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HBD-3 induces NK Cell activation, IFN-γ secretion and mDC dependent cytolytic function

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Abstract

We previously showed that human beta defensin-3 (hBD-3) activates mDC via TLR1/2. Here we investigated the effects of hBD-3 on NK cell activation state and effector functions. We observed that hBD-3 activates PBMC to secrete IFN-γ and kill K562 and HUH hepatoma target cells in an NK dependent fashion, and both TLR1/2 and CCR2 are involved. TLR1, TLR2 and CCR2 were expressed on NK cells, and in purified NK culture experiments we observed hBD-3 to directly act on NK cells, resulting in CD69 upregulation and IFNγ secretion. We also observed mDC-hBD-3 enhanced NK cytolytic activity and IFNγ production. These results implicate hBD-3 in its ability to directly activate NK cells and increase NK cell effector function, as well as promote mDC-dependent NK activity. HBD-3 may therefore act as a mediator of innate cell interactions that result in bridging of innate and adaptive immunity.

INTRODUCTION

NK cells make up 5–10% of the peripheral blood mononuclear cell fraction in humans (1). In addition to killer activity, recent data indicate that these cells play a role in forming the adaptive immune response through immune modulation (2). Human NK cells can be categorized into three subsets based on expression of CD56 and CD16 (2). Within the lymph node and other secondary lymphoid organs, $CD16^{-}56^{bright}$ NK cells are the most prevalent NK subset found, and are capable of secreting large amounts of cytokines that can modulate formation of the adaptive immune response (1,2). In the peripheral blood, $CD16^{+}CD56^{dim}$

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NK cells are the dominant NK subset, and they provide greater cytotoxicity activity than $CD16^{-}56^{bright}$ or $CD16^{+}56^{-}$ subsets (1,2).

Immature dendritic cells (DCs) are located in peripheral tissue and mucosal surfaces, where they may be exposed to pathogens or microbial products that lead to activation and initiation of the adaptive immune response (1). Key to this process is the recognition of pathogen-associated molecular patterns, mediated in part by toll-like receptors (TLRs)(1). Myeloid dendritic cells (mDC) are a main peripheral DC subset that, in addition to having potent naive T cell activating activity, also are capable of activating natural killer (NK) cells (3,4).

Human β-defensins (HBDs) are antimicrobial peptides (AMPs) found at mucosal surfaces that are most commonly produced by epithelial cells (5–8). Some hBDs are constitutively expressed (eg. hBD-1), while others, such as hBD-2 and hBD-3, are induced by microbial products, inflammatory cytokines, or epidermal growth factor (9). HBD functions include direct killing of microorganisms and chemo-attraction of immature dendritic cells and T cells or monocytes via binding to chemokine receptors CCR6 and CCR2 respectively (10–12). These properties of hBDs imply a role for these AMPs in bridging innate and adaptive immunity (5,8,13). We previously showed that hBD-3 activates mDC via TLR1/2 (5). NK cells are also reported to express these TLRs and can respond to TLR ligands, resulting in IFNγ secretion and cytotoxic function (1,2,9,14–18). Additionally, NK cells express CCR2 (19–21). NK-mDC bidirectional interaction, or cross-talk, involves both cell contact and cytokine-mediated communication, which can occur upon pathogen or tumor exposure (1,22,23). This interaction results in NK activation and enhanced NK effector function. We examined here how hBD-3 affects NK cell activation, cytolytic activity, and IFNγ production, focusing on CCR2 and TLR1/2. Our data indicate that hBD-3 can act as a direct positive modulator of NK cell activation and IFNγ production, and facilitate mDC-dependent NK cytolytic function.

METHODS

HBD-3-induced PBMC IFNγ and killer activity

This work was approved by the University Hospitals of Cleveland Institutional review board. Peripheral blood samples (100ml) were obtained from healthy individuals who provided written informed consent.

Freshly Ficoll (Fisher Scientific, Hudson, NH) prepared PBMC (3×10^5) were cultured overnight at 37°C with or without recombinant or synthetic hBD-3 (10–20μg/mL) or R-848 (imidazoquinoline compound, Resiquimod, 1μg/ml, InvivoGen, San Diego CA USA). This concentration range for hBD-3 was chosen based upon our prior analysis of TLR-dependent myeloid cell activation (5), while R-848 concentration was selected based upon our prior experience with this TLR ligand and activation of DC (24,25). Recombinant hBD-3 was prepared as previously described (5), as a single band on SDS-PAGE, and when analyzed for LPS contaminant, <0.001 EU LPS/ug hBD-3 was observed. Synthetic hBD-3 (Peptides International, Louisville, KY) was utilized as a source of protein prepared without bacterial culture.

Culture supernatant IFNγ levels were measured by ELISA. PBMC (2×10^5) were also cultured in the presence of 1×10^4 Huh7.5 hepatoma cells (provided by CM Rice, Apath LLC, St. Louis, MO) for 5 hours, and supernatants were collected to evaluate cytotoxic function via M30 ELISA (DiaPharma), which detects epithelial cell caspase-cleaved cytokeratin-18. 2.5×10^4 K562 target cells (ATCC, Manassas, VA) were also cultured with 5×10^5 PBMC to measure cytotoxic function. For the latter, K562 target cells were stained with PKH26 (Sigma, St. Louis, MO) and added to the pre-cultured PBMC at 20:1 effector to target (E:T) ratio and cultured an additional 2 hr at 37°C. Cells were then removed and stained 15 minutes at room temperature with Annexin V (BD Biosciences Pharmingen, San Diego, CA). Killing activity was measured by quantifying the proportion of PKH26 positive target cells that were Annexin V positive by flow cytometric analysis using an LSRII flow cytometer (Becton Dickinson, San Jose, CA). For depletion experiments, mDC were depleted from PBMC via BDCA-1 bead negative selection or NK cells were depleted from PBMC by CD56 negative selection (Miltenyii Biotech, Auburn, CA) (>90% depletion efficiency for each).

