet (ND) or n-3 PUFAs-enriched diet (n-3 PUFAs) for 3 weeks ($n = 5$ each). Histology of liver **(E)** and serum ALT **(F)** were examined 24 h following Con A administration. Scale bars = 100 μm. $^{**}p < 0.01$. Data are representative of two independent experiments with similar results.

Con A injection (**Figure 1B**). Liver histological changes were examined at 16 h after Con A challenge. The result showed that liver tissues from *fat-1* transgenic mice displayed less hepatocyte necrosis and disseminated hemorrhage than those from WT mice (**Figure 1C**). Besides, the TUNEL positive cells were more abundant in the livers of Con A-treated WT mice than those from *fat-1* transgenic mice, indicating endogenous n-3 PUFAs could suppress the severe necrosis and apoptosis in the liver (**Figure 1D**). To further verify the protective effect of n-3 PUFAs on Con A-induced hepatitis, WT mice were fed with n-3 PUFAs-enriched diet for 3 weeks prior to Con A administration. In contrast to mice with normal diet (ND), mice receiving n-3 PUFAs-enriched diet showed minor liver damage after Con A injection as reflected by hepatic pathology (**Figure 1E**) and serum ALT levels (**Figure 1F**).

Next, we compared the serum levels of cytokines between Con A-treated WT and *fat-1* transgenic mice to determine the protective role of endogenous n-3 PUFAs in Con A-induced hepatitis. Attenuated liver damage in *fat-1* transgenic mice was accompanied by a pronounced reduction of pro-inflammatory cytokines (i.e., TNF-α, IFN-γ, IL-6, and IL-17A) and increment of the anti-inflammatory cytokine IL-10 in serum, while no significant difference in IL-4 was observed between Con A-treated WT and *fat-1* transgenic mice (**Figure 2A**). Accordingly, intrahepatic mRNA expression of the pro-inflammatory cytokines was significantly suppressed in *fat-1* transgenic mice compared with those in WT mice upon Con A administration (**Figure 2B**). Also, the trend of change in the hepatic expression of IL-10 and IL-4 was similar with those in serum between WT and *fat-1* transgenic mice (**Figure 2B**).

Activation of T Cells and the Differentiation of Th1 Cells Are Suppressed in *Fat-1* Transgenic Mice Treated with Con A

Activation of T cells or NKT cells was also determined in Con A-challenged mice. The frequency of T cells and NKT cells in the liver was decreased in both WT and *fat-1* transgenic mice after Con A injection, and no significant difference was observed between the two groups (**Figure 3A**). However, hepatic T cells and NKT cells displayed reduced activation in Con A-administrated *fat-1* transgenic mice compared with those in WT counterparts, as indicated by a decreased expression of CD69 (**Figure 3A**).

Since IFN-γ and $CD4^+$ Th1 cells differentiation plays a dominant role in Con A-induced liver damage (4, 7), we therefore evaluated the IFN-γ-producing immune cells in liver. As shown in **Figure 3B**, Con A-treated *fat-1* transgenic mice showed a marked decrease in the frequency of IFN-γ-producing $CD4^+$ T cells in the livers compared to WT controls. Accordingly, liver STAT1 phosphorylation at Tyr701 induced by Con A was much lower in *fat-1* transgenic mice than that in WT littermates (**Figure 3C**). Meanwhile, reduced STAT3 activation was also observed in the liver of Con A-administrated *fat-1* transgenic mice (**Figure 3C**), which was possibly due to the reduced IL-6 level in serum of *fat-1* transgenic mice. T-bet is a transcription factor critical to the development of $CD4^+$ Th1 cells (22). We demonstrated that T-bet was greatly suppressed in the liver of *fat-1* transgenic mice compared to their WT counterparts, suggesting that endogenous n-3 PUFAs could suppress the differentiation of Th1 cells in Con A-induced hepatitis. By contrast, the Th2 transcript factor GATA3 expression was comparable in these two groups with or without Con A administration. Surprisingly, the Th17 transcription factor RORγt expression was lower, while the Treg transcript factor Foxp3 expression was higher in liver from *fat-1* transgenic mice than those from WT mice after Con A injection (**Figure 3D**).

Suppression of the *In vitro* Activation of Hepatic T Cells by n-3 PUFAs

To determine whether endogenous n-3 PUFAs influence the Con A-induced T cell activation *in vitro*, hepatic MNCs isolated from WT and *fat-1* transgenic mice were stimulated with different concentrations of Con A. As shown in **Figure 4A**, [^{3}H] thymidine

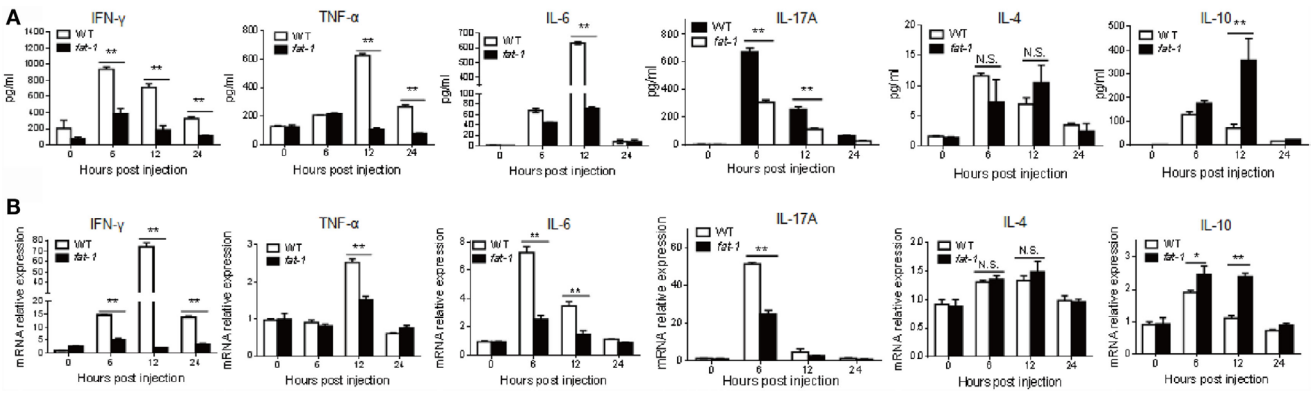

FIGURE 2 | Endogenous n-3 PUFAs differentially regulate the expression of cytokines during Con A-induced liver injury. **(A)** Serum levels of IFN-γ, TNF-α, IL-6, IL-17A, IL-4, and IL-10 were determined at 0, 6, 12, and 24 h post-Con A administration ($n = 5$). **(B)** Relative intrahepatic mRNA expression levels of IFN-γ, TNF-α, IL-6, IL-17A, IL-4, and IL-10 were measured by quantitative RT-PCR analysis at 0, 6, 12, and 24 h post-Con A administration and expressed as a ratio to GAPDH ($n = 5$). *$p < 0.05$, **$p < 0.01$. Data shown represent three independent experiments with similar results.

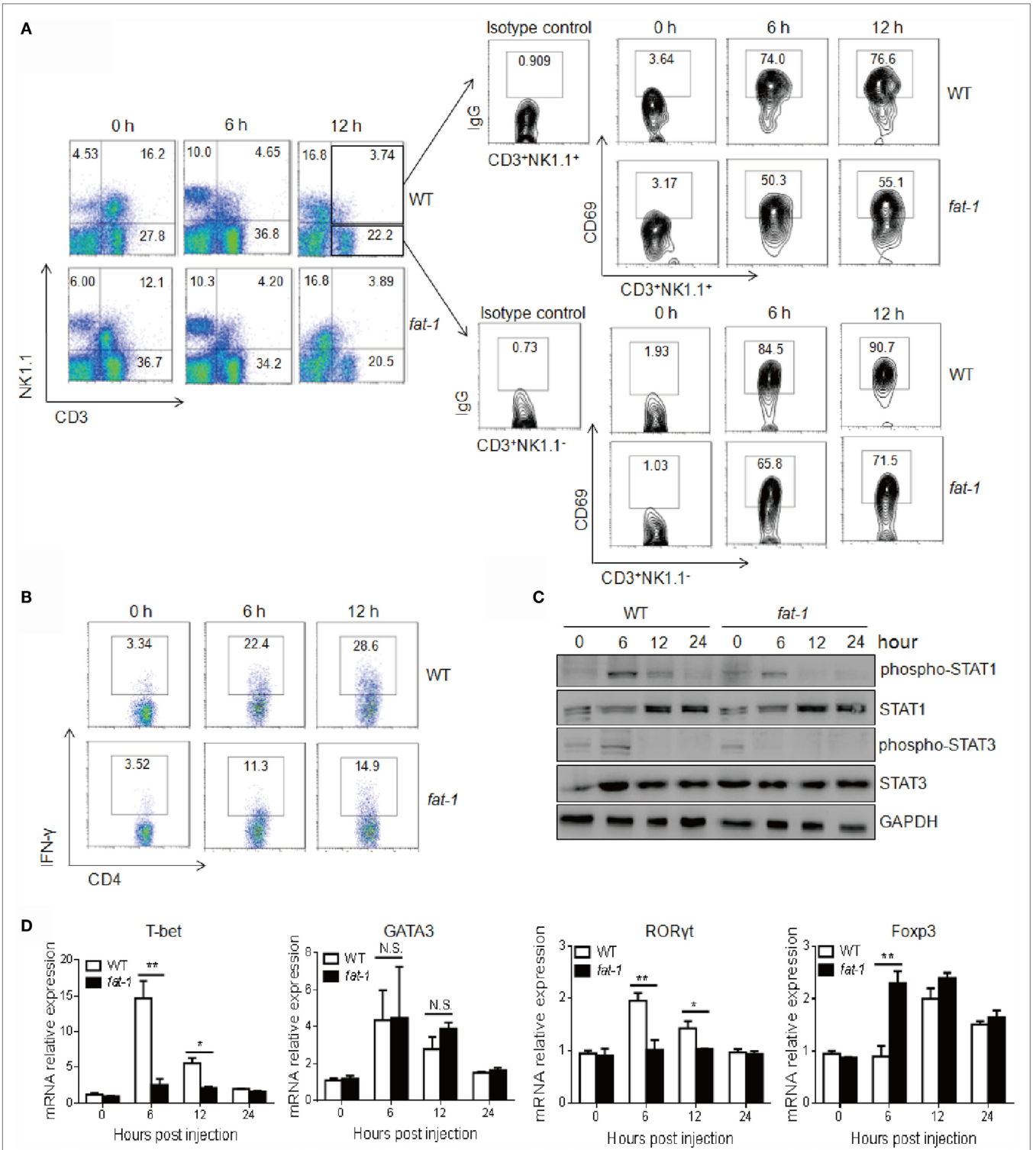

FIGURE 3 | **Endogenous n-3 PUFAs suppress intrahepatic NKT and T cells activation and Th1 cells differentiation upon Con A administration**. Hepatic MNCs were isolated from the liver of WT and *fat-1* transgenic mice ($n = 5$) at indicated time after Con A (15 mg/kg) injection. **(A)** The lymphocyte population and its activation were analyzed by FACS using anti-NK1.1, anti-CD3, and anti-CD69 antibodies. **(B)** Intracellular IFN-γ production of hepatic $CD4^+$ T cells was compared by FACS analysis. **(C)** Levels of STAT1 and STAT3 proteins and phospho-STAT proteins in liver lysates from WT and *fat-1* transgenic mice after injection of Con A were analyzed by Western blotting. GAPDH was served as a loading control. **(D)** Relative intrahepatic mRNA expression of T cell transcript factors, including T-bet, GATA3, RORγt, and Foxp3, was measured by quantitative RT-PCR analysis at 0, 6, 12, and 24 h post-Con A administration and expressed as a ratio to GAPDH. $^*p < 0.05$, $^{**}p < 0.01$, N.S., not significant. One of three independent experiments is shown.

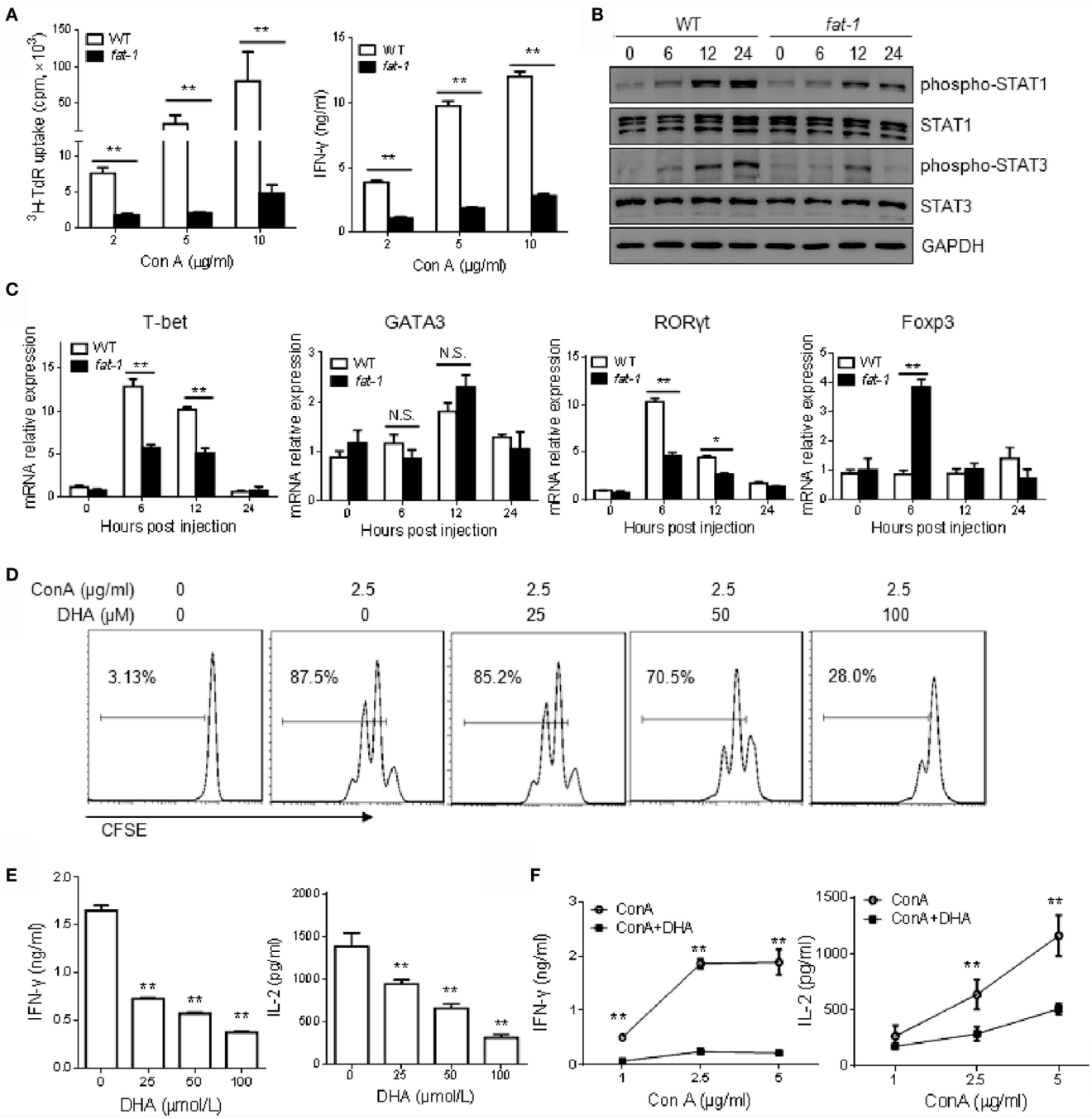

FIGURE 4 | **n-3 PUFAs inhibit Con A-stimulated T cell activation *in vitro***. **(A)** Hepatic MNCs from WT and *fat-1* transgenic mice were stimulated with different concentration of Con A *in vitro*. The proliferation response and cytokine production of T cells were determined by [^{3}H] thymidine uptake and ELISA assay, respectively. $^{**}p < 0.01$. Data are representative of three independent experiments. **(B,C)** Hepatic MNCs were treated with Con A (5 μg/ml) for various time. Cell extracts were then subjected to western blotting analysis with the antibodies against phospho-STAT1, phospho-STAT3, STAT1, and STAT3. One of the three independent experiments is shown **(B)**. Relative mRNA expression of T cell transcript factors, including T-bet, GATA3, RORγt, and Foxp3, was measured by quantitative RT-PCR analysis and expressed as a ratio to GAPDH. $^{*}p < 0.05$, $^{**}p < 0.01$, N.S., not significant. One of the three independent experiments is shown **(C)**. **(D,E)** Liver MNCs isolated from WT mice were cultured with Con A (2.5 μg/ml) in the presence of indicated doses of DHA. T cell proliferation was measured by CFSE dilution **(D)**, and cytokine levels were determined by ELISA assays **(E)**. $^{**}p < 0.01$, compared to the group without DHA. Data are representative of three independent experiments. **(F)** Liver MNCs isolated from WT mice were stimulated with Con A at different concentrations with or without DHA (50 μmol/L) incubation, and the cytokine production was evaluated by ELISA. $^{**}p < 0.01$, compared to the group without DHA. Data shown represent three independent experiments with similar results.

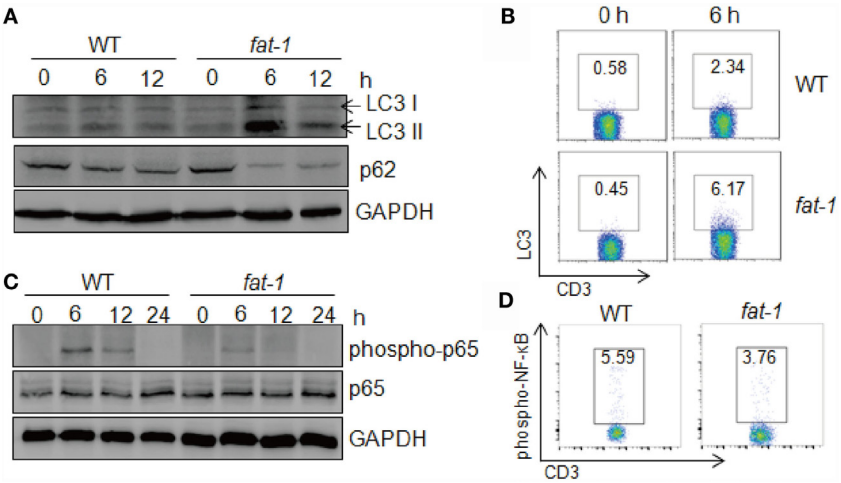

FIGURE 5 | Endogenous n-3 PUFAs enhance autophagy activity in immune cells upon Con A administration. **(A)** Autophagy associated protein LC3 and p62 in liver tissues were analyzed by western blotting analysis at indicated time after Con A (15 mg/kg) injection into WT and *fat-1* transgenic mice ($n = 5$). **(B)** The frequency of LC3-producing hepatic T cells was assessed at 6 h after Con A (15 mg/kg) injection by intercellular staining and FACS analysis. **(C,D)** Reduced NF-κB activation in the livers of Con A-administrated *fat-1* transgenic mice ($n = 5$). Levels of phospho-NF-κB p65 and total NF-κB p65 protein in liver lysates from WT and *fat-1* transgenic mice were examined by immunoblotting at indicated time following Con A (15 mg/kg) injection **(C)**. The activity of NF-κB in hepatic T cells from WT and *fat-1* transgenic mice was evaluated at 6 h after Con A (15 mg/kg) injection by FACS analysis **(D)**. Data are representative of three independent experiments.

uptake and IFN-γ production were reduced in Con A-stimulated liver MNCs from *fat-1* transgenic mice compared to those from WT mice. Moreover, the phosphorylation of STAT1 or STAT3 was much lower in Con A-stimulated liver MNCs derived from *fat-1* transgenic mice than those in WT controls (**Figure 4B**). Besides, the mRNA expression of T-bet and RORγt was greatly suppressed, whereas Foxp3 mRNA expression was increased in the Con A-treated liver MNCs from *fat-1* transgenic mice compared to those from WT mice. In contrast, the GATA3 expression was comparable in both groups with or without Con A treatment (**Figure 4C**).

