Polarization and Improved Maturation of Dendritic Cells with Th1 Polarization and Improved Migration

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Errata
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*Helicobacter pylori* neutrophil-activating protein (HP-NAP) is a major virulence factor involved in *H. pylori* infection. Both HP-NAP protein and oncolytic viruses encoding HP-NAP have been suggested as immunotherapeutic anticancer agents and adjuvants for vaccination but with little known about its mode of action to activate adaptive immunity. Dendritic cells (DCs) are key players in bridging innate and adaptive immune responses, and in this study we aim to evaluate the effect of HP-NAP on DC maturation, migration, and induction of adaptive immune response. Maturation markers CD83, CD80, CD86, HLA-DR, CD40, and CCR7 were upregulated on human DCs after treatment with supernatants from HP-NAP adenosine−infected cells. HP-NAP−activated DCs had a Th1 cytokine secretion profile, with high IL-12 and relatively low IL-10 secretion, and migrated toward CCL19. Ag-specific T cells were efficiently expanded by Ag-presenting HP-NAP−activated DCs, which is an important property of functionally mature DCs. Furthermore, intradermal injections of HP-NAP−encoding adenosine in C57BL/6 mice enhanced resident DC migration to draining lymph nodes, which was verified by imaging lymph nodes by two-photon microscopy and by phenotyping migrating cells by flow cytometry. In conclusion, therapeutic effects of HP-NAP are mediated by maturation of DCs and subsequent activation of Ag-specific T cells in addition to provoking innate immunity.

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*Helicobacter pylori* is a gastric pathogenic Gram-negative bacterium, usually found in the gastric mucus layer (1). *H. pylori* infection is associated with various gastric conditions such as chronic superficial gastritis, chronic active gastritis, peptic ulcer disease, and gastric cancer (2). *H. pylori* virulence factors interact with the innate and adaptive immune system, thereby eliciting a complex immune response (3, 4). *H. pylori* neutrophil-activating protein (HP-NAP), one of the major virulence factors of the bacteria, has gained much interest as a therapeutic agent. It is used as a potent protective vaccination agent or as an adjuvant to boost poorly immunogenic Ags (5, 6), and both the protein alone and HP-NAP−encoding oncolytic viruses are used in experimental cancer therapy (7–9).

HP-NAP is a 150-kDa dodecameric protein formed by twelve 15-kDa subunits that can be purified from water extracts of *H. pylori* bacteria (10). It is a TLR-2 agonist, is chemotactic for neutrophils and monocytes, and activates them to produce reactive oxygen species (5, 10, 11). HP-NAP also induces a Th1-polarized immune response by stimulating the secretion of IL-12 and IL-23 (12), and also other proinflammatory cytokines such as TNF-α and IL-8 (13). Most studies on HP-NAP have investigated its role on innate immunity (10, 14–16). But, to act as an anticancer agent, it needs to induce a potent and specific adaptive immune response. HP-NAP reverses or suppresses Th2-type immune polarization, causes eosinophilia, and also reduces serum levels of IgE in allergic disease models (17, 18). Treatment of mice bearing bladder cancer with purified HP-NAP protein induces T cell infiltration to the site of infection, which aids in tumor eradication (7). However, the above-mentioned responses need activation of adaptive immunity by HP-NAP, which has not yet been fully described.

Dendritic cells (DCs) are professional APCs and usually the key player in bridging the innate and adaptive immune system by initiating primary B and T cell−mediated immune responses (19). The property of DCs to initiate and modulate immune responses to effector immunity or immune tolerance depends on their maturation state and the microenvironment in which they are present (20–22). Microbial pathogens and Ags present at the site of infection can interact and stimulate maturation of resident DCs, which is characterized by high surface expression of MHC-II, CD80, CD83, CD86, and CCR7 together with production of cytokines (22, 23). Depending on the maturation signal received by the DCs, they can promote effector T cell responses with polarized cytokine secretion pattern like cytokine-polarized Th1, Th2, Th9, Th17, or regulatory T cell responses (23, 24), which is very essential for successful outcome of vaccination or Ag-specific immunotherapy. *H. pylori* bacteria and its virulence factors such as cytotoxin-associated gene A (CagA) induce a semimatured tolerogenic DC phenotype secreting cytokines, including IL-6, IL-10, IL-8, and IL-12 (25, 26). However, the role of HP-NAP alone on DC maturation and subsequent activation of the immune system have not been completely studied.

We have previously developed an oncolytic adenosine expressing secreted HP-NAP, which had improved results in experimental tumor treatment (9). In this study, we demonstrate that adenovirus vector-encoded HP-NAP can mature monocyte-derived DCs, induce a Th1-polarized immune response, improve Ag uptake and
presented by mature DCs (mDCs), and also improve migration of DCs to draining lymph nodes in vivo.