IFNγ Enzyme-linked Immunospot Assay (ELISPOT)

PBMCs (3×10^5) or NK cells (1×10^5) were plated with or without the presence of hBD-3 or R848 or IL12 (1ng/mL) and IL15(10ng/mL) in ELISPOT plates precoated with IFNγ capture mAb (Human IFNγMAb, clone 2G1, 4μg/ml, Thermo Scientific, Rockford, IL) and cells were cultured 20 hrs at 37°C. TLR1/2 blocking antibodies (anti-TLR-1, GD2.F4, anti-TLR-2, T2.5, 20μg/ml, eBioscience, San Diego, CA), isotype control, and CCR2 antagonist (RS 102895 hydrochloride, 20mM, Sigma-Aldrich, St. Louis, MO), or solvent (DMSO) control, were included throughout the culture when indicated. Secondary antibody was added and spots were detected as previously described (26).

mDC-NK co-culture assays

mDC were purified from PBMC by bead selection method using the BDCA1 isolation kit (Miltenyi Biotech, Auburn, CA) (>85% purity), and NK cells were prepared from PBMC by bead negative selection method (Miltenyi Biotech, Auburn, CA) (>median 94% purity) as previously described (27), or by bead purification followed by staining with anti-CD3-APC, CD56-PE and CD16-FITC (BD Biosciences), followed by flow cytometry assisted cell sorting (FACS Aria cell sorter, BD Bioscience) of $CD3^-CD16^+CD56^+$ cells (>99% purity). 2×10^4 mDC and 1×10^5 NK cells were either cultured at 37°C 16h alone or co-cultured with or without the presence of hBD-3. Supernatant IFNγ levels were measured by ELISA. hBD-3 treated NK cells and mDC were also cultured in the presence of 1×10^4 Huh7.5 hepatoma cells for 5 hours, and supernatants were collected to evaluate cytotoxic function via M30 ELISA (DiaPharma) as previously described (28). NK isolated populations were also cultured with or without the presence of hBD-3. In additional experiments, NK cells were also cultured with Pam3cysk (model TLR1/2 ligand, 10ng/mL) and MCP1 (CCR2 ligand, 100ng/mL). Anti-TLR1/2 (20μg/mL) blocking antibodies, isotype control, CCR2 antagonist (20mM), or solvent control were included as indicated. After culture, NK cells were stained with CD56-PE-Cy7 (NCAM16.2) and CD69-PE (L78) (BD Biosciences) and analyzed for CD69 expression by flow cytometry.

TLR1/2 and CCR2 Expression

PBMCs were stained with anti-HLA-DR-PerCP (L243), -LIN-1-FITC (CD3, CD14, CD16, CD19, CD20, CD56), -CD11c-AlexaFluor700 (B-ly6), -CD3-PerCP (SK7), -CD56-PE-Cy7 (NCAM16.2), -CD16-FITC (3G8) (BD Biosciences), -TLR1-PE (GD2.F4), -TLR2-APC (TL2.1) (ebioscience) and -CCR2-PerCP-Cy5.5 (TG5, Biolegend, San Diego, CA) monoclonal antibodies to detect mDC and NK cell TLR1, TLR2 and CCR2 expression, and analyzed by flow cytometry on a BD LSRII using FACS Diva software. TLR1, TLR2, and CCR2 expression was analyzed based on fluorescence minus one (FMO).

Statistical methods

Statistical analyses were performed with SPSS for Windows V. 21.0 (SPSS Inc). We used Wilcoxon Signed Rank related samples test for non-parametric comparison of related continuous variables within groups, and Spearman rank correlation coefficient to analyze associations between continuous variables. All tests of significance were two-sided and *p* values < 0.05 were considered significant.

RESULTS

HBD-3 induces PBMC cytotoxicity activity and NK dependent IFNγ production

Given that our previous work has shown that hBD-3 activates mDC via TLR1/2 (5), and that NK cells are also found at mucosal sites and express TLRs (1,14–18), we first examined whether hBD-3 can promote NK cell effector function by evaluating hBD-3-treated PBMC for IFNγ production and cytotoxic function. Since R848 is a TLR7/8 agonist that is known to induce accessory cell dependent NK effector functions (29–32), we included this ligand as a control. PBMC were treated with media, hBD-3 or R848, and supernatants were analyzed for IFNγ by ELISA. We found that both R848 (p=0.001) and hBD-3 (p=0.02) induced PBMC IFNγ secretion (Fig. 1A). To address the possibility of whether contaminant microbial products within recombinant hBD-3 contributed to activity, we compared recombinant hBD-3-induced PBMC IFNγ activity to PBMC IFNγ production in response to chemically synthesized [synthetic hBD-3 (shBD-3)]. Both shBD-3 and recombinant peptide induced activities strongly correlated with each other (r = 0.89, p = 0.001, supplemental Figure 1), indicating the hBD-3 peptide itself is responsible for the activity.

We further analyzed the effect of hBD-3 on PBMC cytolytic activity. Similar to R848, when PBMC from subjects with an hBD3 induced IFN-γ response were treated with hBD-3 prior to target cell co-culture, hBD-3 stimulation resulted in a modest trend towards an increase in K562-target cell death (p=0.2, Fig. 1B), and a significant increase in Huh7.5 cell death (p=0.04, Fig. 1C). These results indicated that hBD-3 induces PBMC effector functions. We next inquired to what extent NK cells played a role in these hBD-3 enhanced PBMC activities. Upon NK cell-depletion, hBD-3 mediated IFNγ activity was completely eliminated (Fig. 1D). These data indicate that NK cells contribute to the hBD-3 induced PBMC function.