Furthermore, we stimulated liver MNCs from WT mice with Con A in the absence or presence of DHA, which is a form of n-3 PUFAs. DHA was found strongly to inhibit the T cell proliferation (**Figure 4D**) and cytokines (i.e., IFN-γ and IL-2) production (**Figure 4E**) in a dose-dependent manner. Likewise, we evaluated the function of DHA on hepatic T cells activation induced by different concentration of Con A. The result also showed that DHA has an inhibitory effect on T cell activation, as indicated by decreased secretion of IFN-γ and IL-2 (**Figure 4F**).

n-3 PUFAs Enhance Autophagy Activity in Immune Cells upon Con A Administration

Autophagy serves as a cellular protective mechanism, helping maintain normal cellular functioning and homeostasis (23). In acute liver injury, autophagy plays a protective role, and autophagic cell death occurs in the failure of adaptation (24). To investigate whether autophagy is associated with the protective effect of n-3 PUFAs in Con A-induced hepatitis, we assessed the expression of proteins related to autophagy in the liver post-Con A injection. The result showed that LC3-II level dramatically increased in liver tissue from Con A-treated *fat-1* transgenic mice compared with that from WT mice (**Figure 5A**). Also, the expression of p62 was low in *fat-1* transgenic mice as compared with that in WT mice. Indeed, FACS analysis showed a much higher frequency of LC3 positive T cells in the liver of *fat-1* transgenic mice compared to their WT counterparts (**Figure 5B**). These results suggested that n-3 PUFAs enhanced the autophagy activity in hepatic T cells from mice with Con A treatment. Given the interplay between autophagy process and NF-κB signaling pathway is necessary for the maintenance of cellular homeostasis (25), we examined the NF-κB activity in the liver tissue and hepatic T cell upon Con A stimulation. Reduced phosphorylation of NF-κB p65 was observed in the liver of Con A-treated *fat-1* transgenic mice (**Figure 5C**). Also, NF-κB activity in hepatic T cells from Con A-injected *fat-1* transgenic mice was lower than those from WT counterparts (**Figure 5D**).

Next, we evaluated the autophagy levels in hepatic MNCs stimulated with Con A *in vitro*. Isolated hepatic MNCs and T cells from *fat-1* transgenic mice exhibited higher autophagy activity compared with those from WT mice upon Con A stimulation (**Figures 6A,B**). Additionally, DHA elevated the expression of LC3 in hepatic MNCs and T cells induced by Con A incubation, as showed by immunoblotting and FACS analysis (**Figures 6C,D**). To further define whether n-3 PUFAs directly affect autophagy activity in T cells, hepatic T cells were purified from WT mice and stimulated with Con A *in vitro* in the presence or absence of DHA. The result showed that DHA significantly increased autophagy activity and suppressed NF-κB p65 phosphorylation in Con A-stimulated hepatic T cells (**Figure 6E**). Autophagy has

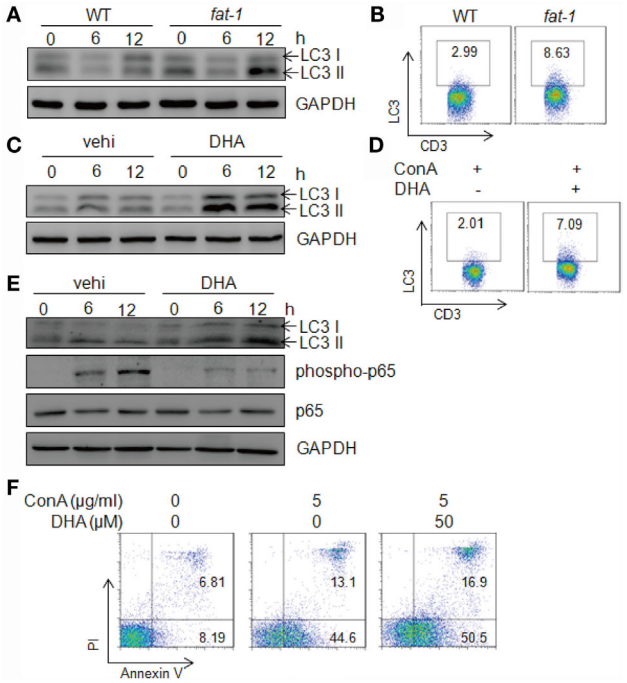

FIGURE 6 | n-3 PUFAs increase autophagy levels in hepatic T cells stimulated with Con A *in vitro*. (A) Autophagy-associated protein LC3 in Con A (5 μg/ml)-stimulated hepatic MNCs from WT and *fat-1* transgenic mice were analyzed by western blotting analysis at indicated time. **(B)** The frequency of LC3-producing hepatic T cells was assessed at 12 h after Con A stimulation by intercellular staining and then FACS analysis. **(C)** Autophagy-associated protein LC3 in Con A (5 μg/ml)-cultured hepatic MNCs in the presence or absence of DHA (50 μmol/L) by western blotting analysis. **(D)** The frequency of LC3-producing hepatic T cells was assessed at 12 h after Con A stimulation with or without DHA treatment by intercellular staining and then FACS analysis. Data shown represent three independent experiments with similar results. **(E)** Autophagy-associated protein LC3 and NF-κB p65 in Con A (5 μg/ml)-stimulated hepatic T cells with or without DHA (50 μmol/L) treatment were analyzed by western blotting analysis at indicated time. Data are representative of three independent experiments. **(F)** Hepatic T cells purified from WT mice were incubated with Con A (5 μg/ml) in the presence or absence of DHA (50 μmol/L) for 24 h. Cells apoptosis were determined by Annexin V/PI staining. Data shown represent three independent experiments with similar results.

also been associated with the regulation of various cell death pathways, most notably apoptosis (26). Thus, we evaluated the effect of n-3 PUFAs on Con A-induced cell apoptosis in hepatic T cells, and the result showed that DHA increased apoptosis of Con A-activated T cells (**Figure 6F**). It indicated that n-3 PUFAs limit T cell immune response at least partially by promoting cell apoptosis of activated T cells.

Inhibition of Autophagy with Chloroquine Abrogates the Protective Function of n-3 PUFAs in Con A-Induced Hepatitis

Chloroquine inhibits autophagy as it raises the lysosomal pH, thus blocking the fusion between autophagosomes and lysosomes (27). To further study the role of autophagy in the protective function of n-3 PUFAs in Con A-induced liver damage, WT and *fat-1* transgenic mice received 40 mg/kg of chloroquine to block autophagy before Con A administration, followed by histological and serum analysis. The severity of liver damage was comparable between Con A-injected WT and *fat-1* transgenic mice with chloroquine pretreatment (**Figure 7A**). Additionally, the sera collected from WT and *fat-1* transgenic mice showed no significant difference in the levels of ALT activity (**Figure 7B**) and pro-inflammatory cytokines (i.e., IFN-γ and TNF-α) (**Figure 7C**). Analysis of hepatic MNCs showed that the frequency and the activation of NKT cells or T cells were similar between WT and *fat-1* transgenic mice upon Con A challenge with autophagy inhibition (**Figure 7D**). The percentage of IFN-γ-producing $CD4^+$ Th1 cells in *fat-1* transgenic mice was almost the same with those in WT mice (**Figure 7E**). Moreover, no significant difference was seen between WT and *fat-1* transgenic mice in the phosphorylation of STAT1 and STAT3 as well as the NF-κB activity (**Figure 7F**). These data suggested that the protective effect of endogenous n-3 PUFAs on Con A-induced hepatitis is dependent on autophagy in regulating T cell activation.

DISCUSSION

Omega-3 polyunsaturated fatty acids have been reported to attenuate various liver diseases and cancer (15–17, 28). However, the function of n-3 PUFAs in autoimmune hepatitis remains unclear. In the present study, we demonstrate that endogenous production of n-3 PUFAs in *fat-1* transgenic mice reduces the Con A-induced T cell-mediated hepatic injury. Our studies revealed the inhibitory effect of endogenous n-3 PUFAs on Con A-induced T cell activation and $CD4^+$ Th1 differentiation. Furthermore, we also provided the evidence that n-3 PUFAs enhanced autophagy activity, resulting in protection from severe liver damage due to the acute overreaction of the immune system.

Previous *in vitro* and *in vivo* studies showed that n-3 PUFAs reduced pro-inflammatory cytokines production secreted from monocytes and macrophages. For example, cell culture studies showed that n-3 PUFAs can suppress the production of TNF-α by MNCs (29) and IL-6 and IL-8 by endothelial cells (9, 30). Schmöcker et al. demonstrated that *fat-1* transgenic mice developed less severe macrophage-mediated liver injury induced by D-GalN/LPS than WT mice, as indicated by decreased plasma TNF-α level, ALT activity, and liver pathology (15). Here, we demonstrated that endogenous n-3 PUFAs also inhibited the pro-inflammatory cytokines produced by activated T cells. These results extended our understanding of the negative regulatory function of n-3 PUFAs on inflammatory responses triggered by various immune cells. It is quite important to notice that the liver macrophages are responsible for the proliferation of $CD4^+$ T cells and generation of IFN-γ-producing Th1 cells in Con A-induced hepatitis (31). Therefore, it is possible that modulation of T cell function *in vivo* partially owing to the effect of n-3 PUFAs on liver macrophages, and further investigations are warranted.

Since T cells activation plays a significant role in Con A-induced hepatitis (18, 21), the amelioration of Con A-induced liver injury in *fat-1* transgenic mice may also be related to the selective inhibition of activated T lymphocytes by n-3 PUFAs.

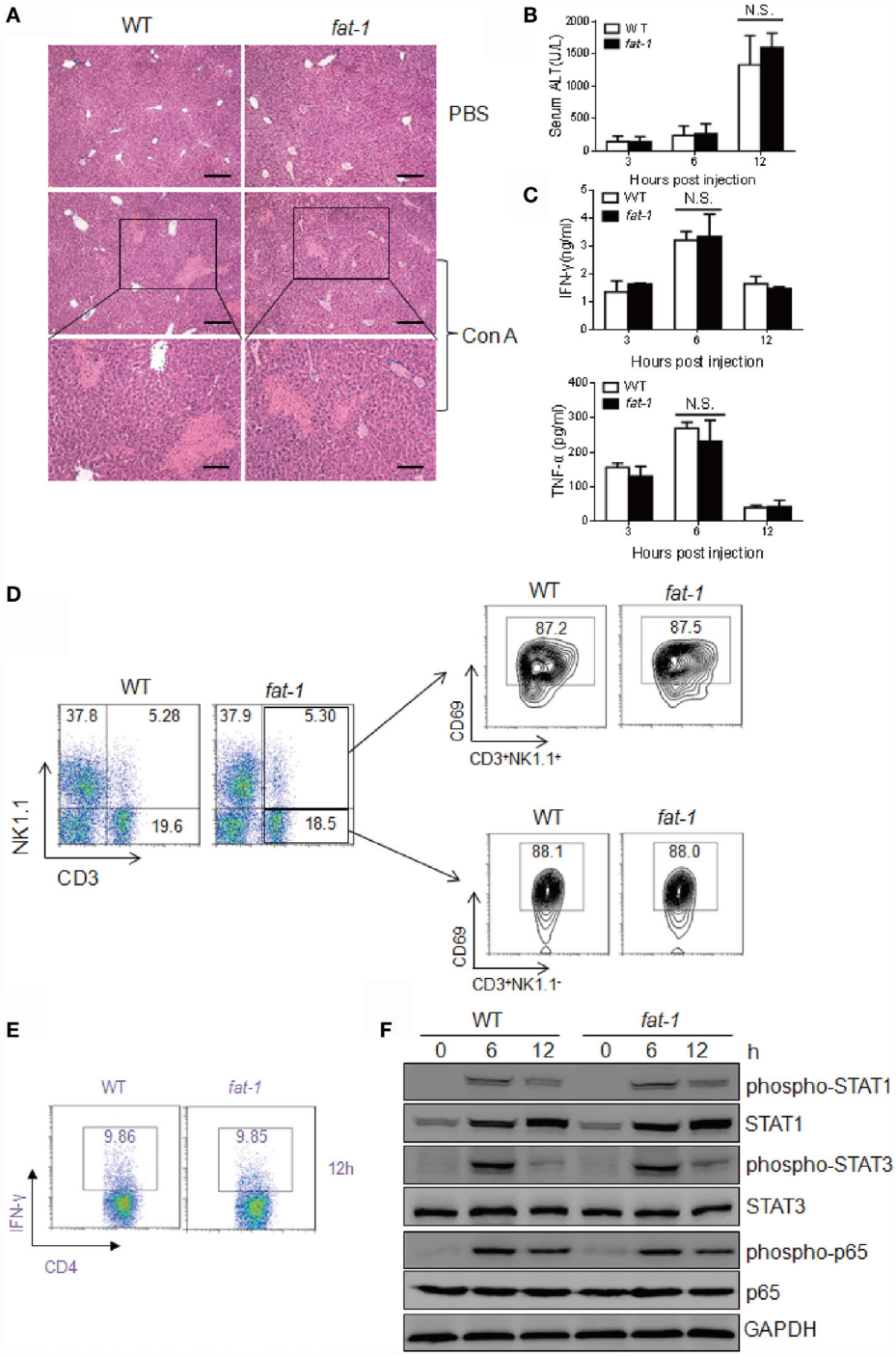

FIGURE 7 | **Chloroquine abolishes the protective function of endogenous n-3 PUFAs in Con A-induced liver injury**. WT and *fat-1* transgenic mice ($n = 5$) received 40 mg/kg body weight of chloroquine by intraperitoneal injection before Con A (15 mg/kg) challenge. **(A–C)** Histology of liver was examined at 24 h following Con A administration. Scale bars = 100 μm. Bottom panels showed the higher-magnification views of the necrotic area. Scale bars = 50 μm **(A)**. Serum ALT **(B)** and cytokine levels **(C)** were evaluated at indicated time. N.S., not significant. One of the three independent experiments is shown. **(D,E)** The percentage of $CD69^+$ T cells and NKT cells **(D)** as well as the frequency of IFN-γ-producing $CD4^+$ T cells **(E)** were assessed at 12 h after Con A injection by FACS analysis of hepatic MNCs. Data shown represent three independent experiments with similar results. **(F)** Levels of phospho-STAT1, phospho-STAT3, phospho-NF-κB p65, STAT1, STAT3, and NF-κB p65 protein in the liver from Con A-administrated mice with chloroquine pretreatment were analyzed using immunoblotting assay at indicated time. One of the two independent experiments is shown.

We determined that endogenous n-3 PUFAs suppressed the activation of T cells and NKT cells during Con A challenge. On autoimmune hepatitis, $CD4^+$ T cells represent the predominant population of T cells infiltrating into the liver, and Th1-like cytokines (e.g., IFN-γ and TNF-α) contribute to hepatic injury (4, 19). It has been noted that n-3 PUFAs supplementation attenuated Th1 cell-mediated delayed type hypersensitivity responses in healthy human volunteers (32). Here, we observed remarkably suppressed polarization of Th1 cells and reduced expression of Th1 transcription factor, T-bet, in liver from Con A-injected *fat-1* transgenic mice. Additionally, we also observed reduced activation of Th17-related STAT3 as well as expression of Th17 transcript factor (RORγt) in liver and decreased IL-17A level in serum from Con A-administrated *fat-1* transgenic mice. A recent study showed that long-term administration of n-3 PUFAs amplified the number of Tregs in the liver, and abundant IL-10 expression in n-3 PUFAs-feed mice might be partially contributing to the generation of hepatic Tregs (33). It is worth noting that the expressions of Treg transcript factor Foxp3 and immunomodulatory cytokine IL-10 were significantly increased in *fat-1* transgenic mice compared to their WT littermates after Con A administration. Our observations support the conclusion that hepatic n-3 PUFAs could influence the differentiation of $CD4^+$ T cells into various effector subsets in the liver during immune-mediated hepatitis, and therefore modulate the pathogenesis of autoimmune and chronic hepatitis.

The autophagy process is often used to eliminate damaged or unwanted organelles and also remove intracellular microbial pathogens (23), which play a significant role in the normal liver physiology, as well as in the pathogenesis of liver disease (24, 34). However, the influence of autophagy in the pathogenesis of T cell-mediated hepatitis remains controversial. In the present study, we provided the evidence that autophagy activity is associated with the function of endogenous n-3 PUFAs in modulating the severity of liver injury induced by Con A. NF-κB signaling, which regulates the transcription of critical effector T cell genes, is crucial for T cell activation (35). Autophagy has been described to downregulate NF-κB activity in effector T cells, *via* degrading the adaptor protein Bcl10, which assembles a signaling complex with Carma-1 and the paracaspase Malt1 for NF-κB activation (36). We found that n-3 PUFAs promoted autophagy activity and inhibited NF-κB p65 phosphorylation in T cells. This effect may be associated with the reduced T cell activation in *fat-1* transgenic mice upon Con A administration. Nevertheless, additional studies are required to elucidate the precise mechanism of autophagy in modulating T cell-NF-κB activity during Con A-induced hepatitis.

Importantly, mammalian target of rapamycin (mTOR) has been identified as a crucial regulator of cell metabolism regulating metabolism and plays a critical role in driving T cell differentiation and function (37). Inhibition of mTOR activity by rapamycin promotes T cell anergy even in the presence of costimulatory activation (38). Emerging evidence showed that increased autophagy is linked to mTOR inhibition in senescent cells (39). The previous study demonstrated that n-3 PUFAs rapidly and efficiently suppressed both mTOR complex 1 (mTORC1) and mTORC2 and their downstream signaling (20). Hence, we speculate that hepatic n-3 PUFAs may regulate mTOR-autophagy axis in the liver during immune-mediated hepatitis.

In summary, we demonstrate a role for endogenous n-3 PUFAs in the alleviation of T cell-mediated hepatic injury. Increased T cell autophagy activity induced by n-3 PUFAs restrains Con A-induced T cell activation and inflammatory liver injury. These findings substantiate the concept that n-3 PUFAs have potential clinical application to attenuate immune-mediated liver injury.

AUTHOR CONTRIBUTIONS

DZ and ZC designed research. YL, YT, SW, Jing Zhou, Jia Zhou, and XL performed the experiments. YL, Jia Zhou, XB, and DZ analyzed the data. DZ, ZC, and X-YW wrote the manuscript.

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A Subset of Patients with Acute Myeloid Leukemia Has Leukemia Cells Characterized by Chemokine Responsiveness and Altered Expression of Transcriptional as well as Angiogenic Regulators

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Acute myeloid leukemia (AML) is an aggressive and heterogeneous bone marrow malignancy, the only curative treatment being intensive chemotherapy eventually in combination with allogeneic stem cell transplantation. Both the AML and their neighboring stromal cells show constitutive chemokine release, but chemokines seem to function as regulators of AML cell proliferation only for a subset of patients. Chemokine targeting is therefore considered not only for immunosuppression in allotransplanted patients but also as a possible antileukemic strategy in combination with intensive chemotherapy or as part of disease-stabilizing treatment at least for the subset of patients with chemokine-responsive AML cells. In this study, we characterized more in detail the leukemia cell phenotype of the chemokine-responsive patients. We investigated primary AML cells derived from 79 unselected patients. Standardized *in vitro* suspension cultures were used to investigate AML cell proliferation, and global gene expression profiles were compared for chemokine responders and non-responders identified through the proliferation assays. CCL28-induced growth modulation was used as marker of chemokine responsiveness, and 38 patients were then classified as chemokine-responsive. The effects of exogenous CCL28 (growth inhibition/enhancement/no effect) thus differed among patients and was also dependent on the presence of exogenous hematopoietic growth factors as well as constitutive AML cell cytokine release. The effect of CCR1 inhibition in the presence of chemokine-secreting mesenchymal stem cells also differed among patients. Chemokine-responsive AML cells showed altered expression of genes important for (i) epigenetic transcriptional regulation, particularly lysine acetylation; (ii) helicase activity, especially DExD/H RNA helicases; and (iii) angioregulatory proteins important for integrin binding. Thus, chemokine responsiveness is part of a complex

Abbreviations: AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; cpm, counts per minute; ELISA, enzyme-linked immunosorbent assay; FAB, French–American–British; Flt3, FMS-like tyrosine kinase 3; Flt3L, Flt3 ligand; GM-CSF, granulocyte macrophage colony-stimulating factor; GO, gene ontology; GPCR, G-protein-coupled receptor; GVHD, graft vs. host disease; NPM1, nucleophosmin; SCF, stem cell factor; SNF/SW1, sucrose non-fermentable/SWItch.

AML cell phenotype with regard to extracellular communication and transcriptional regulation. Chemokine targeting in chemokine-responsive patients may thereby alter AML cell trafficking and increase their susceptibility toward antileukemic treatment, e.g., conventional chemotherapy or targeting of other phenotypic characteristics of the chemokine-responsive cells.