Materials and Methods

Ethics statement and biosafety level

The Uppsala Research Animal Ethics Committee (C21512) and the Northern Stockholm Research Animal Ethics Committee (N170/13) have approved the animal studies. The Swedish Work Environment Authority has approved the work with genetic modification of the infectious capacity of human adenovirus serotype 5 (identification number 202100-2932 v66a13 and v67a9) and genetic modification of replication capacity of human adenovirus serotype 5 (identification number 202100-2932 v66a11 and v67a7). All experiments regarding modified adenovirus were conducted under biosafety level 2.

Cell lines and culture conditions

The human prostate adenocarcinoma LNCaP and the T2 hybrid cell lines were purchased from American Type Culture Collection (Rockville, MD). The transformed embryonic retina cell line 911 was obtained from Crucell (Leiden, the Netherlands). LNCaP cells were cultured in RPMI 1640 supplemented with 10% FBS, 2 mM l-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, and 0.5% penicillin/streptomycin (PST). T2 cells were cultured in RPMI 1640 supplemented with 10% FBS and 0.5% PEST. The 911 cells were cultured in DMEM Glutamax supplemented with 10% FBS, 1 mM sodium pyruvate, and 0.5% PEST. Primary blood cells were cultured in DC medium (RPMI 1640 supplemented with 10% FBS, 10 mM HEPES, 1% PEST, 2 mM l-glutamine, and 20 μM 2-ME). All reagents were purchased from Invitrogen (Carlsbad, CA).

Recombinant adenoviral vectors

Recombinant viruses used in this study were produced by the AdEasy system. A synthetic HP-NAP transgene sequence was purchased from GenScript (Piscataway, NJ). It was codon optimized for Homo sapiens; it contains the sequence for an artificial signal peptide for efficient secretion (27) and the sequence for an N-terminal tag (6-His); and it is flanked by Hpal and XbaI restriction sites. This transgene was introduced and replaced E1A in the pShuttle-i/PPT-E1A (28). Furthermore, the original 870-bp prostate-specific Ag enhancer in the PPT sequence was shortened to 359 bp, thus creating a new plasmid, pShuttle-i/pPT-NAP. The adenovirus genome Ad5[i/ptt-sNAP], with an intact E3 region and deleted E1B, was obtained through homologous recombination between the new shuttle plasmid and the Ad5 backbone AdEasy (in B5183H bacteria). The other viruses used Ad5[Mock]; Ad5PTDf35-[i24] and Ad5PTDf35-[i24-sNAP] have been described earlier (9).

Virus production and transduction

High titer adenoviral adenoviruses were produced in 911 cells by several rounds of amplification. Viruses were purified by CsCl gradient ultracentrifugation at 25,000 rpm at 4°C for 2 h, dialyzed against a dialysis buffer (10 mM Tris-HCl [pH 7.9], 2 mM MgCl2, and 4% w/v sucrose), and stored in aliquots at −80°C. Virus titers were determined as encapsidated viral genomes by quantitative PCR and by a fluorescence-forming unit assay on 911 cells, as described earlier (9). LNCaP cells were transduced with Ad5[i/ptt-sNAP] and Ad5[Mock] in suspension at a multiplicity of infection of 10 for 2 h. The cells were washed and cultured on CellBind plates (Corning). Supernatants from virus-transduced LNCaP cells were collected 24 h posttransduction and used for maturation of DC or Western blot experiment to detect secreted HP-NAP, as previously described (9).

Generation of monocyte-derived DCs

Human primary BMDCs were isolated by Ficoll-Paque (GE Healthcare, Uppsala, Sweden) density gradient centrifugation. Monocytes were separated from PBLs by CD14+ magnetic beads isolation (Miltenyi Biotec) or by adherence of monocytes to cell culture dish. The nonadherent PBLs were cryopreserved. The monocytes were differentiated to immature DCs by adherence of monocytes to cell culture dish. The nonadherent PBLs were cultured on CellBind plates (Corning). Supernatants from virus-transduced LNCaP cells were collected 24 h posttransduction and used for maturation of DC or Western blot experiment to detect secreted HP-NAP, as previously described (9).