HBD-3 directly activates NK cells

Since NK cells were necessary for hBD-3-mediated PBMC IFNγ, we next evaluated whether hBD-3 can also directly induce expression of the activation marker CD69 on bead purified cells (Fig. 2a–b). We found that treatment of purified (median purity 94%, Fig. 2a) NK cells with hBD-3 consistently upregulated CD69 expression (p<0.001, Fig. 2b). These results suggest that hBD-3 directly activates NK cells. In experiments of concentration dependence, we observed 10μg/mL of rhBD-3 to be optimal (data not shown). To address the possibility that minor cell contaminants of the NK cell preparations could be playing a role in mediating hBD-3 induced NK activity, we also analyzed hBD-3 mediated activity of highly pure (>99%) flow sorted $CD16^{+}56^{dim}$ NK cells in select subjects who displayed the strongest hBD-3-mediated CD69 upregulation seen in Fig. 2b. We observed that hBD-3 also induced CD69 expression on these highly pure flow sorted NK samples (p=0.016, data not shown), further in support of direct cellular activation.

NK cell IFNγ production is enhanced by HBD-3

We next evaluated purified NK cell IFNγ production upon treatment with hBD3. We compared NK cells treated with hBD-3 to NK cells stimulated with IL-12 and IL-15, potent inducers of NK cell IFNγ production (2). As expected, IL-12 and IL-15 dual treatment significantly enhanced NK cell IFNγ production, with a mean magnitude of 162 IFNγ spot-forming units (SFU), compared to 1.25 SFU in media treated cells (n=6, p=0.03, data not shown). We further found that hBD-3 treatment leads to a direct enhancement of NK cell IFNγ production by ELISPOT assay (p=0.0156, Fig.3a). We also measured hBD3 induced NK IFNγ secretion by ELISA, and found a trend towards hBD3-mediated NK IFNγ production (p=0.074, data not shown). We were interested to see if there was enhancement of this hBD3-induced NK IFNγ production by presence of mDCs. Indeed, we found that the presence of mDCs in hBD3-treated NK cultures significantly enhanced the ability of NK cells to secrete IFNγ (p=0.016, Fig.3b). Furthermore, hBD3 was able to increase IFNγ produced by NK-mDC cocultures (p=0.039, Fig.3c).

hBD3 promotes mDC-dependent NK cell cytotoxic function

Since we observed hBD3-mediated PBMC cytotlytic activity against both K562 and Huh7.5 target cells (Fig. 1b–c, respectively), we next focused on the potential ability of hBD-3 to enhance NK cell cytolytic activity. hBD-3 treatment of purified NK cell cultures did not increase NK cell lysis of Huh7.5 target cells (Fig. 4a). To determine if the observed hBD-3-induced PBMC killer activity was dependent on mDC-NK interactions, we compared cytolytic activity of hBD-3 treated NK cell cultures to hBD-3 treated NK-mDC co-cultures. Interestingly, we found that the presence of mDCs significantly enhanced hBD-3 induced NK cell cytolytic function (p=0.002, Fig.4b). Likewise, hBD-3 also enhanced mDC-dependent NK cytolytic activity (p=0.0273, Fig. 4c). Together, these data support that hBD3 promotes mDC-dependent NK cell cytotoxic function.

HBD-3 induced PBMC and purified NK activity are dependent on TLR1/2 and CCR2

hBD-3 is known to activate cells via TLR1/2 and CCR2 (5,10,33) and NK cells have been shown to express these receptors (1,29,14–21). To verify the expression level of TLR1, TLR2 and/or CCR2 on freshly prepared NK cells in our system, we analyzed cell surface expression by flow cytometry (Fig. 5a–d). We first focused on CD3^{-}CD16^{+}56^{dim} NK cells, the dominant subset in the peripheral blood (Fig.5b). NK cells routinely displayed expression of CCR2 in similar fashion to that observed on mDC (Supplemental Fig. 2). In contrast, TLR1 and TLR2 expression were more variable on NK cells than on mDC, with some subjects showing little TLR1 expression, while others demonstrating higher levels of expression (Fig. 5b,d). Similar data regarding TLR1/2 and CCR2 expression were observed on the CD16^{-}56^{bright} NK subset, while the CD16^{+}56^{-} subset exhibited less variation in expression of these receptors compared to the other two NK subsets (Fig. 5c–d). These data suggest TLR1/2 and CCR2 expression could potentially play a role in hBD-3 induced NK activity.

To test the role of TLR1/2 and CCR2 in hBD-3 induced NK activity, purified NK cell monocultures (n=11) were treated with hBD-3 in the presence or absence of TLR1/2 or CCR2 blockade. Both TLR1/2 and CCR2 inhibition were able to reduce hBD-3 induced CD69 expression (p=0.009 and p=0.001, respectively, Fig. 6a). Notably, in control experiments R848 induced CD69 expression was not abrogated by TLR1/2 blockade or CCR2 antagonist (not shown). These data are consistent with both TLR1/2 and CCR2 playing a role in hBD-3 mediated NK cell activation.

We also performed similar blockade experiments in unfractionated PBMC (n=5) IFNγ ELISPOT assays. We observed hBD-3-induced IFNγ production was commonly abrogated by either CCR2 or TLR1/2 blockade (not shown). Again, R848 induced PBMC IFNγ was not abrogated by the presence of CCR2 antagonist, indicating the antagonist does not display off target effects (not shown). Overall, it appears that hBD-3 induced PBMC, mDC, and NK activity are all TLR1/2 and/or CCR2 dependent.

To further understand the relation between hBD-3 mediated NK activation and TLR1/2 or CCR2 pathway engagement, we performed purified NK cell assays comparing hBD-3 induced activity to Pam3cysk (model TLR1/2 ligand) and MCP1 (CCR2 ligand) induced activity. We observed that hBD-3, and Pam3Cysk both induced NK cell CD69 expression, but MCP1 did not induce CD69 expression (not shown). Additionally, hBD-3 induced activity strongly correlated with Pam3Cysk induced activity (r=0.65 p=0.006, Fig. 6b). We also inquired if NK TLR1 or TLR2 expression had an association with hBD-3-mediated NK activity. In fact, we found a trend towards a positive correlation between CD56dim16+ TLR1 expression and hBD-3-mediated NK IFNγ production (p=0.09, r=0.685, Supplemental Fig. 3). Altogether, these data further support direct hBD-3 induced NK cell activation via TLR1/2, and indicate hBD-3-CCR2 interactions may also play a role in facilitating activity.