Keywords: acute myeloid leukemia, chemokine, CCL28, integrin, RNA, epigenetic, helicase

INTRODUCTION

Acute myeloid leukemia (AML) is an aggressive disease characterized by infiltration of malignant myeloblasts in the bone marrow; the only curative treatment is intensive chemotherapy potentially in combination with allogeneic stem cell transplantation (1). However, such intensive treatment is not applicable for elderly or unfit younger patients due to an unacceptable risk of severe toxicity and treatment-related mortality; immunological complications are important causes of the transplant-related mortality that can be due to acute graft vs. host disease (GVHD) as well as severe infections due to the immunocompromised state of patients with chronic GVHD (1).

Several strategies for targeted therapy in AML are now considered (2, 3). One possibility is to target the AML-supporting communication between AML cells and their neighboring stromal cells that mediate growth support through their release of soluble mediators, including several chemokines (4–11). Chemokines are involved in this communication; they may function as growth regulators or be important for keeping the leukemic cells in their permissive microenvironment and thereby render them less susceptible to antileukemic therapy. However, *in vitro* studies suggest that chemokines function as growth regulators in leukemic hematopoiesis only for a subset of AML patients, and a wide range of both CCL and CXCL chemokines can then modulate leukemia cell proliferation (4). One of these chemokines is CCL28 (4) that is released by non-leukemic bone marrow stromal cells, and that preserves the functional integrity of normal hematopoietic progenitor cells (12) through binding to the G-protein-coupled receptors (GPCRs) CCR3 and CCR10 (13–15). CCR3 is a promiscuous receptor, which can bind several ligands in addition to CCL28, whereas CCR10 can only bind CCL27 and CCL28 (16).

Our previous studies have identified a subset of patients whose AML cells show altered proliferation in the presence of exogenous chemokines, and the aim of the present study was to give a broader and more detailed characterization of the AML cell phenotype for these chemokine-responsive patients. First, chemokine-responsive patients show growth modulation in the presence of several chemokines, including CCL28. We therefore used CCL28 responsiveness to identify the chemokine-responsive subset among 79 unselected patients, and because CCL28 is important in normal hematopoiesis, we in addition wanted to characterize both the effects of exogenous CCL28 and chemokine receptor inhibition in leukemic hematopoiesis as parts of our phenotype studies. Second, the phenotype of the chemokine-responsive patient subset was further characterized by comparison of global gene expression profiles for chemokine-responsive and non-responsive patients. A more detailed characterization of this phenotype would be necessary in order to design clinical studies and decide optimal clinical use of targeted therapy in this subset of AML patients.

MATERIALS AND METHODS

AML Patients and AML Cell Preparation

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the local Ethics Committee (Regional Ethics Committee III, University of Bergen). Samples were collected after written informed consent. AML blasts were derived from 79 consecutive patients (34 females and 45 males; median age 67 years with range 18–87 years). Six patients had AML relapse (**Table 1**) and 11 patients had acquired AML secondary to previous hematological disease (10 patients) or chemotherapy (1 patient). Cytogenetic analyses were available for 71 patients; 9 patients had favorable, 6 patients intermediate, 15 patients adverse, and 41 patients normal cytogenetics, respectively. Our selection of patients and the methods for preparation (gradient separation alone) and characterization of AML cells have been described in detail previously (17).

Prior to the study, we decided to include 80 unselected patients (one patient was left out due to technical reasons); based on a previous study (4), we would then expect to identify at least 25 chemokine responders. This number would be sufficient for statistical comparisons of functional characteristics as well as global gene expression profiles.

The study included only patients with a high percentage (>80%) and a high absolute number ($>15 \times 10^9$/L) of leukemia cells among the peripheral blood leukocytes (18). A cell population, including >95% AML cells, could then be prepared by density gradient separation alone (19). Cells were cryopreserved and stored in liquid nitrogen until used in the experiments (19).

Functional *In Vitro* Characterization of Primary Human AML Cells

All cultures of AML cell alone were prepared in serum-free medium (Stem Span, Stem Cell Technologies, Vancouver, BC, Canada), and all recombinant cytokines were supplied by PeproTech (Rocky Hill, NJ, USA). All exogenous cytokines were added at 20 ng/mL, i.e., corresponding to an excess of the added cytokine. Our methods for flow-cytometric characterization of AML cell viability (20), spontaneous and cytokine-dependent proliferation in suspension cultures determined by ^{3}H-thymidine incorporation (4, 17), constitutive cytokine release (4), and analysis of AML cell viability and proliferation (^{3}H-thymidine

TABLE 1 | Clinical and biological characteristics of the 79 unselected patients admitted to our hospital for AML treatment and included in the present study.

Patient characteristics		Cell morphology		Cell genetics	
Age		FAB classification		Cytogenetics[a]	
Median (years)	67	M0	6	Favorable	9
Range (years)	18–87	M1	21	Intermediate	6
		M2	11	Normal	41
Gender		M3	2	Adverse	15
Females	34	M4	18	n.d.	8
Males	45	M5	15		
		n.d.	6	Flt3 mutations	
Secondary AML				ITD[b]	28
MDS	7	CD34 receptor		Wild-type	37
Chemotherapy	1	Negative (≤20%)	21	n.d.	14
CM(M)L	3	Positive (>20%)	53		
		n.d.	5	NPM1 mutations	
AML relapse	6			Mutated	26
				Wild-type	40
				n.d.	13

[a]*One of the patients had cytogenetic abnormalities with different prognostic impact; inv(16) is associated with a favorable prognosis and +8 with an adverse prognosis. Inv(16) is regarded to have the strongest impact and therefore to neutralize the negative impact of +8, the patient was classified as having a favorable karyotype.*

[b]*One of the patients has an additional point mutation at D835.*

n.d., not determined.

incorporation) in transwell cocultures with bone marrow mesenchymal stromal cells [(MSC); MSC24539 purchased from Lonza, Cambrex BioScience, Walkersville, MD, USA] (8, 9) have been described in detail previously. CCL28 levels were determined by enzyme-linked immunosorbent assay (ELISA) analysis (R&D Systems, Abingdon, UK), the minimal detectable level being 45 pg/mL. CXCL2 levels were also determined by ELISA analysis, whereas the levels of the other chemokines were investigated by Luminex analyses (R&D Systems). The combined CCR1 and CCR3 antagonist J113863 (R&D Systems) was used at a final concentration of 1.5 μM for chemokine receptor inhibition.

RNA Preparation and Analysis of Global Gene Expression

All microarray data were performed using the Illumina iScan Reader, which is based upon fluorescent detection of biotin-labeled cRNA; 300 ng total RNA from each sample was reversibly transcribed, amplified, and labeled with Biotin-16-UTP using the Illumina TotalPrep RNA Amplification Kit (Applied Biosystems/Ambion, Foster City, CA, USA). Amount and quality of the biotin-labeled cRNA were controlled both by NanoDrop spectrophotometer and Agilent 2100 Bioanalyzer, before 750 ng of biotin-labeled cRNA was hybridized to the HumanHT-12 V4 Expression BeadChip according to the manufacturer's instructions. The chip targets 47,231 probes derived primarily from genes in the NCBI RefSeq database (Release 38).

Bioinformatical and Statistical Analyses

Bioinformatical analyses were performed using the J-Express 2012 software (MolMine AS, Bergen, Norway). For hierarchical clustering of the cytokine secretion, all values were median-normalized and log(10)-transformed. Complete linkage and Pearson correlation were used as linkage method and distance measurement, respectively. The statistical analyses were performed with the IBM Statistical Package for the Social Sciences (SPSS) version 23 (Chicago, IL, USA). The Mann–Whitney *U*-test was used to compare value distributions among different patient groups, and χ^2 tests (Pearson's χ^2 test and the likelihood ratio) were used to determine correlations between different categories. Additionally, Kendall's tau-*b* correlation tests were performed to determine the agreement between cytokine expression levels in cell supernatants and the mRNA levels of these cytokines. *P*-values <0.05 were regarded as statistically significant.

RESULTS

Identification of a Chemokine-Responsive Patient Subset: Exogenous CCL28 Does Not Alter Spontaneous *In Vitro* Apoptosis but Modulates AML Cell Proliferation for a Subset of Patients

There was a wide variation among the 79 AML patients with regard to the viability of the leukemic cells after 40 h of *in vitro* culture in medium alone (median viability 36%, range 2–81%). The presence of 20 ng/mL exogenous CCL28 during culture did not significantly alter AML cell viability (median viability 35%, range 3–78%) when comparing the overall results (data not shown). Thus, exogenous CCL28 does not have any major effect on the regulation of spontaneous *in vitro* apoptosis for primary human AML cells.

Proliferation was analyzed using the ^{3}H-thymidine incorporation assay, where the nuclide was added after 6 days and nuclear activity assayed 24 h later. A median value of at least 1,000 counts per minute (cpm) in triplicate cultures was defined as *detectable proliferation*, whereas a *significant alteration* of ^{3}H-thymidine incorporation was defined as (i) an increase/decrease corresponding to at least 20% of the control culture, and the absolute value of this change being at least 2,000 cpm for patients with detectable proliferation or alternatively, (ii) a change from detectable to undetectable proliferation or *vice versa* (4, 17). We first compared AML cell proliferation for cultures prepared in medium alone and medium supplemented with 20 ng/mL of CCL28 for all 79 patients. The proliferation in medium alone varied among the 79 patients (range <1,000–9,191 cpm), and only 17 patients showed detectable autocrine (i.e., spontaneous) proliferation. Exogenous CCL28 increased the proliferation for 14 of the 79 patients compared with the corresponding control cultures prepared in medium alone, whereas decreased proliferation was seen for 3 patients.

The Chemokine-Responsive AML Cell Phenotype: CCL28-Associated Growth Modulation Depends On the Local Cytokine Network

We investigated the effect of CCL28 on growth factor-dependent proliferation of primary human AML cells for 56 patients that represent a consecutive and thereby unselected subset of the 79

patients examined in the autocrine proliferation studies described above. The effect of CCL28 was tested in suspension cultures prepared either in medium alone or medium supplemented with exogenous granulocyte macrophage colony-stimulating factor (GM-CSF), stem cell factor (SCF), or FMS-like tyrosine kinase 3 ligand (Flt3L). A detectable proliferative response for at least one of these three growth factors was detected for 42 of the 56 patients, and the results for these patients are summarized in the hierarchical cluster analysis presented in **Figure 1** (the analysis also includes the effect of CCL28 for cells cultured in medium alone, see above). We defined a significant alteration as a difference between the CCL28-containing and the corresponding control culture as either (i) a change from/to undetectable levels or (ii) a difference corresponding to an absolute value of at least 2,000 cpm and in addition corresponding to >20% of the respective control culture. It can be seen that exogenous CCL28 had divergent effects on AML cell proliferation depending on the local cytokine network. When using these definitions, we observed a significant growth inhibition for 21 and a growth enhancement for 3 out of the 42 patients in the presence of GM-CSF. In contrast, in the presence of Flt3L, significant growth enhancement was seen for 11 patients, and growth inhibition was observed for 3 patients. Finally, CCL28 had generally weaker effects in the presence of SCF; significant inhibition was seen only for six patients and enhancement for two patients. To summarize, CCL28 can alter the proliferation of primary human AML cells for a considerable number of patients, this effect is highly dependent on the local cytokine network and the presence of hematopoietic growth factors, and exogenous CCL28 altered either autocrine or cytokine-dependent (GM-CSF, Flt3L, and SCF) proliferation for 36 of the 56 patients.

CCL28 can bind to the CCR3 and CCR10 receptors (13–15). We used our global gene expression profiles (see below) to compare the expression of CCR3 and CCR10, but we could not detect any significant difference in receptor expression between patients with and without CCL28-associated growth modulation (data not shown). These observations suggest that the differences in chemokine responsiveness between patients is not caused by different regulation of CCR3/CCR10 expression at the mRNA level.

Additional Phenotypic Characteristics of Chemokine-Responsive AML Cells: Constitutive CCL28 Release by AML Cells Is Detected for a Minority of Patients, Showing Weak Association with the Release of Other Chemokines and No Association with Chemokine Responsiveness

The constitutive release of 11 chemokines was investigated for all 79 patients (2×10^6 cells/mL, 48 h of *in vitro* culture). Detectable CCL28 release was observed for only 18 patients (median supernatant level 126 pg/mL, range 49–812 pg/mL), whereas the other chemokines were released for most patients (**Table 2**). When comparing the overall results, no significant correlation was seen between detectable CCL28 release and cell viability (i.e., spontaneous *in vitro* apoptosis), differentiation [morphological signs

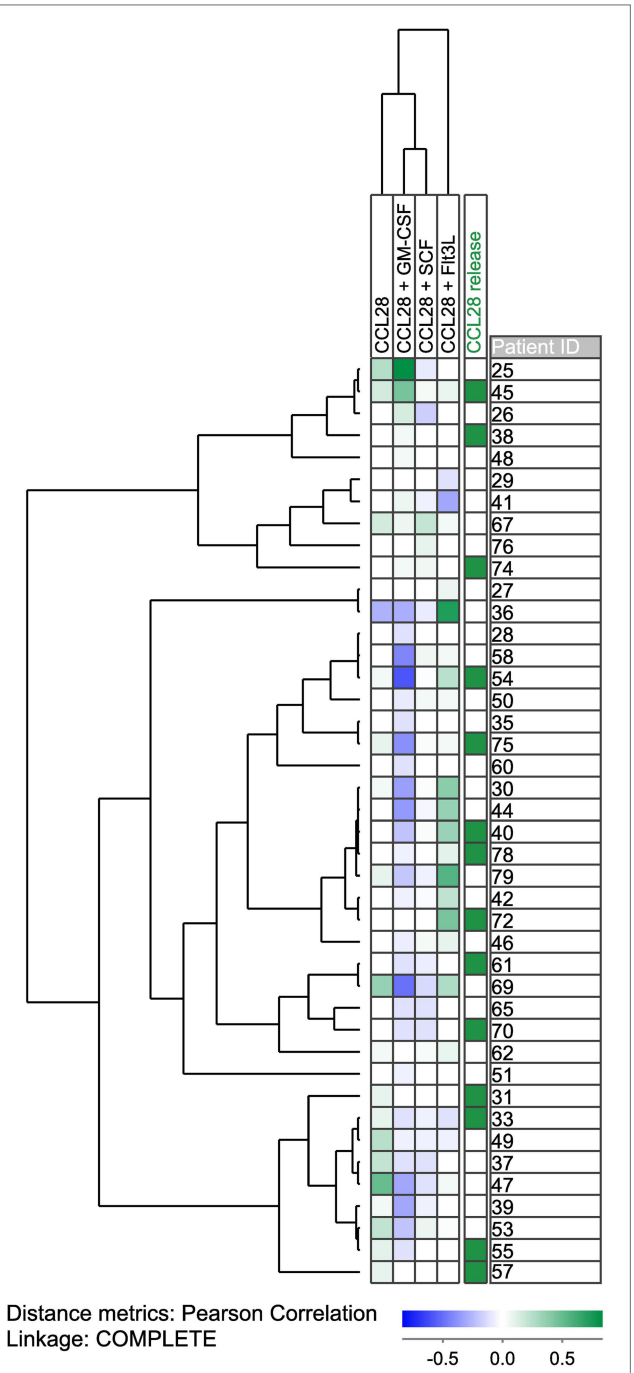

FIGURE 1 | CCL28-associated growth modulation in primary human AML cells; hierarchical clustering analysis of the effects of exogenous CCL28 on the spontaneous and GM-CSF/SCF/Flt3L-dependent proliferation of AML blasts derived from 56 unselected patients. AML cells were cultured in medium alone or medium supplemented with exogenous GM-CSF, SCF, or Flt3L; for all four types of culture, we compared proliferation for cells cultured with and without exogenous CCL28. All cytokines were tested at a final concentration of 20 ng/mL. For 14 patients, undetectable proliferation (corresponding to a median incorporation of <1,000 cpm for triplicate determinations) was detected for all cultures; for this reason, we only present the results for the remaining 42 patients. The leukemic cells were cultured in suspension cultures, and the proliferation was measured as ^{3}H-thymidine incorporation after 7 days of culture.

TABLE 2 | Constitutive chemokine release by primary human AML cells; a summary of the results for the 79 unselected patients included in the present study.

Chemokine	# patients with detectable release	Median conc. (pg/mL)	Range (pg/mL)
CCL2	67	110	n.d.–9,807
CCL3	79	200	119 to >30,000
CCL4	79	92	21–11,744
CCL5	79	39	7.4–2,481
CCL28	*18*	*n.d.*	*n.d.–812*
CXCL1	79	79	41–22,772
CXCL2	69	16	n.d.–11,594
CXCL5	78	95	n.d. to >14,500
CXCL8	79	535	0.3 to >18,500
CXCL10	79	12	1.4–24,642
CXCL11	79	83	25–246

The levels were determined in the culture supernatants after 48 h of in vitro culture for the primary AML cells, the supernatant levels of 11 chemokines were determined, and the results are presented as the number of patients with detectable release, the median level for all 79 patients, and the variation range. CCL28 is highlighted.
n.d., not detected, i.e., below detection limit.

according to French–American–British (FAB) classification, expression of the CD34 stem cell marker], karyotype, and *Flt3* or *NPM1* mutations (data not shown). Finally, there was no significant association between constitutive CCL28 release and spontaneous/autocrine *in vitro* AML cell proliferation, and constitutive CCL28 release was seen both for patients with and without CCL28-associated growth modulation.

We have previously investigated the constitutive chemokine release for a consecutive patient group, and in the present study, we also determined the supernatant levels for 10 additional chemokines commonly released by primary human AML cells at relatively high levels (79 patients). The chemokine levels for each individual patient was median-normalized and log(10)-transformed prior to Pearson clustering in J-Express. The analysis is presented in **Figure 2**. Several chemokines were then grouped together in defined subsets. Our previous studies have shown that there are three different chemokine subsets characterized by close clustering and significant correlations between supernatant levels (4); this was also confirmed by our present clustering analysis of constitutive chemokine release: (i) CXCL10/11 clustered close to CCL5; (ii) CCL2/CXCL1/5/8 clustered close to each other; whereas (iii) CXCL2 was the only chemokine tested out of the five members of the third cluster and did not cluster close to CCL28 (4). CCL28 as well as CCL5 clustered as outliers without any strong association with other members of the main chemokine clusters.

It can be seen from **Figure 2** that the patients could be divided into four main subsets/clusters; the two upper subsets generally showed high constitutive chemokine release. CCL28-induced growth inhibition for cultures prepared with GM-CSF and SCF was significantly more frequent in this high chemokine release group (15 and 6 out of the upper 42 patients, respectively) compared with the low-release group (6/37 and 0/37; likelihood ratios = 0.023 and 0.003, respectively). This observation further supports our hypothesis from the studies of CCL28 effects in the presence of exogenous cytokines (GM-CSF, Flt3L, and SCF, see above); the final effects of CCL28 on AML cell proliferation depends on the local cytokine network determined by the constitutive cytokine release by the leukemic cells (**Figure 2**) and exogenous cytokines (i.e., cytokines not released by AML cells).

Additional Phenotypic Characterization of AML Cells: Normal Blood Cell Counts at Diagnosis Do Not Differ among Patients with and without Constitutive CCL28 Release

Bone marrow failure with pancytopenia in peripheral blood is common at the time of diagnosis for AML patients (1). Furthermore, CCL28 is a growth and survival factor for normal hematopoietic cells (12), and for this reason we compared peripheral blood hemoglobin levels and platelet counts at the time of diagnosis for patients with and without constitutive CCL28 release by their AML cells. We could not detect any significant difference between the two groups with regard to Hb levels or platelet counts at the time of diagnosis (data not shown).

Additional Phenotypic Characterization of Chemokine-Responsive Patients: Chemokine Receptor Blocking Has Antileukemic Effects Only for Certain Patients

We investigated the effects of endogenous chemokines using an *in vitro* model, where primary human AML cells and normal bone marrow MSCs derived from a healthy donor were separated by a semipermeable membrane during coculture (9); the MSCs can then influence AML cell proliferation only through their release of soluble mediators. We tested the effects of the combined CCR1 (ligands being CCL2/3/5/7/14/15/16/23/28) and CCR3 (ligands being CCL5/7/8/11/13/15/24/28) antagonist J113863 on AML cell viability and proliferation; viability was investigated by a flow-cytometric assay and proliferation as ^{3}H-thymidine incorporation (8, 9). We investigated this pharmacological effect for primary AML cells derived from five CCL28/chemokine responders. The inhibitor had divergent effects on AML cell proliferation during coculture with chemokine-releasing MSCs; for one patient, growth inhibition was seen (median proliferation corresponding to 9,457 vs. 3,502 cpm), for three other patients, increased proliferation was observed (21,323 vs. 26,253 cpm, 10,934 vs. 19,713 cpm, and 55,257 vs. 74,956 cpm, respectively), and for the last patient, only a minor effect of CCL28 was seen (4,800 vs. 4,392 cpm) in the cocultures. These experiments show that chemokines mediate divergent effects on AML cell proliferation that differs among the chemokine responders also, when tested in the presence of cytokine/chemokine-releasing bone marrow MSCs.