Flow cytometry

The following Abs were used for cell surface phenotyping analysis: HLA-DR-allophycocyanin/CD7 (MHC class II), CD1a-BV510 (BD Biosciences, Franklin Lakes, NJ), CD14-PE, CD40-FITC, CD80-PE, CD83-allophycocyanin, CD86-BV421, CCR7-BV421, and CD3-allophycocyanin. All Abs were purchased from BioLegend (San Diego, CA), unless specified elsewhere. Briefly, cells were centrifuged and resuspended in staining buffer (1× PBS with 2 mM EDTA). Abs were added (amount according to manufacturer’s instructions) to cells, which were incubated at room temperature for 20 min. Cells were washed with 1× PBS and resuspended in 1× PBS. Data acquisition was performed using a FACSVerse II (BD Biosciences) flow cytometer, and the analysis was performed using FlowJo software (version 7.5.5, Tree Star, Ashland, OR). Differentiation of monocytes to DCs in Ad24 was characterized by gating of CD1α+ and CD14+ cells from the bulk population. Maturation markers on DCs were quantified on CD1α+ gated cells in the flow cytometer.

Quantitative PCR analysis

mRNA was isolated from all experimental DCs with RNAEasy kit (Qiagen, Hilden, Germany), and mRNA was reverse transcribed to cDNA with iScript cDNA synthesis kit (Bio-Rad, Hercules, CA), according to manufacturers’ instructions. The cDNAs were evaluated by quantitative PCR, and the CCR7 gene-specific PCR product was continuously measured by the CFX96 real-time detection system (Bio-Rad) during 39 cycles using iTaq Universal SYBR Green Supermix kit (Bio-Rad). The primers (Sigma-Aldrich) used for CCR7 detection were pF-CCR7, 5’-CAGCTTCCTGGTGTTTTT-3’ and pR-CCR7, 5’-TGACACAGGCTACTCGGAGAAG-3’, and for GAPDH detection were pF-GAPDH, 5’-TGTCGTCGACAGGCTGT-3’ and pR-GAPDH, 5’-CAGCCCTCATTGTTTGAG-3’. Relative expression of CCR7 was calculated using the ΔΔ cycle threshold method and related to GAPDH expression levels for each sample.

DC Transwell migration

DC migration was assessed using a 96-well microplate chemotaxis chamber (Neuro Probe, Gaithersburg, MD) containing a polycarbonate membrane filter of 8 μm pore size (Neuro Probe). Differently treated DCs (2 × 105) were added to the upper chamber. Medium, with or without 250 ng/ml CCL19 (R&D Systems, Minneapolis, MN), was added to the lower chamber. Migration of DCs was assessed after a 2-h incubation period at 37°C by collecting the DCs from the lower chamber and counting them using a TC20 automated cell counter (Bio-Rad). To further verify whether the migration was CCR7 dependent, the different DCs were preincubated with 5 μg/ml blocking anti-human CCR7 Ab (BioLegend) or 5 μg/ml matched isotype control (mouse IgG2a, κ) (BioLegend) and then used in the migration assay described above. The migration efficiency was calculated as percentage of DCs loaded to the top well that had migrated.

Cytokine profiling

Differently matured DCs were stimulated by coculturing with T cells at 1:1 ratio or unstimulated to evaluate the effect of different cytokines secreted by the DCs and T cells when cultured together. Supernatants were collected after 24 h and stored at −20°C. Th1/Th2 cytokines were quantified using a multiplex kit (Bio-Rad), according to manufacturer’s instructions. The samples were analyzed in a MAGPIX Luminex machine (Lumixx, Austin, TX).

Ag presentation and T cell restimulation assays

Monocyte-derived DCs from CMV-seropositive, HLA-A2–positive blood donors were chosen for the Ag presentation assay. During maturation, DCs were loaded with 10 μg/ml CMV-pp65–905–903 peptide (GenScript) in the presence of 1 μg/ml β2-microglobulin (Sigma-Aldrich). After maturation, they were washed with 1× PBS to remove the unloaded peptide. The loaded DCs were then cocultured with autologous T cells at a ratio of 1:5 for 12 to 24 h to stimulate and expand CMV-pp65–specific T cells in the presence of low-dose IL-2 (20 IU/ml, Proleukin; Novartis, Basel, Switzerland). CMV-pp65–specific T cells were detected by staining with HLA-A*0201/pp65–905–903 tetramer (PE conjugated; Beckman Coulter, San Diego, CA) and analyzed by flow cytometry. Expanded CMV-pp65–specific T cells were restimulated for 24 h with T2 cells loaded with 5 μg/ml CMV-pp65–905–903 peptide. Amount of IFN-γ secretion by T cells was recorded as a measure of T cell activity. IFN-γ was measured from the supernatant using a sandwich ELISA kit (MABexpress, Nacka Strand, Sweden).