DISCUSSION

We have previously observed that hBD-3 activates mDC in a TLR1/2 dependent fashion (5). Here we extend these findings to characterization of hBD-3 mediated NK cell activation, IFNγ secretion, and cytotoxic function.

We first demonstrated hBD-3 mediates PBMC cytotoxic function and IFNγ production, both key NK cell effector functions. In fact, NK cell depletion completely abrogated hBD-3-induced PBMC IFNγ production (Fig. 1d). Furthermore, in purified NK cell monoculture, we observed hBD-3 upregulated CD69 expression (Fig. 2a–c) and induced IFNγ production (Fig. 4a). These data indicate that hBD-3 can directly act on NK cells, independently of mDCs.

To address the potential effect of mDC-dependent NK cell IFNγ production, we also evaluated the effect of mDC depletion on hBD-3-induced PBMC IFNγ production. While we observed a partial abrogation of this hBD-3 activity (data not shown), there was not complete elimination as with NK cell depletion. Interestingly, hBD-3-induced NK IFNγ secretion could be enhanced by presence of mDCs (Fig. 4b), and promoted mDC-dependent NK cell IFNγ activity (Fig. 4c). While we did not observe consistent hBD-3 induced NK cell cytolytic activity in the absence of mDC, the presence of mDCs significantly increased hBD-3 treated NK cell cytolytic activity (Fig.3a–b). Additionally, hBD-3 induced mDC-dependent NK cell target cell killing (Fig. 3c). hBD-3 may therefore act as a direct stimulant of NK cells and mediator of mDC-NK interactions that promote NK cell effector functions.

We further found direct activation of NK cells by hBD-3 to be TLR1/2 and CCR2 dependent (Fig. 6a). Finally, there is variability in hBD-3 induced activity when we compared samples of one subject to another, potentially in part mediated by variability in NK cell subset expression of TLR1, TLR2 and/or CCR2. Furthermore, TLR1 expression of CD56dim16+ NK cells, the predominant NK cell subset of peripheral WBC, has a trending positive correlation to hBD-3-mediated NK IFNγ production (Supplemental Fig. 3).

NK cells are known to express CCR2, previously shown quite clearly at the mRNA level (20,21). Our findings here are in agreement with other recent data confirming CCR2 protein expression on the NK cell surface (19,20). NK cells have also been described to express TLR1 and TLR2, with some variability among reports (14–18). Murine studies have clearly shown TLR2 expression on NK cells and TLR2 dependent NK cell activation (14,18). Analysis of human NK cell TLR1 and TLR2 mRNA expression has yielded somewhat more variable results, with some data indicating greater TLR1 expression compared to TLR2 (16,17), while other data suggesting TLR1 mRNA is expressed at lower levels (14). Results in different studies may reflect variable expression from one subject to the next. In fact, our flow cytometry analysis reveals variable NK cell surface expression of both TLR1 and TLR2 among healthy individuals (Fig. 5d).

We previously showed that both TLR1 and TLR2 are required for hBD-3 activity in promoting APC maturation (5); TLR2 dimerizing with TLR1 in order to interact with the ligand (15,34). Variability in TLR1/2 expression may be due to heterogeneity in states of NK cell activation, in part due to environmental factors, such as prior exposure to infection. In regards to the latter, NK cells have been described as having some aspects of memory, with changed phenotype lasting long after exposure to infection (35,36). In addition to detectible receptor expression, NK cells were shown here to respond to the TLR1/2 agonist, Pam3sk4, and this induced activity correlated with hBD-3 induced activity (Fig. 6b). This correlation is in agreement with data in mice that suggests that TLR2 expression can facilitate NK cell activation (18). Furthermore, despite the variation in receptor expression, we have shown that hBD-3 can consistently upregulate CD69 and IFNγ secretion on bead purified NK cells (Fig. 2 and 4a). Finally, while CCR2 antagonism reduces hBD-3 induced activity, MCP1 stimulation did not result in direct cellular activation. While mechanisms accounting for these separate observations are not clear, one possibility is that CCR2 binding facilitates hBD-3 induced activity mediated through another receptor/pathway. Taken together, these observations indicate hBD-3 induced NK activity is likely regulated in part by NK cell TLR1/2 and CCR2 expression.

In summary, results here indicate hBD-3 directly activates NK cells and facilitates NK cell IFNγ production. Furthermore, we demonstrated that hBD-3 also promotes mDC-dependent NK cell cytolytic function. This interaction is likely in part mediated by TLR1, TLR2 and CCR2, and level of expression of these receptors on NK cells may regulate level of activity. These activities may facilitate the abilities of the innate immune response and bridge the functions of innate and adaptive immunity at inflamed tissue sites, where these factors are present.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Highlights

- hBD-3 activates PBMC to secrete IFN-γ and kill target cells in mDC and NK dependent fashion
- TLR1, TLR2 and CCR2 are expressed on both mDC and NK cells and are involved in hBD-3 mediated activity
- hBD-3 activates NK cells, mDC, and likely facilitates mDC-NK interactions