Additional Phenotypic Characterization of Chemokine-Responsive Patients: Chemokine Responsiveness Is Associated with Altered Expression of Genes Important for Protein Acetylation, Helicase Activity, and Integrin Binding

In a previous study, we observed that CCL28 could modulate AML cell proliferation only for a subset of patients (4); this was

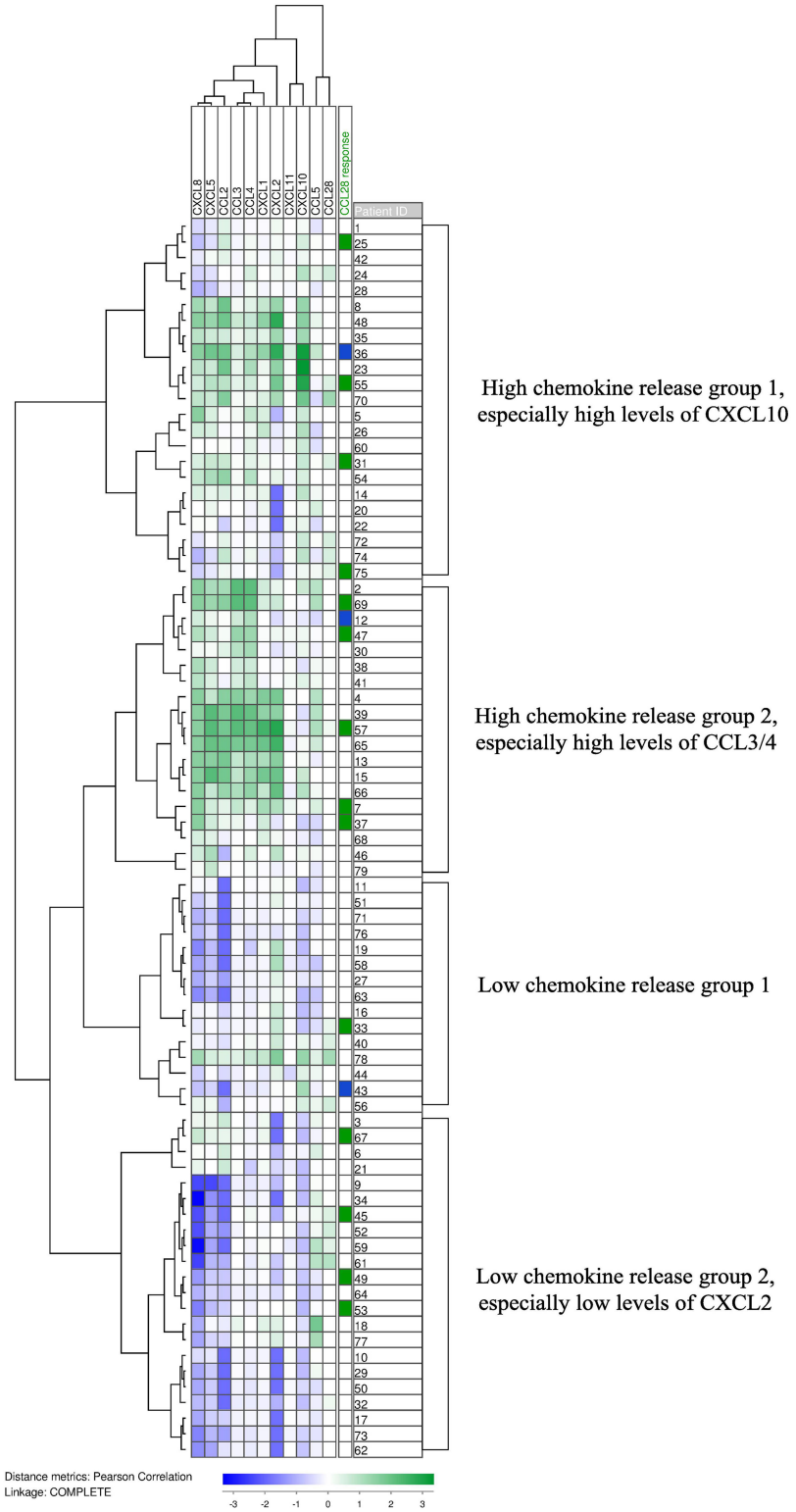

FIGURE 2 | **The constitutive chemokine release profile by primary human AML cells**. The constitutive release of 11 chemokines, including CCL28, during *in vitro* culture was examined for primary human AML cells derived from 79 consecutive patients. Chemokine release was determined as the protein level in culture supernatants. Based on the hierarchical clustering analysis, the patients could be divided into four subclusters. CCL28, together with CCL5, formed an out-group and did not correlate with the expression of the other nine chemokines. The column on the right shows the 14 patients whose cells responded with increased proliferation to CCL28 (green) in medium alone without supplementation of exogenous growth factors, and the 3 patients whose blasts showed reduced proliferation in the presence of CCL28 compared to cultures containing only medium (blue).

also confirmed in our present study (**Figure 1**). The previous study showed that for this particular patient subset, AML cell proliferation could be modulated not only by CCL28 but also by several other chemokines binding to different chemokine receptors (4). Thus, CCL28 growth modulation should be regarded as a marker of a patient subset that, in contrast to other patients, are characterized by chemokine-induced (GPCR-mediated) growth modulation. We then compared the global gene expression profiles for 37 unselected patients; 15 of these patients being classified as responders, according to the definitions used above for the effects of exogenous CCL28 on spontaneous and/or growth factor-dependent (GM-CSF, Flt3L, and SCF) proliferation. However, with regard to the classification of patients as responders/non-responders and the change to/from undetectable from/to detectable proliferation (i.e., proliferation corresponding to >1,000 cpm defined as detectable, see above), this definition is arbitrary and four of the seven patients with such a change showed a relatively small absolute difference between cultures with and without exogenous CCL28. Even though there was a change from undetectable to detectable with CCL28, two of these four patients therefore were by chance classified as responders and the two others as non-responders.

We first identified those genes showing a difference between the two patient subsets corresponding to $p < 0.001$ and a false discovery rate (FDR) of <0.01. The identified genes (21) were included in 20 gene ontology (GO)-terms; a major part of these terms were associated with integrin binding (**Table 3**, Table S1 in Supplementary Material), protein/lysine/histone acetylation (**Table 4**, Table S2 in Supplementary Material), or helicase activity (**Table 5**, Table S3 in Supplementary Material). The term integrin binding included 27 differentially expressed genes; among them were several proteases and angioregulatory mediators, seven genes encoding extracellular matrix molecules, and several adhesion molecules. The large part of the proteins encoded by these genes seems to play a role in the regulation of local angiogenesis, a process important both for the development and chemosensitivity of human AML (8). Integrins may also be important for modulation of intracellular signaling initiated by ligation of the CCR3 chemokine receptor (22). Second, the terms histone acetylation, lysine-protein acetylation, and internal peptidyl lysine acetylation were overlapping and included 48 genes; a large number of them are involved in histone modulation/acetylation and/or in transcriptional regulation (**Table 4**, Table S2 in Supplementary Material). Finally, the largest group of differentially regulated genes were included in the GO term Helicase Activity (**Table 5**, Table S3 in Supplementary Material). This group included mainly RNA helicases (e.g., 23 members of the DEAD box family RNA helicases together with several DEAH box family RNA helicases), mini-chromosome maintenance proteins, and members of the sucrose non-fermentable/SWItch (SNF/SW1) family.

TABLE 3 | Comparison of global gene expression profiles for primary human AML cells with and without chemokine-induced growth modulation – a summary of the differentially expressed genes encoding proteins important for integrin binding (for additional details, see Table S1 in Supplementary Material).

Classification of genes based on the protein function	Angioregulation vascular biology
Proteases	
ADAM2, a disintegrin and metallopeptidase (ADAM) domain 2	+
ADAM23, ADAM metallopeptidase domain 23	+
ADAM22, ADAM metallopeptidae domain 22	+
ADAMTS8, ADAM metallopeptidase with thrombospondin type 1 motif 8	+
Extracellular matrix molecules	
COL3A1, collagen type III alpha 1	+
COL5A1, collagen type V alpha 1 (vascular stability)	
COL16A1, collagen type XVI alpha 1	
FBLN5, fibulin 5	+
LAMA5, laminin alpha 5	+
LAMB2, laminin beta 2 (angioregulator)	+
TNN, tenascin N	+
Soluble mediators involved in angiogenesis	
ANGPTL1, angiopoietin-like 1	+
CYR61, cysteine-rich angiogenic inducer 61	+
JAM3, junctional adhesion molecule 3 (the soluble form)	
VWF, von Willebrand factor	+
Cell-to-cell and cell-to-matrix adhesion	
ICAM3, intercellular adhesion molecule 3 (LFA-1 ligand)	+
ITGB1BP1, integrin beta 1 binding protein 1	+
ITGB6, integrin beta 6	+
JAM3, junctional adhesion molecule 3	+
THBS4, thrombospondin 4	+
THY1, Thy-1 cell surface antigen	+
Other genes encoding proteins involved in angiogenes	
EMP2, epithelial membrane protein 2 (integrin modulator)	+
KDR, kinase insert domain receptor (VEGF receptor)	+
NMB (neuromedin B)	+
SOD1, superoxide dismutase 1, soluble	+
Other genes	
IMPAD1, inositole monophosphatase domain containing 1	
OXCT1, 3-oxoacid transferase 1 (mitchondrial metabolism)	

DISCUSSION

Several recent studies have emphasized the importance of investigating the cancer cell phenotype for understanding carcinogenesis, defining patient subsets in heterogeneous malignancies, and identifying possible therapeutic targets (23–25). The aim of the present study was therefore to characterize the AML cell phenotype more in detail for the subset of patients having chemokine-responsive leukemic cells. Our present observations suggest that these patients respond to exogenous chemokines and combination of chemokine/chemokine receptor targeting either with other targeted therapies (e.g., epigenetic or integrin-targeting) or conventional chemotherapy may therefore be considered especially for the identified patient subset.

Chemokine receptor blockers can be used as an immunosuppressive strategy, e.g., as GVHD therapy in AML patients receiving allogeneic stem cell transplantation (21). Chemokine targeting may thus be used in these patients (i) as a pre-/posttransplant antileukemic strategy with direct effects on the *leukemic* cells at least for patients with chemokine-responsive cells or (ii) as an immunosuppressive treatment. GVHD has antileukemic effects, and immunosuppression due to severe GVHD will reduce this effect. However, at least for patients with chemokine-responsive

TABLE 4 | A comparison of the global gene expression profiling of primary human AML cells with and without chemokine-induced growth modulation – a summary of the differentially expressed genes that encode proteins important for histone acetylation (for additional details, see Table S2 in Supplementary Material).

Main classification based on the protein function	Gene identity
Chromatin modulation/histone acetylation and transcriptional regulation	ACTL6A, CHD9, DMAP1, EPC1, ING3, KAT2A, KAT2B, KIAA1267, MEAF6, MLL, MYST1, MYST2, MYST4, SRCAP, SMARCA4, TRRAP, YEATS2
Transcriptional regulation only	BRD8, BRPF1, CHD9, GTF3C4, EP400, HCFC1, LDB1, MECP2, MYOD1, OGT, PHD15, PHD16, PHF17, PHF20, TAF1, TAF1L, TAF6L, TAF15, TCF3
DNA repair	BRCA2, HCFC1, PHF20, TRRAP
Cell cycle regulation	HCFC20
Genes with other or unknown functions	BAT3 (apoptosis), CCDC101, CPA3 (protease), KIAA1310, MBIP, MSL2, USP22

TABLE 5 | Comparison of global gene expression profiles of primary human AML cells with and without chemokine-induced growth modulation – a summary of the differentially expressed genes encoding proteins important for helicase activity.

Classification of genes based on the protein structure and/or function	DNA	RNA	Trans.
DEAD box family of proteins (RNA helicases)			
DDX4, DDX10 (involved in leukemogenesis?), DDX17, DDX18 (activated by Myc)		+	
DDX27/50/51/52/54/58/60/60L			
DOX19A		+	
IFIH1		+	
DDX3X/6/28		+	+
DEAH box family of proteins			
DHX8/9/40		+	
DHX29			
WRN (DNA repair?)	+		
Mini-chromosome maintenance proteins (genome replication)			
MCMDC1 (chromatin modulation)			
MCM7/9			
MCM8	+		
SNF/SW1 family of protein (chromatin remodeling)			
ATRX			+
HLTF			+
SMARCA2, SMARCA4, SMARCAL1			+
Other helicases			
CHD3 (histone deacetylation)			+
CHD4 (histone deacetylation)	+		+
CHD6 (chromatin remodeling)			+
CHD8 (chromatin remodeling)	+		+
CHD9	+		
MOV10		+	
HELZ		+	
Proteins involved in DNA methylation, chromatin remodeling, and DNA repair			
ASCC3 (DNA repair)			
ERCC8 (DNA repair), SHPRH (DNA repair?), SRCAP (histone remodeling)			+
Other genes/proteins			
EIF4A1			
EP400 (protein synthesis?)			
GTF2F2			+
JARID2 (mutations associated with myeloid malignancies)			+
PRIC285			
RAD54L2			
TDRD12			
YTHDC2			
ZRANB3 (DNA repair?)			

Additional characteristics that possibly are important for leukemogenesis are given in parenthesis at the end of the functional description (for additional details, see Table S3 in Supplementary Material). To the right in the figure is indicated whether the proteins are important for the function of DNA, RNA function/metabolism, or transcriptional regulation.

AML cells, the use of chemokine targeting may represent a therapeutic alternative that reduces the GVHD-associated antileukemic reactivity but at the same time has an additional antileukemic effect by itself against residual AML.

Acute myeloid leukemia is a heterogeneous and aggressive disease, and in a previous study of another smaller patient population, we observed that exogenous CCL28 could modulate cytokine-dependent AML cell proliferation for a minority of patients. For this patient subset, CCL28 was one out of several chemokines with growth-modulating effect (4). Several chemokine receptor inhibitors are now available, and this strategy is considered in the treatment of AML both as an AML-directed antileukemic treatment or as an immunosuppressive strategy for patients with immune-mediated complications following allogeneic stem cell transplantation (21). In the present article, we have studied a large group of patients more in detail, and for these patients, the growth modulation seems to be a part of a wider phenotype that also includes altered epigenetic/transcriptional regulation, RNA metabolism/splicing, and local regulation of angiogenesis. Many chemokines and chemokine receptors show promiscuous receptor/ligand binding, and targeting of single chemokines may therefore have limited effect because a wide range of chemokines are present in the bone marrow microenvironment (9). Combined targeting with different GPCR antagonists or combination with agents that target other aspects of this complex phenotype may then be considered for this particular subset of patients.

CCL28 is a chemokine involved in normal hematopoiesis (12). We now describe that it can also be involved in leukemic hematopoiesis and modulate the growth of primary human AML cells for a subset of patients. However, its final effect depends on the local cytokine network. Growth inhibition was observed in the presence of the exogenous cytokines, SCF and especially GM-CSF, whereas growth enhancement was seen in the presence of exogenous Flt3L. Finally, CCL28 can be constitutively released by primary human AML cells and thereby be a part of the cross talk between leukemic cells and their neighboring bone marrow cells, but the ability of constitutive CCL28 release showed no association with autocrine proliferation or the effects of exogenous CCL28.

We included 79 patients in the present study, and they represent a consecutive and thereby unselected group, except that all of them showed relatively high levels of circulating AML cells. This strategy was used to allow a standardized preparation of highly enriched AML cells by gradient separation alone, thereby avoiding the risk of inducing functional alterations in the cells by more extensive separation procedures (17). The use of this strategy has been discussed previously (17), and our study population does not differ from the overall patient population regarding important biological, clinical, and prognostic parameters (4) (see below).

In our study, we investigated primary AML cells from consecutive/unselected patients. These cell populations have a hierarchical organization with (i) a majority of more mature cells undergoing stress-induced apoptosis during *in vitro* culture; (ii) an intermediate subset of clonogenic progenitors (often <1%); and (iii) a small minority of AML stem cells (18, 26). Studies of the total cell population should still be regarded as relevant despite this organization, especially with regard to antileukemic therapy, because: (i) early morphological disease control with remission induction (i.e., reduction of the total AML cell population) is an important prognostic parameter (1); (ii) clinical studies have shown that biological characteristics of the total AML cell population reflect general leukemic cell characteristics that have prognostic impact and are essential for leukemogenesis and chemosensitivity (26); and (iii) previous studies have also shown that the AML stem cells can be detected in various subsets, usually in the $CD34^+CD38^-$ subset and also in the $CD34^-$ and $CD34^+CD38^+$ subsets, and stem cell characteristics are also reflected in the overall cell population (18, 26).

All our methods for cell preparation, *in vitro* culture, and AML cell characterization have been described in previous methodological investigations. First, our approach with inclusion of only patients with relatively high peripheral blood AML cell counts have been characterized previously when consecutive patients with high blood AML cell counts were compared with the overall consecutive AML patient population (17). The two groups did not differ with regard to other prognostic (i.e., chemosensitivity) parameters, but despite this, one should be careful when generalizing from our results (17). Second, the use of cryopreservation has also been discussed previously, and its effect on AML cell viability has been described in detail (19, 20). Third, we used serum-free medium for culture of AML cells (27), the only exception being the cocultures with MSCs, because these mesenchymal cells require an enriched medium (9). Finally, as described above, the AML cell population has a hierarchical organization (26), and our proliferation assay based on ^{3}H-thymidine incorporation allows us to analyze only the proliferation of long-term surviving cells able to proliferate after 7 days of culture; these cells represents an enrichment of clonogenic cells (18). Thus, our study is based on well-characterized and standardized methodological strategies.

Chemokine receptor inhibitors are now being developed (28, 29). However, patients showing chemokine-dependent growth modulation usually respond to a wide range of chemokines that bind different receptors (4), and both the constitutive chemokine/cytokine release by AML cells and the release by neighboring bone marrow stromal cells will determine the final effect of chemokine receptor binding on AML cell proliferation. These observations suggest that combined targeting of promiscuous chemokine receptors would be most effective in AML. However, the intracellular signaling downstream to the various chemokine receptors is difficult to predict (16, 22), and our present results show that even such broad targeting of a promiscuous receptor has divergent effects for chemokine-responsive patients. It is not known whether combined targeting of the other phenotypic characteristics of these patients will be more effective.

Constitutive CCL28 release was observed for a minority of patients and showed no correlation with autocrine proliferation, chemokine responsiveness, or gene expression profiles. Despite these observations, CCL28 may contribute to leukemogenesis or chemosensitivity through its effect on neighboring stromal cells. Recent studies have demonstrated that CCR3 and CCR10 receptors are expressed by bone marrow stromal cells (30). The bone marrow microenvironment is hypoxic, and studies in experimental models strongly suggest that CCL28 is important for cell trafficking and angiogenesis in hypoxic microenvironments associated with carcinogenesis (31). CCL28 is also released by endothelial cells and may thereby be a part of the cross talk between AML and neighboring stromal cells. Through its release by non-leukemic stromal cells in the bone marrow microenvironment (7, 32), CCL28 may still function as a growth factor for remaining normal hematopoietic cells in AML (12). Finally, as discussed below, there may also be an interaction between integrins and CCL28, as the latter promotes integrin-dependent cell adhesion (33).

We investigated the effect of CCR1/CCR3 inhibition (both being promiscuous receptors) in a more physiological model of AML/MSC cocultures; this model was used to study chemokine effects in a local cytokine network with a major influence of bone marrow stromal elements. Our results show that even in a cytokine environment formed by the constitutive cytokine release and the cytokine-mediated cross talk between primary AML and bone marrow MSCs, the CCR1/CCR3-mediated effects on AML cell proliferation are divergent, and the different effects of CCR1/CCR3 inhibition on AML cell viability and proliferation can be explained by the hierarchical organization of the AML cell population (17). The viability studies then reflect the characteristics of the majority of more mature cells that undergo spontaneous apoptosis during the first days of culture, whereas the proliferation is assayed after several days of culture and reflects the ability of a more immature cell minority capable of maintaining proliferation after several days.