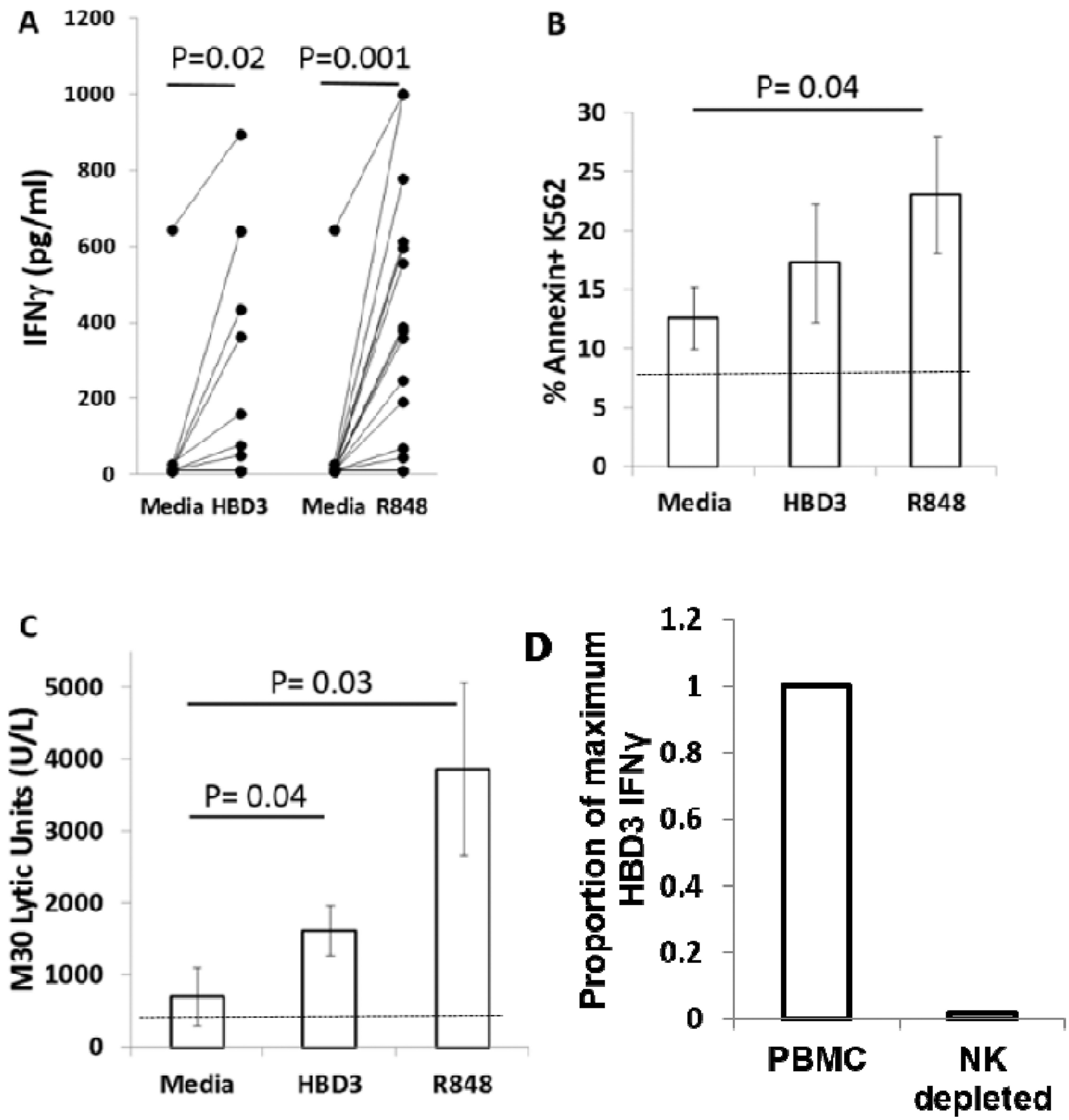

Figure 1. HBD-3 induced PBMC IFNγ Secretion and Cytotoxicity Function is mDC and NK dependent

Panel A: PBMC (5×10^5) from 15 subjects were plated with media-alone or hBD-3 (20ug/ml) and cultured 20hr at 37C. Supernatants were removed and IFNγ levels were measured by ELISA. **Panel B**: PBMC (2×10^5) (n=3) were cultured 20hr at 37C in the presence or absence of hBD-3 (20ug/ml) or R848 (1ug/ml), followed by culture with pkh labeled K562 target cells (10^4) for 2 hours, and target cell death was evaluated by Annexin staining. 20:1 E:T shown here, though 5:1 and 50:1 also performed with similar results. In the absence of effector cells Annexin staining averaged 7.5% (hashed line).**Panel C**: Huh target Killing was measured in the same subject (n=3) PBMC analyzed in panel B by co-culture of activated PBMC with HUH7.5 cells during the final 2 hr of culture, quantifying culture supernatant for cytokeratin cleaved fragment CK18 M30 by ELISA (20:1 E:T shown here, though 5:1 and 50:1 also performed with similar results). In the absence of effector cells, target cells alone treated with media, HBD3 or R848 averaged 200U/L M30 (hashed line). **Panel D**: PBMC and NK-depleted PBMC (n=3) were treated with media or hBD-3, and the proportion of hBD-3 induced IFNγ production is shown.