Even though we could not detect effects of chemokine inhibition on AML cell proliferation *in vitro*, studies in chronic lymphocytic leukemia (CLL) have shown that altered regulation of *in vivo* cell trafficking can contribute significantly to the antileukemic effects of certain drugs (34–39). The microenvironment is important both for survival and proliferation of CLL cells, and local chemokine release is a part of this support. Furthermore, ibrutinib mediates its antileukemic effect in CLL through direct effects on the CLL cells and through inhibition of chemokine-dependent leukemia cell trafficking, thereby keeping the cells out of their growth-enhancing/antiapoptotic microenvironment and making them susceptible to the direct antileukemic effect. Our present study shows that a subset of patients has chemokine-responsive AML

cells, and similar dual-targeting may be possible in AML. This hypothesis is supported by recent studies; ibrutinib has direct antileukemic effects in human AML (40) and is in addition able to alter chemokine-dependent AML cell migration (41). Our present studies therefore suggest combined targeting of AML cell survival, and migration (i.e., chemokine targeting) should be considered for patients with chemokine-responsive AML cells.

In our studies of global gene expression profiles, we observed that AML cells with CCL28-induced growth modulation (i.e., chemokine-responsive) showed an altered expression of genes included in the GO-terms lysine acetylation/integrins/helicases. As discussed above, there is a functional interaction between CCL28 and integrins, but there is also a biological interaction between the integrin system and lysine acetylation. First, histone acetylation is important for regulation of gene expression (2, 42), including the expression of integrins in human AML cells (43). Second, lysine acetylation can also alter integrin expression indirectly; HOX transcription factors are important for regulation of integrin gene expression, and acetylation is then important in the regulation of HOX activity both through effects on the mRNA expression of these transcription factors and through modulation of their transcription factor activity by posttranscriptional modulation of HOX proteins (44, 45). Third, experimental studies suggest that integrins can mediate intracellular signaling that alters histone acetylation and thereby alter the proliferation of malignant cells (46, 47). Through this signaling, integrins can also regulate gene expression in human myeloid cells through the activation of several transcription factors (48). Finally, acetylation of microtubules is important for regulation of the cell surface density of integrins (49). Thus, through their interactions with extracellular matrix and neighboring cells, integrins mediate bidirectional signaling, i.e., inside-out and outside-in signaling (50, 51), and lysine acetylation is important at several steps of this process.

Helicases are also important for transcriptional and translational regulation and thereby seem to represent a functional overlap with lysine acetylation. First, a major part of the helicases with altered expression are members of the DEAD box family of RNA helicases, they belong to the helicase superfamily 2 together with the DEAH helicases and are often referred to as DExD/H box RNA helicases (52, 53). The helicases should be regarded as multifunctional proteins important in regulation of transcription, ribosome biogenesis, translation, and RNA decay/storage/metabolism; they also have ATPase activity and can function as metabolic sensors (53–55). The helicase DDX10 can be involved in leukemogenesis (56). Second, the mini-chromosome maintenance complex forms an important part of the replication machinery, but its loading to chromatin at sites distant from replicating DNA strongly suggests that it has additional functions, possibly as transcriptional regulators (57). Third, SWI/SNF is a multisubunit chromatin-remodeling complex important for regulation of cell cycle progression, apoptosis, differentiation, genomic instability, and DNA repair (58). The function of this molecular complex in AML seems to differ from other malignancies, and it is considered as a possible therapeutic target in human AML (59, 60).

CONCLUSION

Chemokines have growth-modulating effects on the AML cells only for a minority of patients; and these patients are then responsive to several chemokines. However, this chemokine responsiveness is only a part of a more extensive leukemia cell phenotype that includes several partly interacting cellular functions, i.e., altered extracellular communication (chemokines/integrins/acetylation) and transcriptional regulation (histone acetylation, helicases) compared with the other non-responsive AML cells. Furthermore, the direct effects of chemokine targeting on primary human AML cells are difficult to predict because they depend both on differences among patients and on the overall local cytokine network; this is probably true both if chemokine targeting is tried as an antileukemic strategy or as an approach to treat immune-mediated complications in allotransplanted AML. A careful evaluation of the AML cells will possibly be important to avoid the risk of leukemia enhancement and thereby increased relapse risk; this would probably be important if the treatment is tried both as an antileukemic and an immunosuppressive strategy. A possible approach to avoid this risk may be combined targeting of other aspects of this complex phenotype. However, based on the lessons from recent studies in CLL where chemokines are important for the supportive function of neighboring stromal cells by keeping the CLL cells within their permissive microenvironment, one would emphasize that AML cells from chemokine-responsive patients should be further tested in relevant *in vivo* models (e.g., patient-derived xenografts) to see whether chemokine targeting alters AML cell trafficking/homing and thereby increases their chemosensitivity.

AUTHOR CONTRIBUTIONS

ØB conceived and designed the experiments; AB performed the experiments; AB and HR analyzed the data; and ØB and AB wrote the paper.

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SUPPLEMENTARY MATERIAL

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Mesenchymal Stem Cells Support Survival and Proliferation of Primary Human Acute Myeloid Leukemia Cells through Heterogeneous Molecular Mechanisms

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Acute myeloid leukemia (AML) is a bone marrow malignancy, and various bone marrow stromal cells seem to support leukemogenesis, including osteoblasts and endothelial cells. We have investigated how normal bone marrow mesenchymal stem cells (MSCs) support the *in vitro* proliferation of primary human AML cells. Both MSCs and primary AML cells show constitutive release of several soluble mediators, and the mediator repertoires of the two cell types are partly overlapping. The two cell populations were cocultured on transwell plates, and MSC effects on AML cells mediated through the local cytokine/soluble mediator network could thus be evaluated. The presence of normal MSCs had an antiapoptotic and growth-enhancing effect on primary human AML cells when investigating a group of 51 unselected AML patients; this was associated with increased phosphorylation of mTOR and its downstream targets, and the effect was independent of cytogenetic or molecular-genetic abnormalities. The MSCs also supported the long-term proliferation of the AML cells. A subset of the patients also showed an altered cytokine network with supra-additive levels for several cytokines. The presence of cytokine-neutralizing antibodies or receptor inhibitors demonstrated that AML cells derived from different patients were heterogeneous with regard to effects of various cytokines on AML cell proliferation or regulation of apoptosis. We conclude that even though the effects of single cytokines derived from bone marrow MSCs on human AML cells differ among patients, the final cytokine-mediated effects of the MSCs during coculture is growth enhancement and inhibition of apoptosis.

Keywords: acute myeloid leukemia, mesenchymal stem cells, proliferation, apoptosis, cytokines, chemokines

Abbreviations: AML, acute myeloid leukemia; Ang-1, angiopoietin 1; ATM, ataxia telangiectasia, mutated; bFGF, basic fibroblastic growth factor; CFU, colony-forming unit; cpm, counts per minute; ELISA, enzyme-linked immunosorbent assay; EPO, erythropoietin; FBS, fetal bovine serum; Flt3, FMS-related tyrosine kinase 3; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; HGF, hepatocyte growth factor; MMP, matrix metalloproteinase; MSC, mesenchymal stem cell; MSCGM, mesenchymal stem cell growth medium; NPM1, nucleophosmin; PI, propidium iodide; TNFα, tumor necrosis factor α; VEGF, vascular endothelial growth factor.

INTRODUCTION

Acute myeloid leukemia (AML) is an aggressive malignancy that mainly affects the elderly; the disease is characterized by bone marrow infiltration of immature leukemic blasts (1), and it is highly heterogeneous with respect to leukemia cell biology as well as response to therapy (2). Approximately 50–60% of AML patients carry clonal chromosomal abnormalities that reflect the chemosensitivity of the disease (3). The disease is also associated with specific gene mutations exhibiting prognostic impact, where *FMS-related tyrosine kinase 3* internal tandem repeats (*Flt3*-ITD; adverse prognosis) and mutations in *nucleophosmin* (*NPM1*; favorable prognosis) are the most prominent (4).

Mesenchymal stem or stromal cells (MSCs) are capable of self-renewal and differentiation into osteoblasts, chondrocytes, or adipocytes (5), the most immature MSCs can also trans-differentiate into other embryonic lineages (6, 7). The cells can be isolated from almost any kind of connective tissue (8, 9), and bone marrow MSCs provide a microenvironment for growth, differentiation, and survival of both normal (10) and leukemic (11) hematopoietic cells. The bone marrow MSC population seems to be important in leukemogenesis (12) and also to contribute to chemoresistance through its release of specific soluble mediators (13, 14).

In this study, we have therefore characterized the cytokine-mediated crosstalk between AML cells and normal bone marrow MSCs. Due to the heterogeneity of the disease, we have investigated a large group of unselected patients. Our studies suggest that MSC-derived cytokines have antiapoptotic effects and support AML cell proliferation for most patients, but the molecular mechanisms causing these effects differ among patients.

TABLE 1 | Biological and clinical characteristics of the 51 acute myeloid leukemia (AML) patients included in the study.

Patient characteristics		Cell morphology		Cell genetics	
Age		FAB classification[a]		Cytogenetics[b]	
Median (years)	67	M0	3	Favorable	9
Range (years)	19–87	M1	15	Intermediate	6
		M2	7	Normal	23
Gender		M3	3	Adverse	10
Females	22	M4	8	n.d.	3
Males	29	M5	10		
		n.d.	5	*Flt3* mutations	
Secondary AML				ITD	15
MDS	6	CD34 receptor		Wild type	26
CMML	4	Negative (≤20%)	14	n.d.	10
Chemotherapy	1	Positive (>20%)	32		
		n.d.	5	*NPM1* mutations	
AML relapse	4			Insertion	16
				Wild type	26
				n.d.	9

[a]*No patient was diagnosed with FAB M6 or M7.*
[b]*Patients with favorable karyotype had inv(16); t(15;17); and t(8;21). Patients with adverse karyotype had −5; −7; +8 and complex karyotype with at least three abnormalities.*
n.d., not determined.

MATERIALS AND METHODS

AML Patient Population and Leukemic Cell Preparation

The study was approved by the local ethics committee (Regional Ethics Committee III, University of Bergen) and samples collected after written informed consent. AML blasts from peripheral blood were derived from 51 consecutive/unselected patients admitted to our department for AML therapy (22 females, 29 males; median age 67 years; range 19–87 years). A majority of 36 patients had *de novo* AML (**Table 1**), 4 patients had relapsed disease, and 11 patients had secondary AML.

Acute myeloid leukemia cells were isolated from peripheral blood of patients with levels of circulating blasts by density gradient separation (Lymphoprep; Axis-Shield, Oslo, Norway; specific density 1.077 g/mL). The cells were stored in liquid nitrogen until use (15).

Reagents

The following neutralizing antibodies and receptor antagonists (all from R&D Systems, Abingdon, UK) were used at the following concentrations: (i) 100 ng/mL of affinity purified polyclonal antibodies (goat IgG) against the vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), basic fibroblastic growth factor (bFGF), and IL-6. At this concentration, the antibodies will block ≥50% of receptor binding to VEGF (80 ng/mL), HGF (8 ng/mL), bFGF (0.1 ng/mL), and IL-6 (5 ng/mL), thus concentrations higher than the levels in our MSC cultures (16); (ii) 3 μM of the CCR1 antagonists BX471 (17) and BX513 (18); (iii) 1.5 μM of the combined CCR1 and CCR3 antagonist UCB35625 and its stereoisomer J113863 (19, 20); and (iv) 300 μM of the CXCR4 antagonist AMD3100 (13). Normal goat IgG was used in the antibody control cultures. Cytarabine (Sigma-Aldrich, St. Louis, MO, USA) was tested in dose-response experiments using concentrations between 12.5 nM and 2 μM (21).

In Vitro Expansion of MSCs

Human MSCs from three healthy donors (MSC24429, MSC24539, and MSC25200) were purchased from Lonza (Cambrex BioScience, Walkersville, MD, USA). According to the distributor's information, the cells were obtained in passage two and showed the ability to differentiate into the mesenchymal lineages. All cells tested negative for mycoplasma, bacteria, and fungi. The MSCs were expanded in complete mesenchymal stem cell growth medium (MSCGM™; Lonza), which contains 10% fetal bovine serum (FBS) and 4 mM L-glutamine; cells were trypsinized and used for the experiments in passages three or four. Our previous studies of global gene expression profiles of *in vitro* expanded MSCs showed no evidence for differentiation of such expanded MSCs (16).

Analysis of AML Cell Proliferation and Viability in Transwell Cocultures with MSCs

Preparation of MSC-AML Cell Cocultures

Cultures were prepared in transwell plates (Costar 3401 plates; Costar, Cambridge, MA, USA). The MSCs (2×10^4 cells/well)

were seeded in the lower chamber in complete MSCGM™ medium (1 mL/well). After 3 days of culture (37°C, humidified atmosphere, 5% CO_2) the medium was exchanged and subsequently 1×10^6 AML cells were added in 0.5 mL medium to the upper chamber separated from the MSCs by a semipermeable membrane (0.4 μm pore size). The cells were cultured for 3 days, in which the MSCs did not reach confluence.

Analysis of Cell Proliferation by ^{3}H-Thymidine Incorporation

After 2 days of coculture, 275 kBq of ^{3}H-thymidine (PerkinElmer, Waltham, MA, USA) was added to the upper wells and the cells were incubated for another day. The nuclear ^{3}H-thymidine incorporation was then measured by liquid scintillation counting as described in detail previously (16). All cultures were prepared in triplicates and the median counts per minute (cpm) were used for all calculations. A ^{3}H-thymidine incorporation corresponding to an activity of at least 1,000 cpm was defined as detectable proliferation (22).

Analysis of AML Cell Viability

Acute myeloid leukemia cells and MSCs were cocultured in transwell plates for 3 days before the percentage of viable leukemic cells was determined by flow cytometry after staining with propidium iodide (PI) and fluorescein isothiocyanate-conjugated Annexin V antibodies (Tau Technologies BV, Kattendijke, the Netherlands) as described in detail previously (23). Briefly, after staining with PI/anti-Annexin V, the flow cytometric analysis could identify the viable Annexin$^-$PI$^-$, early apoptotic Annexin V$^+$PI$^-$, and late apoptotic/necrotic Annexin V$^+$ PI$^+$ AML cell subsets.

We also cultured primary AML cells from 10 patients in direct contact with MSCs in 6-well tissue culture plates; 20,000 MSCs were precultured for 3 days before 1×10^6 primary AML cells were added to each well. AML cell viability was analyzed 20 h later before the MSCs reached confluence.

Analysis of the Cytokine Profile in MSC-AML Cell Transwell Cocultures

Supernatants were harvested from the lower chambers and stored at −80°C. The following cytokine levels were determined by Luminex analyses or enzyme-linked immunosorbent assay (ELISA) (R&D Systems): (i) the chemokines CCL2-5 and CXCL1/5/8/10/11, (ii) the interleukins IL-1β/1RA/6/10/33, (iii) the matrix metalloproteinases MMP-1 and -2, (iv) tumor necrosis factor-α (TNFα), and (v) granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), HGF, bFGF, VEGF, and soluble angiopoietin 1 (Ang-1) receptor Tie-2.

Long-term *In Vitro* AML-MSC Cocultures and Analysis of Colony-Forming Cells

Cocultures of MSCs with primary AML cells were prepared as described above. The blasts were transferred weekly to new upper chambers of transwell cocultures where fresh MSCs had been seeded in the lower chambers 3 days in advance. The medium was exchanged twice a week throughout the 3 weeks of culture period in transwell cocultures. After 3 weeks, the AML cells were harvested and subsequently seeded in the colony-formation assay to estimate the number of colony-forming units (CFU). We used two different colony-formation assays; MethoCult™ H4434 Classic and H4534 Classic without erythropoietin medium (StemCell Technologies, Vancouver, BC, Canada). The cells were seeded in duplicate in 24-well plates with 0.5 mL medium/well. Colonies containing more than 30 cells were scored using an inverted microscope after 14 days of *in vitro* culture.

Analysis of Intracellular Signaling and H2AX Phosphorylation

Antibodies

The following antibodies were used for analysis of protein phosphorylation: Alexa Fluor® 647 Mouse anti-PDPK1 (pS241), Alexa Fluor® 647 Mouse anti-PKCα (pT497), Alexa Fluor® 647 Mouse anti-Akt (pS473), phycoerythrin (PE) Mouse Anti-Akt (pT308), PE Mouse Anti-mTOR (pS2448), Alexa Fluor® 647 Mouse anti-4E-BP1 (pT36/pT45), Alexa Fluor® 647 Mouse anti-elF4E (pS209), Alexa Fluor® 647 Mouse anti-S6 (pS244), PE Mouse anti-S6 (pS240), V450 Mouse anti-S6 (pS235/pS236) Alexa Fluor® 647 Mouse anti-PKCα (pT497) (all from BD Biosciences, Franklin Lakes, NJ, USA), and mTOR (7C10) Rabbit mAb (Alexa Fluor® 647 Conjugate) (Cell Signaling Technology, Danvers, MA, USA).

Preparation of Conditioned Medium

MSC24539 was cultured as described above and the conditioned medium harvested before the cells reached confluence.

Analysis of Protein Phosphorylation

Thawed, cryopreserved leukemic cells were incubated for 20 min in culture medium, thereafter incubated with conditioned medium (final concentration 50%) for 30 min before being directly fixed in 1.5% paraformaldehyde and thereafter permeabilized with methanol. The cells were then rehydrated by adding 2 mL phosphate-buffered saline (PBS), gently resuspended, and then centrifuged. The cell pellet was washed twice with 2 mL PBS and resuspended in 150 μL PBS + 0.1% BSA (Sigma-Aldrich). The washed cells were blocked with immunoglobulin (Octagam; Octapharma, Jessheim, Norway) and 1% BSA, and split evenly into separate tubes (1×10^5 cells/sample) before staining. All staining panels included the same live dead discriminator FITC Mouse anti-Cleaved PARP (Asp214; BD Biosciences) and Alexa Fluor® 647 Mouse anti-Cleaved PARP (Asp 214; BD Biosciences), as well as one blank sample. Flow cytometric analysis of protein phosphorylation was performed as described in detail previously (24).

Analysis of H2AX Phosphorylation

The percentage of phosphorylated compared to total H2AX was detected using a cell-based ELISA kit [Phospho-Histone H2AX (S139) Immunoassay; R&D Systems]. The assay was performed according to the manufacturer's instruction for suspension cells; 5×10^4 AML cells were incubated for 16 h in both MSC medium and MSC-conditioned medium (final concentration of 50%).

Statistical and Bioinformatical Analyses

The statistical analyses were performed with the IBM Statistical Package for the Social Sciences (SPSS) version 23 (Chicago, IL, USA). The Wilcoxon signed-rank test was used to compare paired samples, whereas Kruskal–Wallis and Mann–Whitney *U*-tests were used to compare different groups. The χ^2 test was used to analyze categorized data and the Kendall's tau-b test for correlation analyses. *P*-values <0.05 were regarded as statistically significant. Supervised hierarchical clustering was performed using the J-Express 2012 software (MolMine AS, Bergen, Norway). The data were log(10) transformed prior to clustering.

RESULTS

Human MSCs Increase the Proliferation of Primary Human AML Cells during *In Vitro* Coculture

Primary AML cells from 18 consecutive patients were cocultured with normal MSCs from the three different donors (MSC24429, MSC24539, and MSC25200) in transwell plates. AML cell proliferation was determined after 3 days of culture. AML cell proliferation was significantly increased by coculture with MSCs compared with the corresponding control cultures with AML cells alone (**Figure 1A**); this increase reached statistical significance for all three MSC donors ($p \leq 0.001$, Wilcoxon's signed-rank test), even though the AML cells showed undetectable proliferation for two patients, both when cultured in medium alone and in the presence of all three MSC donor cells. The other 16 patients showed increased proliferation for at least one of the three MSCs. The median increase in ^{3}H-thymidine incorporation corresponded to approximately fivefold increase for each of the three donors.

Human MSCs Have Antiapoptotic Effect on *In Vitro* Cultured Primary AML Cells

Primary AML cells show spontaneous or stress-induced apoptosis during *in vitro* culture (25). We prepared *in vitro* transwell cocultures for MSCs and AML cells derived from the same 18 patients as tested in the proliferation assay (see above). MSCs were pre-cultured for 3 days before AML cells were added and leukemic cell viability assayed after 3 days of coculture by flow cytometric analysis. The effect of all three human bone marrow MSCs was investigated (MSC24429, MSC24539, and MSC25200). The results are summarized in Table S1 in Supplementary Material and presented in detail in **Figure 1B**. AML cell viability showed a wide variation after three days of *in vitro* coculture (variation range 0.4–84.7% viability). When comparing the overall results, the fraction of viable cells was significantly increased ($p < 0.001$, Wilcoxon's signed-rank test) after coculture in the presence of all three MSCs compared with the corresponding MSC-free controls, and the median fraction of viable cells was approximately doubled for each of the three MSC donors.

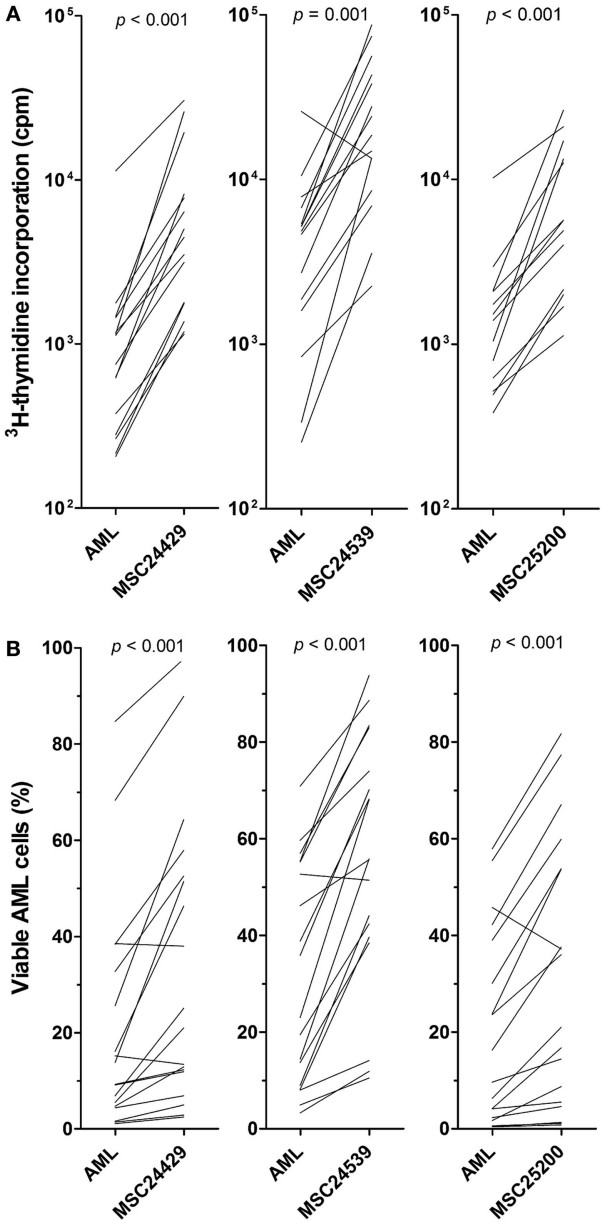

FIGURE 1 | The effect of normal bone marrow mesenchymal stem cells (MSCs) on *in vitro* proliferation and viability of primary human acute myeloid leukemia (AML) cells; a comparison of MSCs derived from three healthy donors (MSC24429, MSC24539, MSC25200). Primary human AML cells derived from 18 consecutive/unselected leukemia patients were cocultured with normal MSCs from the three donors. The MSCs were initially cultured for 3 days to allow them to establish adherent *in vitro* proliferation; primary human AML cells were then added and both leukemia cell proliferation and viability were assayed after additional 3 days of culture. [**(A)**, upper part] Proliferation was assayed as ^{3}H-thymidine incorporation. The results for each patient are presented as nuclear thymidine incorporation (counts per minute, cpm). The *p*-value for the statistical comparison of the overall results is given at the top of the figure for each of the individual MSC. Each line represents the results for one patient. [**(B)**, lower part] Leukemia cell viability was assayed by the Annexin V-PI flow cytometric assay. The *p*-value for the statistical comparison of the overall results for each of the MSCs is given at the top of the figure for the MSCs. Each line represents the results for one patient.

The Growth-Enhancing and Antiapoptotic Effects of Human MSCs on Primary Human AML Cells—A Study of Patient Heterogeneity for a Group of 51 Consecutive/Unselected Patients

Because the proliferation and viability results were consistent among MSC donors, the comparison of MSC effects for a larger group of 51 consecutive patients was performed only for MSC24539. MSC-induced enhancement of AML cell proliferation in transwell cocultures was highly significant also when analyzing the overall results for this larger group of patients (**Figure 2A**; $p < 0.00001$, Wilcoxon's signed-rank test). The growth enhancement showed no significant association with AML cell differentiation (morphology according to FAB classification, CD34 expression), karyotype (favorable/intermediate/adverse/normal), or *Flt3/NPM1* mutations (data not shown). Finally, cells from 11 patients showed detectable proliferation neither in medium alone nor in cocultures with MSC24539. For five additional patients, the presence of MSCs showed no or minimal growth enhancement, corresponding to less than 20% alteration and an absolute change of less than 2,000 cpm in the presence of MSCs. These 16 patients differed significantly from the other 35 patients with regard to cell differentiation as $CD34^-$ cells showed weaker proliferation in the presence of MSCs than $CD34^+$ cells (8/14 $CD34^-$ patients with non-proliferating cells in contrast to only 6/32 $CD34^-$ patients with proliferating cells; $p = 0.011$, χ^2 likelihood ratio) (data not shown).

The same 51 patients were also tested in transwell cocultures with regard to a MSC-associated antiapoptotic effect; viability analyzed by the Annexin V-PI assay was then compared for AML cells cultured in medium alone or cocultured with MSC24539 for 3 days. The presence of MSCs increased AML cell viability significantly also when testing this extended patient group (**Figure 2B**; $p < 0.00001$, Wilcoxon's signed-rank tests). This antiapoptotic effect showed no significant association with AML cell differentiation, karyotype, or *Flt3/NPM1* mutations (data not shown). However, proliferation <1,000 cpm in the presence of MSCs seemed to be associated with weaker antiapoptotic effects of MSCs; i.e., 8/21 patients with viability increase <10% points and 0/20 patients with an increase >20% points showed undetectable proliferation in cocultures ($p = 0.001$, χ^2 likelihood ratio).

Because primary AML cells derived from 16 patients showed undetectable proliferation during coculture with MSCs, we also

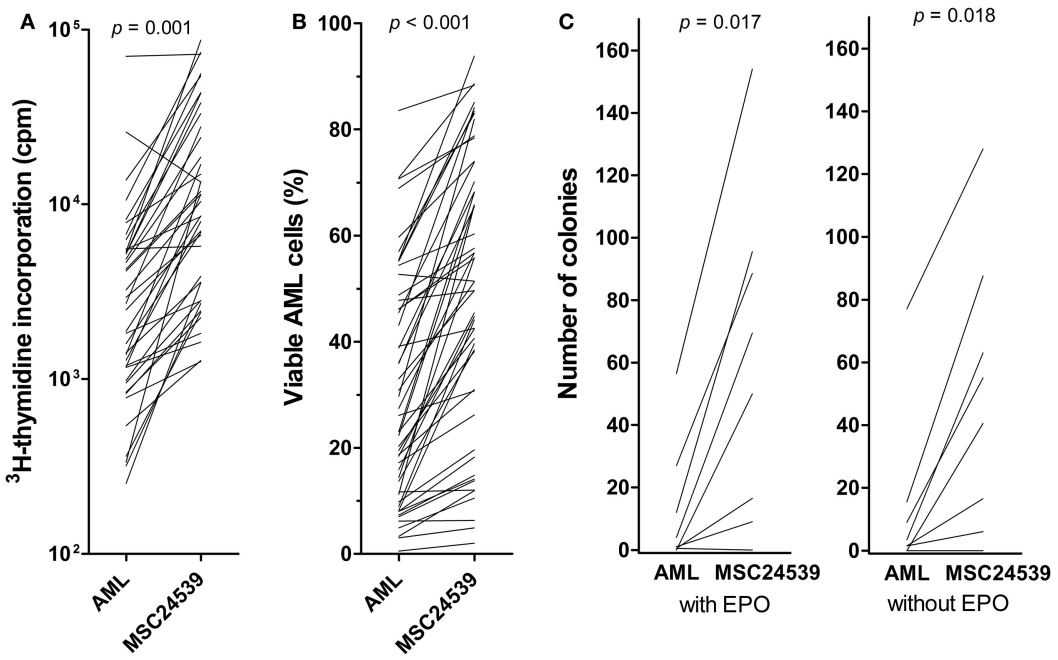

FIGURE 2 | The effects of mesenchymal stem cells (MSCs) on acute myeloid leukemia (AML) cell proliferation/viability in suspension cultures and AML cells tested in a clonogenic assay. The effect of MSCs on the *in vitro* proliferation [**(A)**, left] and viability [**(B)**, middle] of primary human AML cells derived from 51 consecutive patients was examined. The AML cells were then cocultured with normal MSC24539. The MSCs were initially cultured for 3 days to allow them to establish adherent *in vitro* proliferation; primary human AML cells were thereafter added and **(A)** leukemia cell proliferation was assayed as ^{3}H-thymidine incorporation after additional 3 days of culture; additionally, **(B)** AML cell viability was assayed by the Annexin V-PI flow cytometric assay. The *p*-values for the statistical comparison of the overall results are given at the top of the figure. Each line represents the results for one patient. [**(C)**, right] The effects of MSCs on clonogenic AML cells were also investigated. The leukemic cells were cultured either in medium alone or in transwell cocultures together with MSC24593 for 3 weeks; the number of clonogenic cells were thereafter compared for AML cells precultured in medium alone and together with MSCs. The total culture period was thus 5 weeks. The MSCs significantly increased the number of colony-forming units (*p*-values are given on top of the figure) in growth media both with and without erythropoietin. The results are presented as mean of duplicate samples (average deviation from mean corresponding to 1.3 colonies and 8.7% of the total colony number). Each line represents the results for one patient.

analyzed separately the effect of MSCs on AML cell viability for these patients. However, when analyzing the overall results, a highly significant increase in AML cells viability ($p < 0.001$) after MSC coculture was seen also for these 16 patients with non-proliferating AML cells, and a >10% point increase was seen for eight of these patients (see above). Thus, the MSC effect on AML cell viability is not only caused by the increased proliferation but also by additional effects possibly affecting the balance between pro- and antiapoptotic signaling.

Normal Human MSCs Support the Long-term Proliferation of Primary Human AML Cells

To further investigate the MSC effects on AML cell proliferation, we used an *in vitro* model based on 21 days of coculture; this is the same culture period as used previously by Griessinger et al. (26) in their studies of leukemia-initiating AML cells. After this period of transwell cocultures, the number of colony-forming cells was compared for leukemia cells cultured alone and cells cocultured with normal MSCs. We investigated AML cells from eight patients showing both enhanced proliferation and viability in the short-term assays described above. All except one patient showed an increased number of viable cells after 21 days of coculture with the normal MSC24539 cells (data not shown). After the initial 21 days culture period, the cells were seeded in the CFU assays. Even though the culture medium was different in the CFU assays compared with the transwell cocultures containing MSC medium, colony formation could be detected for seven of the eight patients. For all these seven patients, we observed an increased number of CFUs in the AML cell populations previously cocultured with MSCs compared with the corresponding control cultures where AML cells were cultured alone without MSCs in the lower transwell chamber (**Figure 2C**). Thus, the MSC-associated growth enhancement also includes long-term proliferating AML cells.

Normal Human MSCs Have Antipoptotic Effects on Primary Human AML Cells Also in the Presence of Cytarabine

We investigated the effect of 50 nM cytarabine on primary human AML cells derived from 10 patients. All patients showed an increase in leukemia cell viability corresponding to >20% in presence of MSC24539. The cytarabine concentration was chosen based on dose-response (2 μM, 0.5 μM, 125 nM, 50 nM, and 12.5 nM) pilot experiments, which showed that cytarabine at 50 nM decreased AML cell viability for a marked subset of patients when using our *in vitro* models (Figure S1 in Supplementary Material). These results show that there is heterogeneity among patients with regard to the proapoptotic effect of cytarabine, and decreased AML cell viability was seen only for a subset of patients when testing cytarabine at concentrations 12.5–500 nM and with a large overlap between the effects of 50 and 500 nM. At the same time, the drug had a clear antiproliferative effect even at 50 nM and at higher concentrations no detectable cytokine-dependent proliferation could be detected (data not shown). Finally, the concentrations 50–500 nM correspond to levels reached *in vivo* and 50 nM is close to the steady-state concentrations seen during conventional AML induction treatment and higher than the levels reached during low-dose subcutaneous cytarabine treatment (27); both these therapeutic strategies for cytarabine treatment can induce complete remissions (27, 28).

Primary AML cells were cultured in transwell cultures for 20 h either alone or in coculture with MSC24539, and cultures with/without MSCs were prepared with and without 50 nM cytarabine (**Figure 3A**). The present independent experiments confirmed that patients are heterogeneous with regard to susceptibility to cytarabine; a reduction in the number of viable cells exceeding 5% was seen for 5 out of 10 patients in the present experiments and for 16 out of 32 patients in the previous dose-response studies. Furthermore, the percent cytarabine-induced decrease of AML cell viability for the five patients in the present study was significantly higher for cells cultured in medium alone (median decrease 9%, range 6–23%) compared with cells in coculture with MSC24539 (median decrease 4%, range 12–20%; $p = 0.045$). Finally, increased AML cell viability was seen for all 10 patients in MSC cocultures, both in presence and absence of 50 nM cytarabine.

We then compared the overall effects of MSCs on AML cell viability, i.e., leukemic cells cultured in direct contact with MSCs, on the viability of primary human AML cells derived from the same 10 patients as used above. Cytarabine was tested at 50 and 500 nM, the last concentration corresponding to the highest steady-state levels seen during conventional doses of 100–200 mg/m^2 as daily continuous intravenous infusions (27). The presence of MSCs caused a comparable increase in AML cell viability in these direct-contact experiments as described above for the transwell cocultures and similar to the transwell cocultures 50 nM cytarabine induced reduction of cell viability for five patients also in these direct-contact experiments (data not shown). In contrast, 500 nM cytarabine caused a significant reduction in AML cell viability for all patients, and this reduction was partly counteracted by the presence of MSCs as the AML cell viability was significantly increased for cytarabine-containing cultures with MSCs compared with corresponding cultures without MSCs. But the viability was still significantly lower than for drug-free direct cocultures (**Figure 3B**). Thus, the proapoptotic effect of cytarabine can be detected also in our *in vitro* model; this cytarabine effect can be partly counteracted by MSCs and taken together our overall results suggest that cytokine-mediated crosstalk between MSCs and AML cells can contribute to this effect.

Effects of MSCs on H2AX Phosphorylation and mTOR Activation in Primary Human AML Cells

Phosphorylation of Histone H2AX can be seen as part of the DNA damage response and ATM activation; the phosphorylation can then be an early apoptotic event and has been used as an early marker of apoptosis induction (29). We performed cell-based ELISA prepared from cells cultured in MSC medium and in MSC24539-conditioned medium (50% final concentration; for the cytokine profile of this conditioned medium see

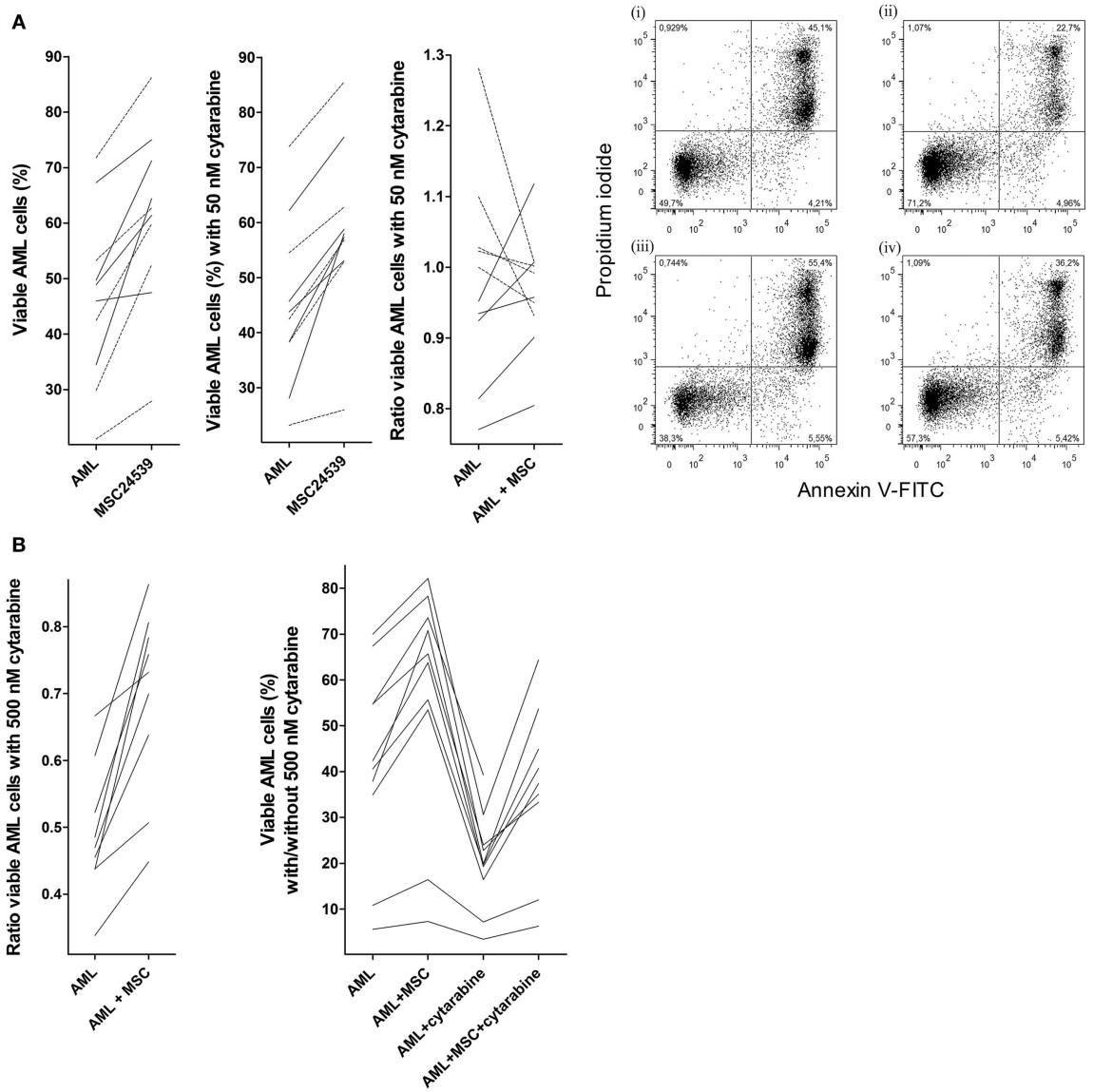

FIGURE 3 | Effects of mesenchymal stem cells (MSCs) on primary human acute myeloid leukemia (AML) cells treated with cytarabine; (A) effect of 50 nM cytarabine-exposure on AML cell viability in transwell cocultures and (B) effect of 500 nM cytarabine tested in direct-contact cocultures. Primary AML cells derived from the same 10 patients were included in all these studies, and MSC24539 was used in all experiments. The AML cell viability was analyzed by flow cytometry. [**(A)**, transwell cocultures] For each patient, we compared cultures containing AML cells alone (AML) or AML cells plus MSCs (AML + MSC). The three figures from left to the middle right show (i) AML cell viability for cells cultured in medium alone with or without MSCs; (ii) AML cell viability in the presence of 50 nM cytarabine for leukemic cells cultured with and without MSCs; and (iii) a comparison of the medium culture ratio (i.e., viable cells in cytarabine-containing cultures versus drug-free controls; medium alone) and MSC culture ratio (i.e., viable cells in cytarabine-containing cultures versus drug-free controls; MSCs being added to both cultures). The results for the five patients (solid lines) for which 50 nM cytarabine exhibited a proapoptotic effect in the presence of MSCs are indicated. The results for one responding patient are presented in detail (right part of the figure; i/ii: drug-free control, iii/iv: 50 nM cytarabine without and with MSCs, respectively); the percentage of viable cells (population at the lower left) is indicated in the figures for each of the four cultures. [**(B)**, direct-contact cultures] The left part of the figure compares the medium culture ratio (i.e., viable cells in cytarabine-containing cultures versus drug-free controls; medium alone) and MSC culture ratio (i.e., viable cells in cytarabine-containing cultures versus drug-free controls; MSCs being added to both cultures) when testing 500 nM cytarabine. The right part of the figure shows the percentage of viable primary AML cells (10 patients tested, one patient not tested for AML cells + cytarabine + MSC) cultured in medium alone, together with MSCs, in medium containing 500 nM cytarabine, and together with 500 nM cytarabine and MSCs.