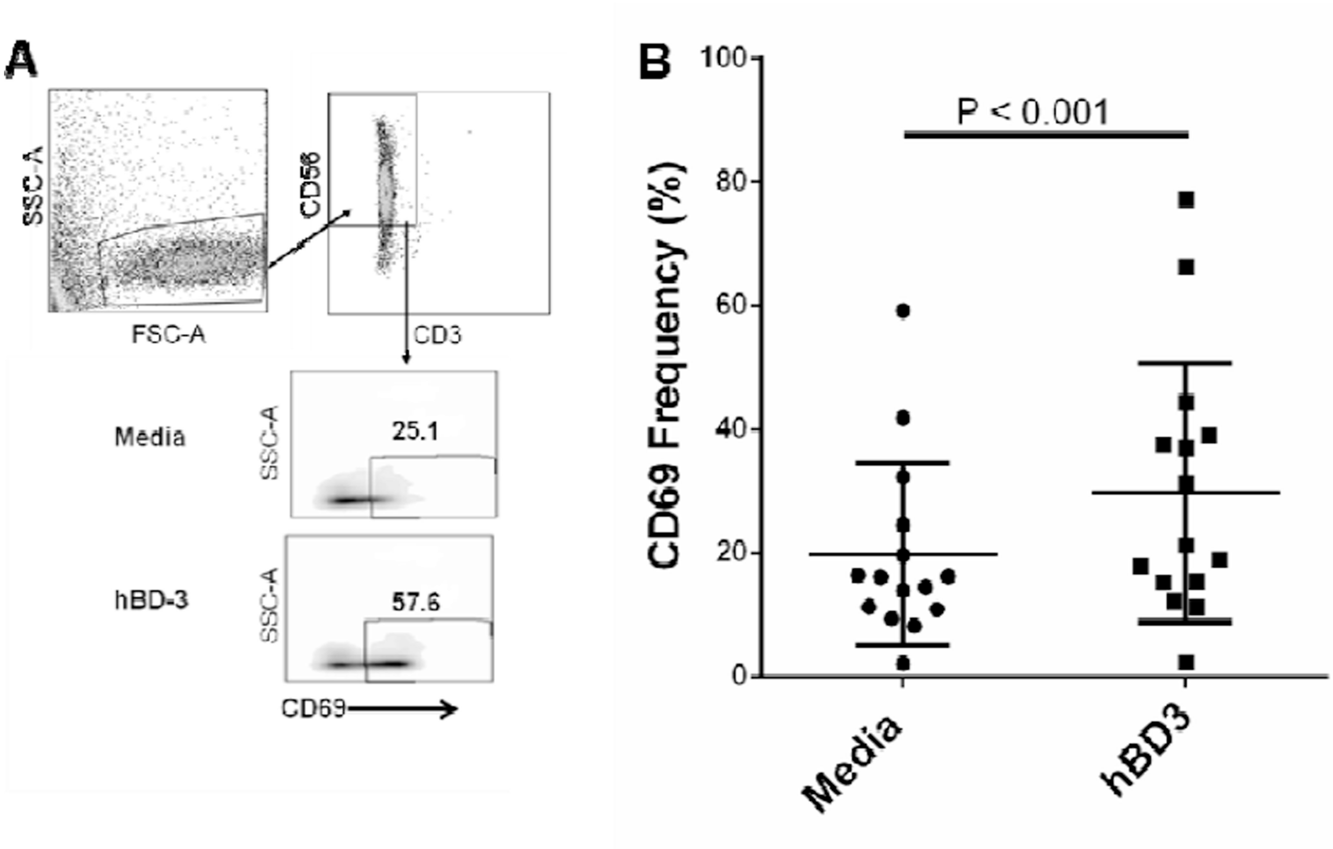

Figure 2. HBD-3-mediated upregulation of CD69 expression on purified NK cells

Purified NK cells (100,000) from 15 subjects were cultured in a 96 well round bottom plate in the presence or absence of hBD-3 (10μg/ml). After 20 hours culture at 37C, cells were stained with fluorochrome labeled monoclonal antibodies for CD3, CD56 and CD69 for NK cell analysis. **(A)** Representative analysis of CD3-56+ gated, purified NK cells (94.1%) were evaluated for CD69 expression by flow cytometry on an LSRII. **(B)** CD69 expression (%) on CD3-56+ NK cells comparing media to hBD-3 treated cells, n=15.

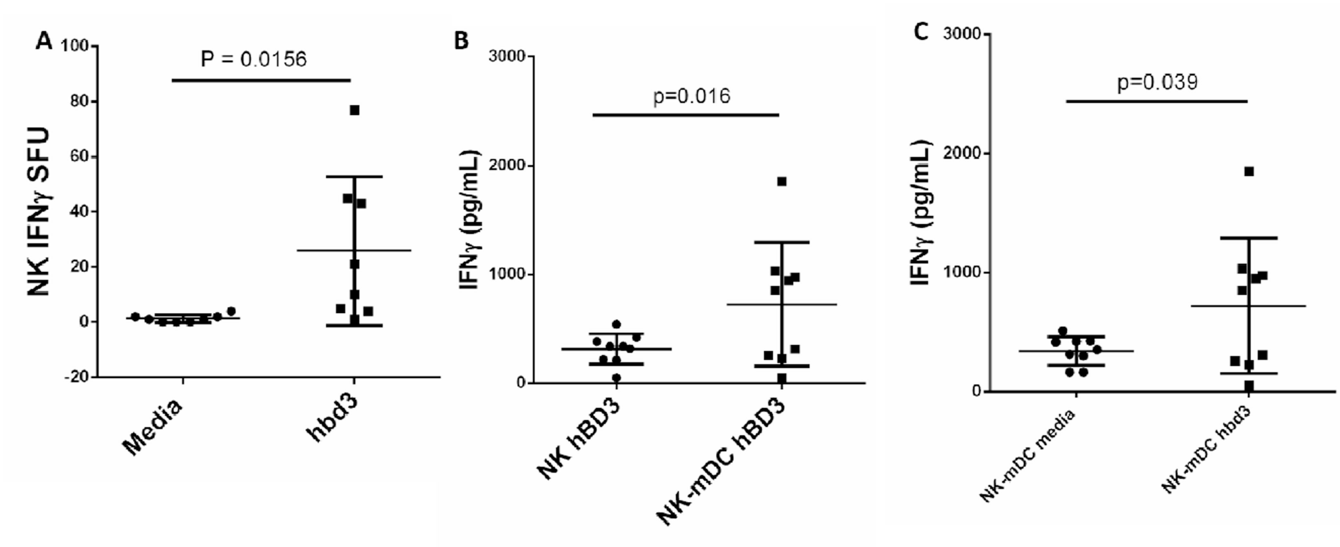

Figure 3. NK cell IFNγ production is enhanced by hBD-3

(A) NK cells (100,000, n =8) were plated without target cells in 96 well round bottom plates with media for 20 hr in presence or absence of hBD-3 (10μg/mL), and IFNγ production was measured by ELISPOT and shown as spot-forming units (SFU). **(B–C)** Huh 7.5 target cells (adherent) were plated (10,000) overnight in 96well flat bottom, then NK cells (500,000) or NK cells and MDC (20,000) prepared from 10 healthy subjects were added for another 20 hr in the presence or absence of hBD-3 as indicated. IFNγ producing function was quantified by ELISA.