Table 2). The percentage of phosphorylated H2AX showed a strong inverse correlation with AML cell viability for AML cells cultured in medium alone (Kendall's tau; $p < 0.0002$), i.e., H2AX phosphorylation seems to be a marker of spontaneous *in vitro* apoptosis during culture of AML cells alone. However, significantly increased H2AX phosphorylation was observed after coculture with MSC-conditioned medium compared with leukemic cells cultured in medium alone (**Figure 4A**), and for

TABLE 2 | Cytokine excretion levels for 23 meditators from 51 acute myeloid leukemia (AML) blast supernatants (AML cells alone), AML blasts in coculture with mesenchymal stem cells (MSCs) (cocultures of AML cells and MSC24539) and only MSCs (MSC24539).

	AML cells alone			Cocultures of AML cells and MSC24539			MSC24539 alone	*p*-Value (supra-additive)
Mediator	**# Patients**	**Median conc. (pg/mL)**	**Range (pg/mL)**	**# Patients**	**Median conc. (pg/mL)**	**Range (pg/mL)**	**Mean conc. (pg/mL)**	
CXCL5	51	368	9.0–155,581	51	3,771	17–>213,550	25	<0.001
CXCL8	51	4,872	257–210,154	51	5,834	457–379,791	204	0.005
GM-CSF	50	3.5	n.d.–834	51	10	0.1–2,673	1.2	<0.001
IL-1RA	50	1,917	5.6–>25,500	50	1,562	5.6–>25,500	n.d.	n.s.
CCL5	50	56	n.d.–728	50	68	n.d.–17,161	2.9	n.s.
CXCL1	49	330	n.d.–35,641	51	11,316	24–42,042	32	<0.001
MMP-1	49	71	n.d.–14,263	51	18,223	157–>37,500	221	<0.001
CCL3	49	209	n.d.–41,519	51	234	8.0–42,167	12	<0.001
CCL4	48	235	n.d.–11,634	51	223	36–62,098	120	0.043
TNFα	45	6.0	n.d.–1,487	46	7.1	n.d.–10,676	0.8	0.037
bFGF	43	8.5	n.d.–57	50	19	n.d.–368	n.d.	<0.001
CCL2	51	992	9.2–22,859	51	3,888	613–20,621	2,643	n.s.
MMP-2	51	3,993	837–18,565	51	23,957	18,594–55,147	17,428	<0.001
CXCL10	39	7.1	n.d.–2,858	43	26	n.d.–2,882	n.d.	0.003
IL-6	39	14	n.d.–3,397	51	2,775	706–10,632	1,895	<0.001
CXCL11	37	4.1	n.d.–486	40	5.1	n.d.–377	1.9	0.014
IL-1β	29	3.5	n.d.–970	35	14	n.d.–21,593	n.d.	<0.001
IL-10	24	n.d.	n.d.–60	30	n.d.	0.8–601	n.d.	<0.001
IL-33	15	n.d.	n.d.–2.9	15	n.d.	n.d.–14	n.d.	n.s.
G-CSF	14	n.d.	n.d.–559	31	8.2	n.d.–>10,500	n.d.	<0.001
HGF	51	51	1.4–2,133	51	100	28–2,277	183	(0.001)
VEGF	50	17	n.d.–290	51	967	483–3,107	1,344	n.s.
Tie-2	45	50	n.d.–145	46	42	n.d.–298	14	(0.004)

n.d., below detection limit; n.s., not significant; GM-CSF, granulocyte macrophage colony-stimulating factor; bFGF, basic fibroblastic growth factor; G-CSF, granulocyte colony-stimulating factor; HGF, hepatocyte growth factor; VEGF, vascular endothelial growth factor.

The soluble mediators are divided into three subgroups: cytokines that are released for a large majority of patients in both medium and coculture (above); mediators that are excreted for a subset of patients when leukemic cells were cultured in medium alone (middle); cytokines that are released to a significantly higher degree in MSCs than in AML cells alone or in coculture with MSCs, or with higher values in AML cells alone compared to cocultures. The background from the growth medium was subtracted from all values. p-Values (Wilcoxon test) for coculture values compared to additive levels of AML cells and MSCs are included on the right; significant p-values thus indicating a supra-additive effect. Values in brackets (HGF and Tie-2) indicate that coculture levels are significantly lower than the expected levels from secretion of AML cells and MSCs alone.

these cultures, no significant association between viability and H2AX phosphorylation could be detected. This effect was also independent of AML cell proliferation and could be seen both for patients with increased proliferation in the presence of MSC-conditioned medium and patients with undetectable proliferation both with and without conditioned medium (data not shown). Thus, as will be discussed later, the increased H2AX phosphorylation in the presence of MSC-derived medium does not reflect increased apoptotic activity but rather a restoration of the decreased DNA damage response known to be present in primary human AML cells (30).

We also investigated the effect of MSC-conditioned medium on Akt-mTOR signaling in primary human AML cells. An increased MSC-associated phosphorylation/activation of mTOR and its downstream targets, S6K1 and 4E-BP1, was then observed (Wilcoxon's signed-rank test; $p = 0.005$; **Figure 4B**). The other upstream mediators did not differ significantly. Thus, MSCs alter H2AX phosphorylation as well as mTOR signaling in primary human AML cells, and as will be discussed later these effects may be important both for the MSC-associated growth enhancement and antiapoptotic effect of primary human AML cells.

The Local Cytokine Network Is Altered during Coculture of Normal MSCs and Primary AML Cells

We determined the supernatant levels for 23 soluble mediators in transwell cocultures of MSC24539 and AML cells derived from all 51 patients (**Table 2**; Tables S2 and S3 in Supplementary Material). Our present study of this larger group confirmed the previous observations from a small group of 18 patients (16). First, the constitutive mediator release by primary AML cells shows a wide variation for each individual mediator, and this patient heterogeneity was maintained in cocultures as we observed significant correlations between the levels for AML cells cultured alone and the corresponding coculture for 22 of the 23 cytokines (Kendall's tau correlation analysis), VEGF being the only exception. Second, relatively high levels were detected for most mediators in transwell cocultures both when compared

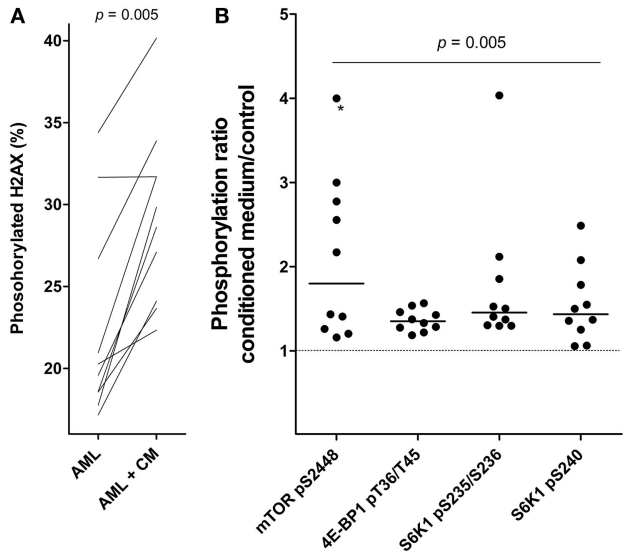

FIGURE 4 | Effects of mesenchymal stem cell (MSC)-conditioned medium on primary human acute myeloid leukemia (AML) cells; a study of H2AX phosphorylation and mTOR signaling. **(A)** The change in percentage of phosphorylated H2AX for AML cells cultured in MSC-conditioned medium (CM, 50%) compared to medium alone. **(B)** The effect of MSC-conditioned medium on the phosphorylation of mTOR and its downstream targets. For each patient, we compared the level of phosphorylation for AML cells cultured with MSC-conditioned medium versus control cells cultured in medium alone. The results are presented as the relative level, i.e., the levels for MSC-conditioned cultures versus control cultures. The value marked with an asterisk represents a value that was undetectable in the control; for simplicity, the ratio was set to the same value as the highest observed ratio in the data series.

with AML cells and MSC24539 cells cultured alone. Only two exceptional cytokines (HGF and VEGF, see **Table 2**) showed lower levels in the cocultures than in cultures of MSC24539 alone, and Tie-2 additionally showed higher levels in AML cell cultures than in cocultures. Third, supra-additive levels (i.e., cytokine levels in coculture supernatants exceeding the summarized levels for MSCs and AML cells cultured alone) were seen for several cytokines and reached statistical significance (Wilcoxon's signed-rank tests, $p \leq 0.005$ if not stated otherwise) when comparing the overall results for CCL3, CCL4 ($p = 0.043$), CXCL1, CXCL5, CXCL8, CXCL10, CXCL11 ($p = 0.014$), IL-1β, IL-6, IL-10, TNFα ($p = 0.037$), bFGF, G-CSF, GM-CSF, MMP-1, and MMP-2. Thus, even though individual differences among patients are maintained during coculture, several mediators show increased levels in cocultures and supra-additive levels are common.

To further investigate the patient heterogeneity, we performed a cluster analysis (**Figure 5**). For each mediator and patient, we determined the ratio between the mediator level in coculture relative to the sum of the levels for MSC24539 and AML cells cultured alone. These ratios were log(10) transformed before the clustering; hence a value >0 indicates a supra-additive effect. The patients then separated into two main clusters: (i) one subgroup of 22 patients characterized by strong supra-additive effects for several cytokines, especially, CXCL1/5, IL-1β/10, TNFα, MMP-1, G-CSF, and GM-CSF and (ii) another patient subset with generally weaker effects. The patient subgroup with strong supra-additive effects was also (according to χ^2 likelihood analyses) characterized by a significantly higher number of patients (17/22 versus 12/29, $p = 0.009$) showing detectable proliferation when cultured in the FBS-containing MSC medium alone without MSCs and also a higher number of patients (10/22 versus 0/29, $p < 0.0001$) with high AML cell proliferation exceeding >20,000 cpm in the cocultures. The supra-additive subset also showed a higher fraction of patients with cell viability >50% after coculture with MSCs (16/22 versus 11/29, $p = 0.012$). Finally, monocytic differentiation (FAB-M4/M5) was also more common among these patients (10/22 versus 5/29, $p = 0.019$). However, even though there was an association between supra-additive cytokine levels and high AML cell viability/proliferation in the cocultures, these supra-additive levels do not simply reflect increased proliferation/viability because supra-additive levels would then be expected for all the mediators and not only for a subset as we observed.

The Cytokines Important for MSC-Mediated Growth Enhancement of AML Cells Differ among Patients

To further study the cytokine-mediated crosstalk between MSCs and AML cells, we investigated the effects of cytokine-neutralizing antibodies and receptor antagonists on AML cell proliferation and viability in transwell cocultures with MSC24539. We then tested inhibitors of cytokines released by MSCs and being able to modulate AML cell proliferation (31–35), including (i) antibodies against VEGF, HGF, bFGF, and IL-6; (ii) the CCR1 antagonists BX471 (17) and BX513 (18); (iii) the combined CCR1 and CCR3 antagonist UCB35625 and its stereoisomer J113863 (19, 20); and (iv) the CXCR4 antagonist AMD3100 (13). The initial experiments included AML cells from eight patients that showed increased proliferation in cocultures corresponding to at least 3,000 cpm; this selection was made to be able to detect an inhibitory effect. Inhibition of AML cell proliferation for at least four patients was seen for anti-bFGF, anti-IL-6, the CCR1/CCR3 inhibitor J113863, and the CCR1 antagonists BX471 and BX513, whereas the CXCR4 inhibitor AMD3100 inhibited MSC24539 proliferation for four patients. These agents were further tested for 24 additional patients with an AML cell proliferation of at least 2,000 cpm in coculture. All but two of these patients then showed MSC-induced growth enhancement. When analyzing the overall results, none of the antibodies/inhibitors had any statistically significant effect, but the following observations were made for single mediators:

- *Normal karyotype*. The patients were heterogeneous with regard to karyotype. The only subset being large enough for statistical analysis was the 15 patients with normal karyotype, and for this subset, anti-IL-6 had a significant antiproliferative effect (**Figure 6A**, Wilcoxon's test, $p = 0.027$) of borderline significance.
- *Patients with and without NPM1 mutations. NPM1* insertions were detected for 13 of the 32 patients, and all of them had normal karyotype. As would then be expected, anti-IL-6 had an antiproliferative effect for *NPM1*-mutated patients,

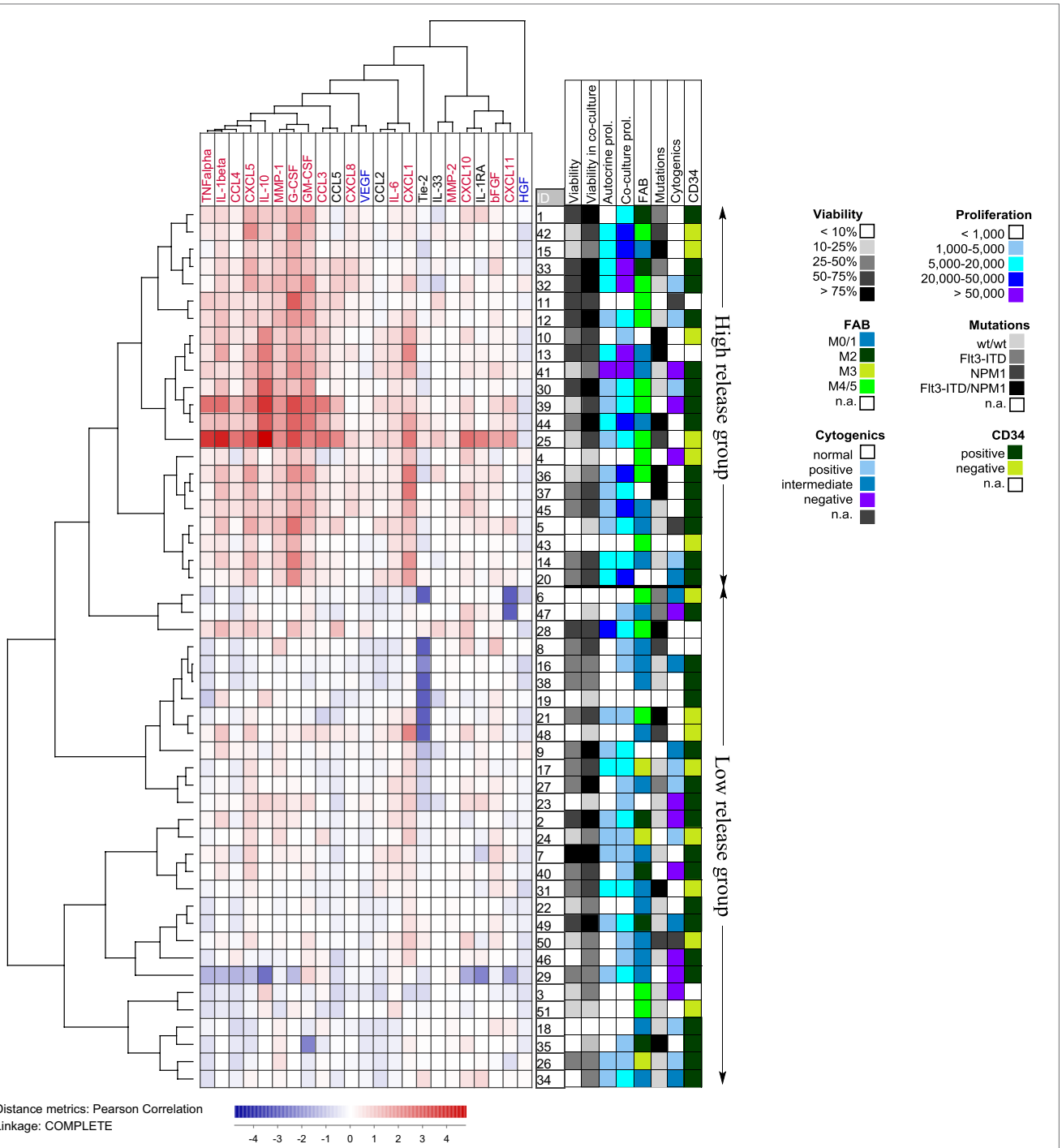

FIGURE 5 | Hierarchical clustering analysis based on the ratio of cytokine levels in acute myeloid leukemia (AML)-mesenchymal stem cell (MSC) cocultures divided by the concentration sums of AML cells and MSCs cultured alone; primary AML cells were derived from 51 patients and cocultured with MSC24539. Each horizontal row in the figure represents the observation for one patient, and the vertical columns represent the observations for the soluble mediators. Red indicates supra-additive effects in coculture. Likewise cytokines marked in red showed supra-additive effect on the total patient cohort, whereas mediators marked in blue showed higher levels in MSC cultures alone than in coculture. The patients clustered into two main groups (see right part of the figure) indicating high and low relative coculture cytokine levels, respectively. The figure also shows the distribution of various biological characteristics between the patient subsets (AML cell viability after 72 h of culture in medium alone and in coculture, proliferative capacity in medium alone and in coculture, FAB classification, genetic abnormalities, and expression of the CD34 stem cell marker).

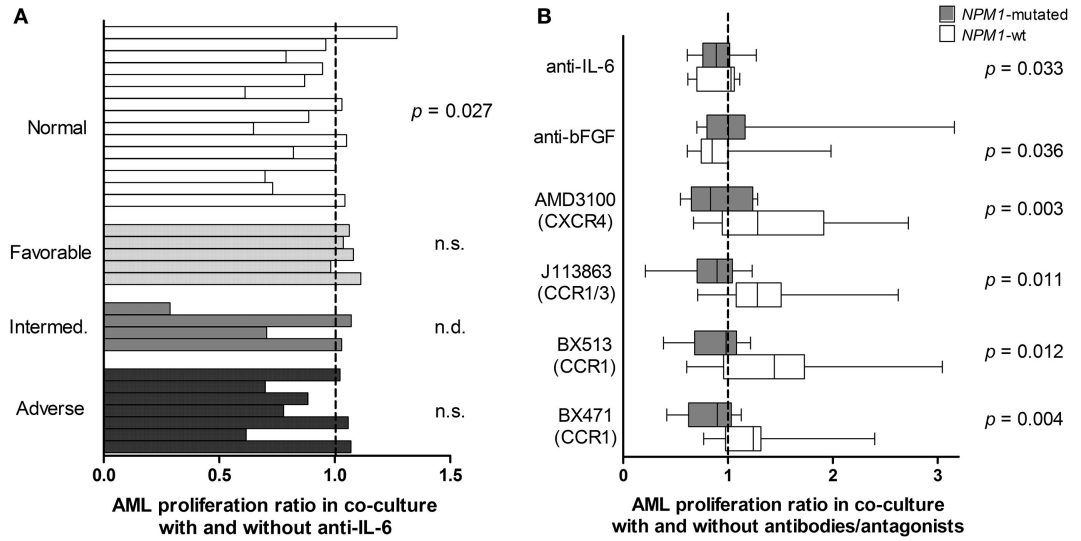

FIGURE 6 | Cytokine-targeted treatment during coculture of primary human acute myeloid leukemia (AML) cells and bone marrow mesenchymal stem cells (MSCs); a comparison of patients with different karyotype (A) and patients with and without *NPM1* mutations. **(A)** Proliferative AML cell responses were compared for cocultures prepared with an IL-6 specific antibody and control antibody. The study included patients with normal AML cell karyotype ($n = 15$), favorable ($n = 5$), intermediate ($n = 4$), and adverse ($n = 7$) cytogenetics. The results for individual patients are presented as the proliferative response in cultures with anti-IL-6 relative to the proliferation in control cultures. The IL-6-specific antibody decreased the proliferation only for patients with normal cytogenetics but not for the other patients (Wilcoxon's test for paired samples). **(B)** AML cell proliferation was also compared for patients with (gray columns) and without *NPM1* mutations (white columns). Decreased AML cell proliferation was seen for several cytokine-directed treatment, especially chemokine-directed interventions (Wilcoxon's test for paired samples for IL-6/bFGF, χ^2 likelihood analyses for the chemokines). The box-plots show the median value, the 25 and 75% quartiles and the variation range (minimum to maximum value).

but the statistical comparison reached only borderline significance (Wilcoxon's test, $p = 0.033$). The same was true for anti-bFGF, which had an antiproliferative effect for patients with *NPM1*-wt (**Figure 6B**, Wilcoxon's test, $p = 0.036$). Because several patients showed a relatively strong effect of chemokine-targeting pharmacological intervention we used statistical analysis based on categorized data when comparing *NPM1*-mutated and *NPM1*-wt patients. A significant alteration of AML cell proliferation was then defined as a difference having an absolute value of >2,000 cpm and in addition being >20% of the corresponding control. *NPM1*-wt was then associated with increased and *NPM1* mutation with decreased proliferation for all four chemokine receptor antagonists: AMD3100 (CXCR4 antagonist; χ^2 likelihood analyses, $p = 0.003$), J113863 (CCR1/CCR3 antagonist, $p = 0.011$), BX513 (CCR1 antagonist, $p = 0.012$), and BX471 (CCR1 antagonist, $p = 0.004$). Thus, growth-modulating effects of several chemokines differed between *NPM1*-wt and *NPM1*-mutated patients.

- *FAB classification, CD34 expression, Flt3 mutations.* The effects of cytokine targeting showed significant associations neither with differentiation nor with *Flt3* mutations (data not shown).

We also did a clustering analysis of the overall results (**Figure 7**) showing that especially the effect of chemokine inhibition differed among patients; growth reduction by CCR1/CXCR4 inhibition was seen especially for a subset of patients with normal cytogenetics and *NPM1* mutations. Based on this analysis, our patients could be classified into three different groups (no or minor effect, decreased proliferation, increased proliferation); these three groups did not significantly differ with regard to constitutive release of supra-additive coculture levels for IL-6, bFGF, or chemokines. Thus, these differences among patients are not caused by differences in cytokine release during coculture. Based on our overall results, we conclude that AML patients are heterogeneous also with regard to the effect (i.e., responsiveness) of single cytokines on AML cell proliferation in the presence of normal bone marrow MSCs, but despite this heterogeneity the final/overall cytokine-mediated MSC effect is increased AML cell proliferation for the majority of patients.

Chemokine-Mediated Signaling Increases MSC Proliferation in the Presence of AML Cells

We further investigated the effects on MSC24539 proliferation of the same antibodies and antagonists as described above during coculture with AML cells from the same 32 patients. Anti-bFGF, anti-IL-6, and the CCR1 antagonist BX513 had only minimal effects (data not shown). In contrast, a significant antiproliferative effect was seen for the CXCR4 inhibitor AMD3100 ($p < 0.0001$), the CCR1/CCR3 inhibitor J113863 ($p = 0.009$), and the CCR1 antagonist BX417 ($p = 0.015$). Statistical analyses based on categorized data (i.e., increased, unaltered, decreased proliferation) showed associations between the CXCR4 mediated

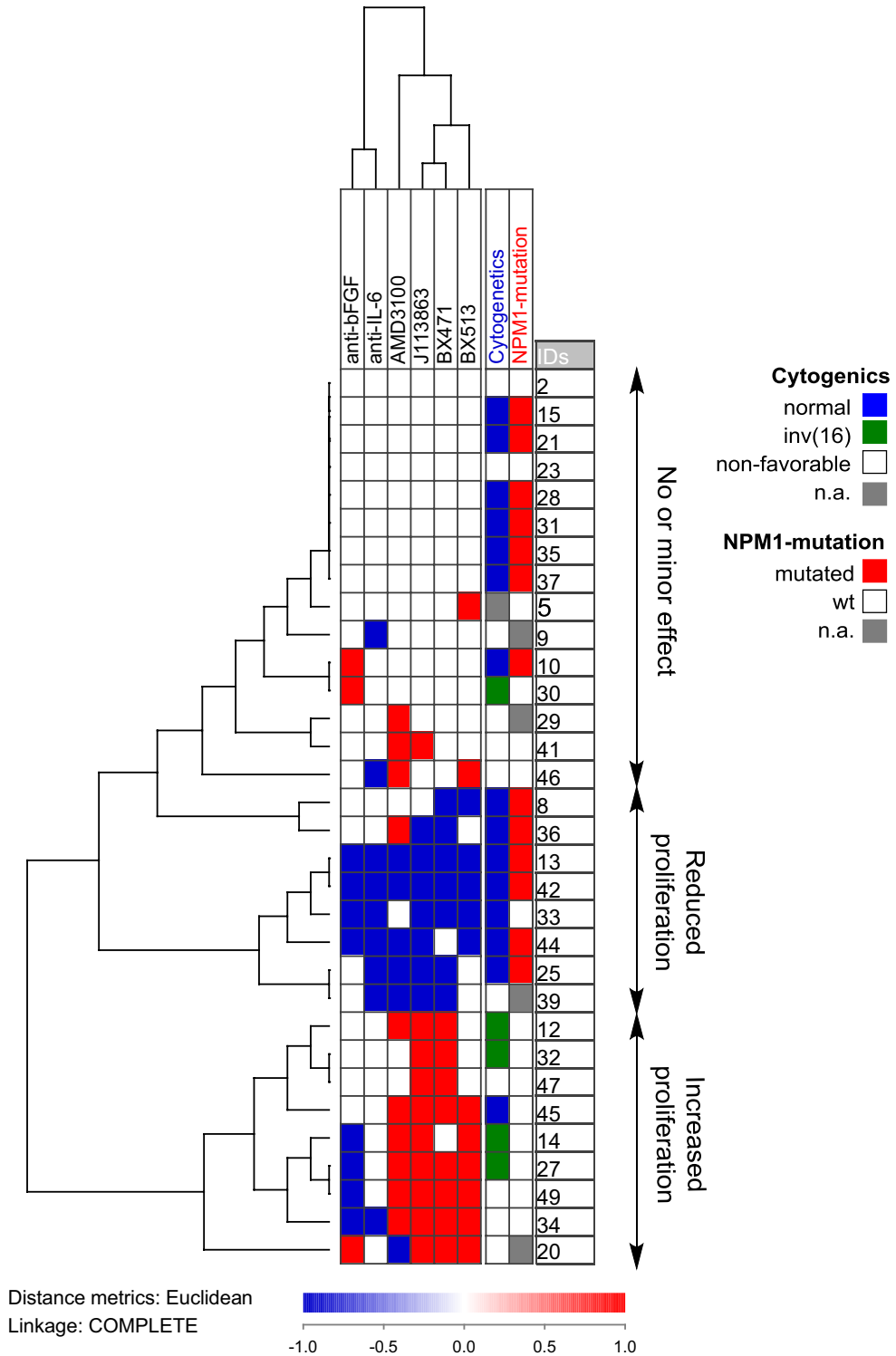

FIGURE 7 | Hierarchical clustering of the effects of anti-cytokine treatment (neutralizing antibodies or receptor blocking) on acute myeloid leukemia (AML) cell proliferation during coculture with normal mesenchymal stem cells. AML cells derived from 32 AML patients were examined. Each horizontal row in the figure represents the observations for one patient, and the vertical columns represent the observations for each of the antibodies and receptor antagonists. Red indicates increased proliferation corresponding to an increase in the absolute value of >2,000 cpm and >20% proliferation increase compared to the control cultures, whereas blue indicates reduced proliferation according to the same definitions. The patients clustered into three main groups (see right part of the figure): one group (above) showing only minor effects, a second group (middle) showing reduced proliferation in the presence of both antibodies and receptor antagonists, and a last group (below) that showed increased proliferation especially for chemokine inhibition.

antiproliferative effect and monocytic AML cell differentiation ($p = 0.009$) and favorable karyotype ($p = 0.025$). These observations suggest that CCR1-, CXCR4-, and possibly CCR3-mediated signaling is important for the regulation of MSC proliferation in cocultures, and these results further illustrate that this bidirectional cytokine-mediated crosstalk between MSCs and AML cells affect not only the AML cells but also the MSCs.

DISCUSSION

Acute myeloid leukemia is a heterogeneous and aggressive malignancy; the long-term AML-free survival is only 50% even for younger patients who receive intensive chemotherapy and the majority of elderly patients who only receive leukemia-stabilizing treatment have a median survival of less than 1 year (36). Recent experimental studies suggest that both adhesion of AML cells to osteoblasts and MSCs (31, 37) and the release of soluble factors from the latter (38) are important for chemoresistance and thereby the risk of leukemia relapse from residual disease (35, 37). Malignant myeloid cells can also alter the functional characteristics of MSCs and thereby create a microenvironment that favors leukemic hematopoiesis (31, 39). Hence, therapeutic targeting of AML-supporting stromal cells may become a possible strategy to indirectly target the leukemia cells. In the present study, we therefore investigated the AML-supporting effects of bone marrow MSCs mediated through the local cytokine network for a large group of unselected leukemia patients.

Effects of stromal cells on primary human AML cells have also been investigated in previous studies (40–43), but none of these studies focused on the cytokine-mediated crosstalk between AML cells and MSCs. There were also several other differences: (i) only a low number of patients (41) or a low number of highly selected patients (42, 43) were examined; it is thereby difficult to address the question of patient heterogeneity; (ii) some of the studies used AML cell lines and not primary leukemic cells in parts of their experiments (42, 43); (iii) the previous studies used a stromal cell line (42, 43) or a single MSC donor (43). Thus, our present study extend the knowledge through its broader focus on the cytokine network and identification of mediators responsible for the leukemia-supporting effect of MSCs, studies of a large and unselected patient population, thereby addressing the question of patient heterogeneity, and the use of bone marrow MSCs derived from several donors.

Our viability assay has been described in detail in a previous publication (25). The AML cell population shows a hierarchical organization with a small number of leukemic stem cells, a minority of colony-forming proliferating cells and a majority of cells that shows spontaneous apoptosis during the first days of *in vitro* culture (25, 44). The number of viable cells will thereby decrease during culture and after four days of culture the viability will often be as low as 10–20% even for patients who show strong proliferative responses. This decreased viability during culture despite detectable proliferation shows that the viability of the total leukemic cell population mainly reflects the characteristics of the non-proliferating majority of AML cells and not the proliferation of a minor subset, i.e., survival and proliferation should be regarded as only partially overlapping characteristics. This is also supported by our recent results where a relatively small increase in AML cell viability during coculture was not exclusively seen for patients with undetectable proliferation but also for several other patients, and even patients with undetectable proliferation showed significantly increased viability (and several of them showed a relatively strong increase) after coculture with MSCs.

We used an experimental model based on transwell cocultures where MSCs and AML cells were separated by a semipermeable membrane; effects mediated through the bidirectional cytokine-mediated crosstalk could thereby be studied without any influence of additional effects mediated through direct cell contact. We used a culture medium supplemented by inactivated FBS and L-glutamine that is suitable for culture of both MSCs and primary AML cells (16). The MSCs then mediated antiapoptotic effects for all but three of the 51 AML patients included in the study. Furthermore, a large majority of patients (40 out of 51) showed growth enhancement in the presence of bone marrow MSCs, and this enhancement was seen both for short- and long-term AML cell proliferation, and for three different healthy MSC donors tested in independent experiments. The last 11 patients did not show detectable AML cell proliferation either in medium alone or during coculture with MSCs.

Primary AML cells show spontaneous or stress-induced apoptosis during *in vitro* culture, and our results demonstrated that MSCs can reduce this apoptosis. Our present results demonstrate that this antiapoptotic effect is also seen in the presence of cytarabine, i.e., the MSCs can also rescue primary AML cells from the combined effect of spontaneous and drug-induced apoptosis. However, we tested a cytarabine concentration that corresponds to systemic (i.e., serum) cytarabine levels reached during *in vivo* chemotherapy (50 nM), and in addition, a concentration corresponding to the steady-state levels during conventional cytarabine treatment with 100–200 mg/m^2. A reduction of cell viability corresponding to more than 5% was seen only for approximately half of the patients when testing 50 nM cytarabine in the initial dose-response experiments in transwell cocultures, and in direct-contact cocultures. Both our experiments with transwell cocultures and cytarabine but especially the direct-contact experiments with 500 nM cytarabine showed that MSCs can counteract the proapoptotic effect of cytarabine, and at least for certain patients the cytokine-mediated crosstalk contributes to this effect. However, further studies are required to characterize and explain this patient heterogeneity and to clarify whether this variation is seen also for other chemotherapeutic agents.

The local cytokine network was also altered during AML-MSC coculture; the levels of three mediators were relatively low (Tie-2, HGF, and VEGF), whereas especially CXCL1/5, IL-1β/10, TNFα, MMP-1, G-CSF, and GM-CSF showed supra-additive effects in cocultures. A strong supra-additive effect for a subset of the mediators was especially seen for a patient subset also characterized by monocytic differentiation, high cell viability in coculture, and strong AML cell proliferation both when cultured in medium alone and in the cocultures with MSCs. The constitutive mediator release by AML cells cultured alone also differed considerably among patients, and despite the supra-additive effect for several cytokines, there was still a significant correlation for all but one mediator between the levels for AML cells cultured alone and

in cocultures. Thus, individual differences between patients with regard to constitutive cytokine release by the AML cells are also maintained during coculture with MSCs.

We characterized the cytokine network in MSC/AML cell transwell cocultures in a previous study that also included a characterization of the effect of the AML-MSC crosstalk on the MSCs (16). The study included a smaller number of patients, but the effects of AML cells on the MSC showed only a limited variation among patients. Both MSCs and AML cells showed to contribute to the altered cytokine network during coculture, and the relative importance of MSCs and leukemic cells differed among cytokines. First, both primary human AML cells and bone marrow MSCs showed constitutive release of several cytokines, but the leukemic cells often showed a broader constitutive release profile than the MSCs (16). Second, the constitutive release by normal MSCs shows relatively small variations among individuals (16), whereas the constitutive leukemia cell release profile differed considerably among patients (16, 45). Third, cocultured MSCs showed increased mRNA expression of several cytokines, especially CCL and CXCL chemokines, as well as increased expression of several mediators of the cytokine-inducing NFκB pathway (16); signaling through this pathway induces increased constitutive release of several cytokines (45). Finally, there is a wide variation among patients in the constitutive release of several cytokines by their AML cells, and this variation is often maintained also in the presence of MSCs. Thus, the cytokine levels in our cocultures are probably determined by an NFκB-induced increase in the constitutive release by the MSCs and by maintenance of differences among patients in the constitutive release by the AML cells.

We investigated possible molecular mechanisms behind the growth-enhancing and antiapoptotic effect of MSCs on primary human AML cells. These experiments included leukemic cells from 10 patients. The PI3K–Akt–mTOR pathway is often constitutively activated in AML cells and is important for cell survival, proliferation and metabolism (46, 47). We therefore compared the activation/phosphorylation of Akt, mTOR, and mediators downstream to mTOR (S6K1, 4E-BP1) after incubation with medium alone or 50% MSC-conditioned medium. Akt phosphorylation did not differ among patients, whereas increased phosphorylation was detected for mTOR and its downstream mediators.

We also compared the effect of MSC-conditioned medium on the phosphorylation of the H2AX histone that is phosphorylated by the ATM kinase as part of the DNA damage response. There will be a background or constitutive level of H2AX phosphorylation, and this level seems to reflect a DNA damage response initiated by endogenous formation of reactive oxidant species as a byproduct from oxidative phosphorylation (48). However, DNA damage response with increased H2AX phosphorylation can also be an early event during apoptosis (29). Primary human AML cells seem to have a suppressed DNA damage response due to increased expression of double-stranded RNA-activated protein kinase, and high expression of this kinase in the AML cells is thereby associated with an inhibited DNA damage response reflected as a low percentage of H2AX phosphorylation (49, 50). H2AX phosphorylation can also be seen without DNA damage (51). In our experiments we observed a significant increase in H2AX phosphorylation after culture with MSC-conditioned medium (Wilcoxon's test; $p = 0.005$). However, this increase of H2AX phosphorylation combined with increased cell viability suggests that the increased H2AX phosphorylation is not an effect of increased apoptosis. In our opinion, the most likely explanation is a reversal of the AML-associated decrease in the DNA damage response; this may be caused by IL-6 that is present at high levels in MSC-conditioned medium (see **Table 2**) and which is known to strengthen the DNA damage response (30). An alternative explanation could be that increased mTOR mediated signaling causes increased oxidative phosphorylation and thereby increased constitutive (i.e., oxidative) DNA damage (52). Thus, both increased mTOR activation and increased DNA damage responsiveness can contribute to the effects of MSCs on primary human AML cells.

Finally, cytokine neutralization and receptor blocking showed that AML cells derived from different patients were heterogeneous with regard to the effects of various cytokines on AML cell viability/proliferation. One of the cytokines with differential effects was CXCL12, which is highly released by MSCs. Even though cells with *Flt3*-ITD have shown increased CXCR4 expression (13) and signaling through the CXCL12-CXCR4 axis, the differential effect of CXCR4 blocking showed no significant association with *Flt3*-ITD.

Several soluble mediators that were detected at relatively high levels in our cocultures have been linked with remodeling of the bone marrow stem cell niche into a leukemia-permissive niche: (i) angiogenic growth factors like VEGF (53, 54), bFGF, Ang-1, and its receptor Tie-2 (31); (ii) IL-6 (32), which also can upregulate VEGF levels (55); (iii) CCL3, which is thought to expand MSCs and drive them into a leukemia-supporting phenotype (39); (iv) CXCL12, which is constitutively expressed by MSCs (37), causes homing of leukemic cells to the bone marrow (56, 57) and functions as a regulator of proliferation, cell cycle progression, and survival of leukemic cells (35, 58). Our previous studies could not detect any evidence for osteoblastic or adipocytic MSC differentiation during MSC/AML cell cocultures, even though the global gene expression profile of normal MSCs was seen after coculture of MSC and AML cells in transwell cultures (16).

MSCs showed constitutive release of several mediators, and we used cytokine-neutralizing antibodies or receptor-blocking agents to identify cytokines that contributed to the antiapoptotic and growth-enhancing effect of MSCs on the AML cells. We then investigated cytokines that were released by MSCs at relatively high levels, showed high levels during coculture and are known to function as growth factors for primary human AML cells. First, antibodies against HGF and VEGF tested separately had no or only minimal effects on AML cell proliferation and were only examined in the initial experiments. Second, IL-6 neutralization inhibited AML cell proliferation for the subset of patients with normal cytogenetics, whereas reduced proliferation upon bFGF neutralization was only observed in *NPM1*-wt cells and this is consistent with previous observations of AML cells cultured alone (33). Third, the effects of several chemokine receptor blockers (including CXCL12/CXCR4 blocking) also differed among patients, especially when comparing patients with and without *NPM1* mutations. Based on the overall results, we conclude that AML patients are heterogeneous with regard to effects

of individual cytokines on AML cell viability and proliferation during MSC/AML cells coculture. However, despite this variation, the final overall cytokine-mediated effects of the MSCs are increased viability and growth enhancement probably caused by a combined effect of several cytokines, and the cytokines contributing to this effect seem to differ among patients.

In contrast to the divergent effects of cytokine neutralization/blocking on AML cells, these interventions had more uniform effects on MSC proliferation during coculture as MSC proliferation significantly decreased in response to chemokine receptor blocking (CCR1, CCR3, CXCR4) agents. Both AML cells and MSCs show constitutive release of several ligands for these receptors, suggesting that autocrine or paracrine loops involving these receptors are important for the regulation of MSC proliferation (59). This hypothesis is also supported by the observation that CCL3 expression by malignant myeloid cells is linked with higher MSC growth rates (39).

Systemic plasma levels of both IL-6, bFGF, and several chemokines have been investigated in human AML. IL-6 levels are increased in patients with untreated AML and high levels seem to be associated with decreased survival (60). In contrast, the results for plasma bFGF levels are conflicting and both normal and increased plasma levels have been described for patients with untreated AML, but none of these studies have described any prognostic impact of bFGF levels (61–63). As reviewed by Reikvam et al. (64), several studies have investigated the systemic (plasma or serum) levels of various chemokines; besides normal, both increased and decreased levels have been described for most of the investigated CCL and CXCL chemokines in patients with untreated AML. Even though only a small minority of patients (<15%) shows detectable CXCL12 release during *in vitro* culture and most of these patients show only low release (45), increased serum CXCL12 levels have been described in patients with untreated AML, including increased levels of the cleaved active form. These observations may suggest that constitutive AML cell release of these cytokines has clinical relevance in human AML. However, the observations should be interpreted with great care because these cytokines can be released by several normal cells and not only AML cells, some of these cytokines may be a part of the acute phase reaction, and the systemic levels reflect the binding between release and binding/degradation, Thus, altered systemic levels may not reflect AML cell release or the local levels in the bone marrow microenvironment.

CONCLUSION

Our present study shows that even though AML is a heterogeneous disease and the response of primary AML cells to the various MSC-derived cytokines during MSC-AML cell coculture differs among patients, the final effect of MSC-derived cytokines on primary AML cells is increased proliferation and viability. Our overall results suggest that therapeutic targeting of the cytokine-mediated AML-supporting effects probably as MSC-directed strategies which inhibit the release of several cytokines, or alternatively receptor blocking could be tried in selected patients.

AUTHOR CONTRIBUTIONS

ØB designed the study and wrote the paper. IN performed the phosphoflow experiments and analyzed the results. AB performed the other experiments, analyzed the results, and wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00106/full#supplementary-material.

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