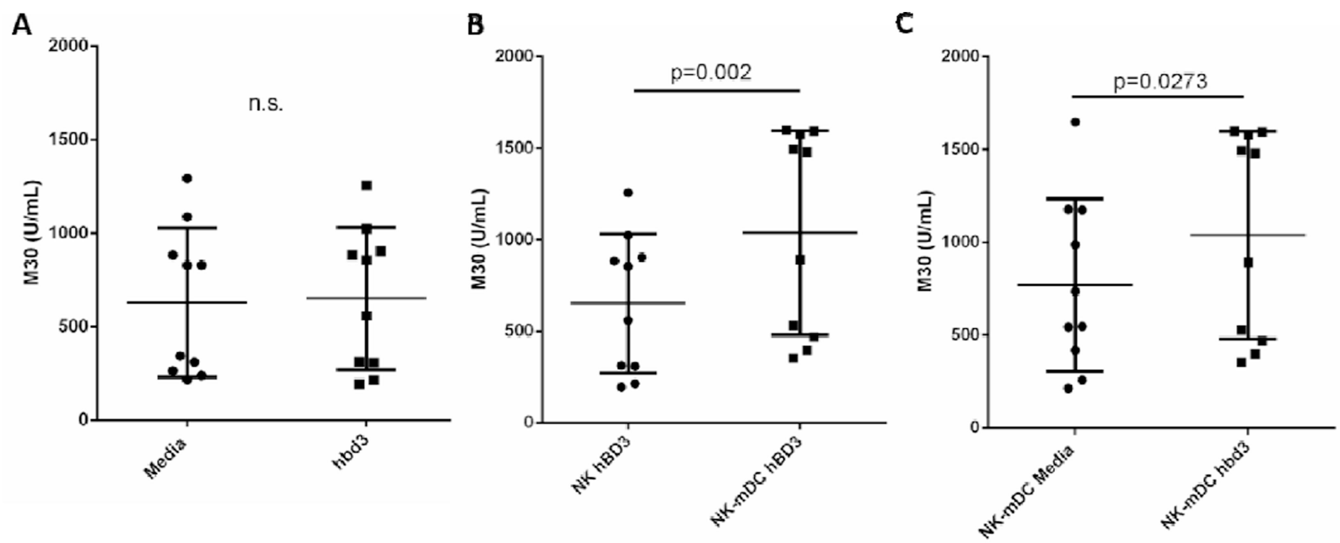

Figure 4. hBD-3 enhances mDC-dependent NK cell cytotoxic function

Huh7.5 target cells (adherent) were plated (10,000) overnight in 96well flat bottom, then NK cells (500,000) and MDC (20,000) prepared from 10 healthy subjects were added for another 20 hr in the presence or absence of hBD-3 (10μg/mL). Cytotoxic function was measured by M30 ELISA (Units/mL, U/mL). **(A)** M30 level in the supernatant of NK cells cultured with Huh7.5 target cells in presence or absence of hBD-3. **(B)** M30 level in the supernatant of NK-Huh7.5 cells treated with hBD-3 in the presence of absence of mDC. **(C)** M30 level in the supernatant of NK cells and mDCs cultured with Huh7.5 cells in presence or absence of hBD-3.

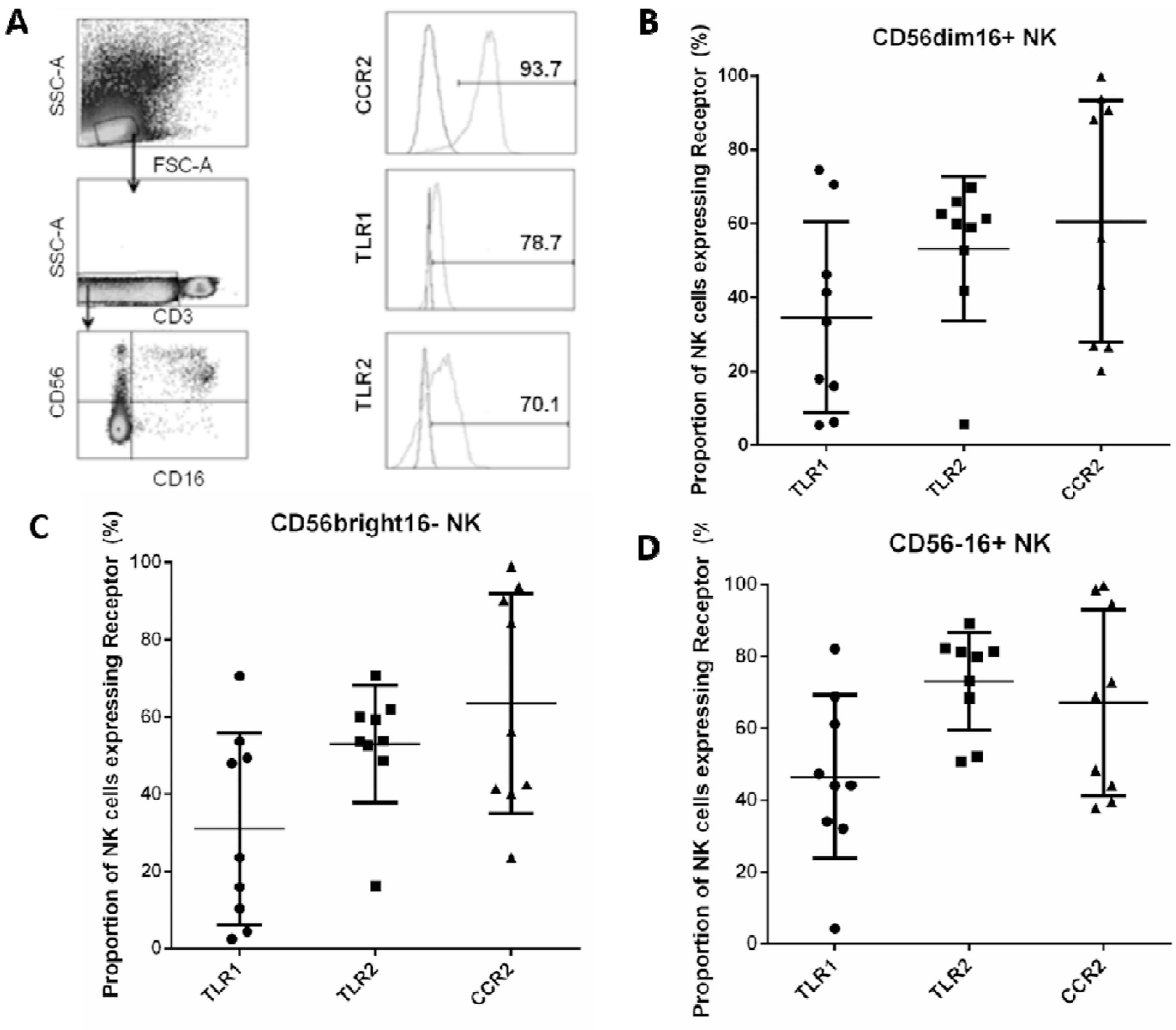

Figure 5. TLR1, TLR2 and CCR2 are expressed on NK cells

(A–D) Freshly obtained PBMC (1×10^6) were stained with fluorochome-labeled monoclonal antibodies for CD3, CD56, CD16, TLR1, TLR2, and CCR2. **(A)** Representative flow cytometric analysis of PBMC for $CD16^+CD56^{dim}$ NK cell TLR1, TLR2, and CCR2 expression, number depicts percentage of cells expressing respective receptor. **(B–D)** Summative data for NK cell subset expression of TLR1, TLR2 and CCR2 (n=9).

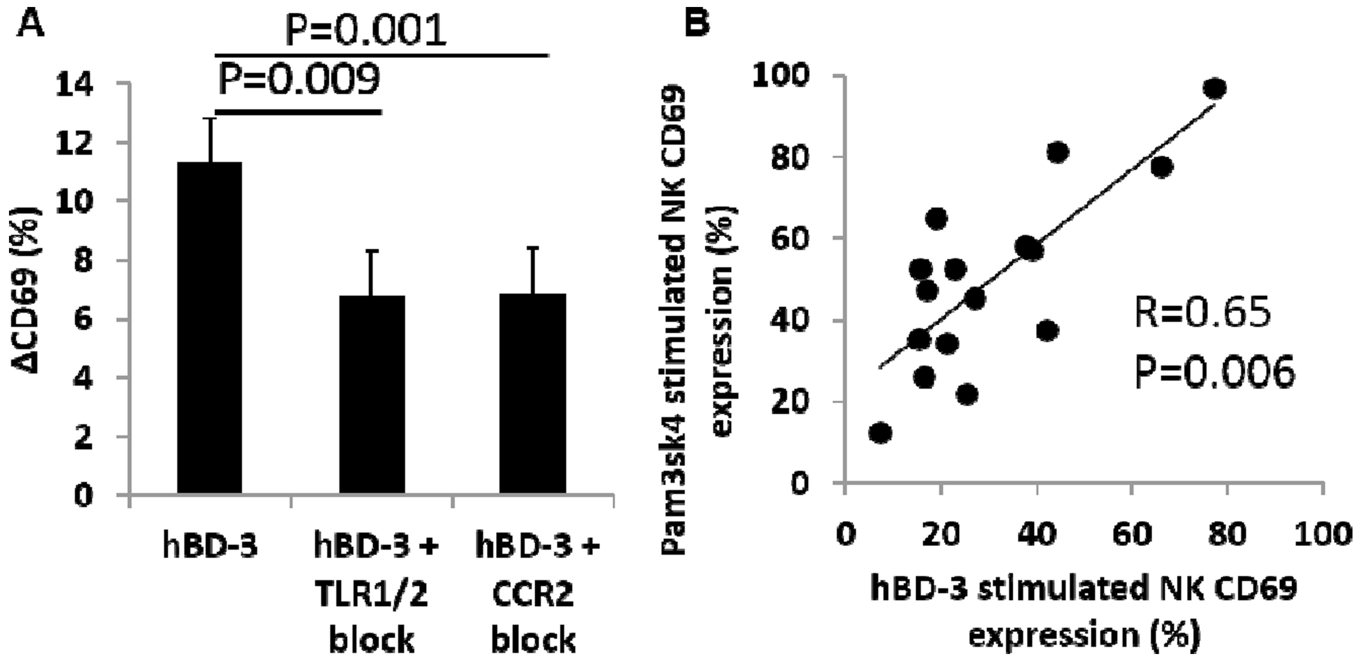

Figure 6. HBD-3-induced IFNγ and CD69 is dependent on TLR1/2 and CCR2

(A) Purified NK cells (100,000) were cultured in the presence or absence of hBD-3 (10μg/mL), in the presence or absence of TLR1/2 blockade or CCR2 inhibitor for 20hours at 37C in a 96 well round-bottom plate. After culture, cells were stained with fluorochrome labeled monoclonal antibodies for CD56 and CD69. CD56+ gated cells were analyzed for CD69 expression by flow cytometry on a LSRII. Data are displayed as the mean and standard deviation of CD69 expression (%) above media treated cell background expression (n=11). **(B)** Purified NK cells (100,000) were treated with media, hBD-3 (10ug/mL) or pam3sk4 (10μg/mL), and hBD-3 treated CD56+ NK cell CD69 vs. pam3sk4 treated NK cell CD69 expression is shown (n=16).