In vivo maturation and migration of endogenous DC

In-house-bred C57BL/6 mice were injected with 20 μl mixture containing Ad5PTDf35-[i24] or Ad5PTDf35-[i24-sNAP] virus (1 × 107 encapsi-
dated viral genomes) and 15–20 μg CellTrace CFSE (Invitrogen). The
injections were performed under anesthesia (isoflurane) and administered
intradermally (i.d.) on the right hind limb of the mice. Draining (inguinal)
lymph nodes were harvested 72–84 h postinjections and either fixed with 4%
paraformaldehyde for 4 h or resuspended in 1x PBS. Fixed lymph nodes
were imaged with a two-photon microscope (Zeiss 710f; Carl Zeiss). Lymph
nodes were two-photon excited at 780 nm, CFSE+–migrated cells were vi-
ualized in green, and second harmonic generation signals were collected to
visualize collagen. Images were analyzed using ZEN software (Carl Zeiss,
Blue edition). PBS-resuspended lymph nodes were digested into single-cell
suspensions and were stained for expression of DC markers CD11b-PerCP,
CD11c-PE, CD86-allophycocyanin, and B220-Pacific blue (BioLegend).
CFSE+ DCs migrating to the lymph nodes were quantified by gating either
CD11b+ B220+ or CD11c+ B220+ cells in flow cytometric analysis.
Activation status of migrated DCs was analyzed by percentage of CFSE+
CD86+ cells of CD11b+ CD11c+ B220+. Nondraining lymph nodes from
the same animals were used as the control. Gates for CFSE detection were
set based on controls with no CFSE added to the injection site. Gates for Ab
staining were set based on controls with unlabeled cells.

**Statistical analysis**

One-way ANOVA with Bonferroni posttest for multiple comparisons was
used for statistical comparison of means between more than two experi-
mental groups in an experiment. For data that are not normally distributed,
Kruskal–Wallis test with Dunn’s posttest for multiple comparisons was
used for statistical comparison of means between more than two experi-
mental groups in an experiment. A detailed description about statistics is
described in the figure legends. Associations with p value <0.05 were
considered as statistically significant. Results were analyzed with Graph-
Pad Prism 6 software (GraphPad Software, San Diego, CA).

**Results**

**Secretion of HP-NAP from cells transduced with adenovirus vector**

To eliminate the outcome of viral particles leading to activation or
maturity of DCs, the adenovirus used for this study was replication
deficient (E1A and E1B deleted) and functioned solely as a gene
delivery vector. The transgene was placed under the control of the
shortened recombinant prostate cell–specific promoter i/ppt (28) (Fig. 1A).
The HP-NAP transgene was tagged with an artificial signal peptide to support efficient secretion of protein from cells
after virus infection. Secretion was verified by performing Western blots on supernatants harvested from Ad5[i/ppt-sNAP]-transduced
LNCaP cells 24 h posttransduction. HP-NAP was detected using a HP-NAP–specific mAb (clone 16F4, provided by I. Iankov, Mayo
Clinic, Rochester, MN), and it can be seen as a 15-kDa protein on
blots on supernatants harvested from Ad5[i/ppt-sNAP]-transduced
LNCaP, or an established maturation
signal peptide to support efficient secretion of protein from cells
after virus infection. Secretion was verified by performing Western blots on supernatants harvested from Ad5[i/ppt-sNAP]-transduced
LNCaP cells 24 h posttransduction. HP-NAP was detected using a HP-NAP–specific mAb (clone 16F4, provided by I. Iankov, Mayo
Clinic, Rochester, MN), and it can be seen as a 15-kDa protein on
blots on supernatants harvested from Ad5[i/ppt-sNAP]-transduced
LNCaP, or an established maturation
secretion of HP-NAP from cells transduced with adenovirus vector

**Secreted HP-NAP induced maturation of monocyte-derived human DCs**

Monocyte-derived human DCs were obtained by differentiation of
CD14+ bead-isolated monocytes in the presence of IL-4 and GM-
CSF for 7 d to obtain immDCs, which was characterized by CD1a+
and CD14+ expression. ImmDCs were left untreated or treated for
24 h with supernatants from either Ad5[i/ppt-sNAP]-transduced LNCaP,
Ad5[i/ppt-sNAP]-transduced LNCaP, or an established maturation
mixture consisting of polyIC, R848, and IFN-γ (29) to induce
DC maturation. The DCs obtained after treatment were denoted
MockDC, NAPDC, and mDC, respectively. NAPDCs had a mature
phenotype expressing high levels of the maturation marker CD83
similar to mDCs, whereas immDC and MockDC had low levels of
CD83, implying that they are still immature (Fig. 2A). NAPDC had upregulated expression of costimulatory molecules CD80
(Fig. 2B), CD86 (Fig. 2C), and CD40 (Fig. 2E) compared with
MockDC. No difference in the expression levels of CD25 was
observed in differently matured DCs (data not shown). These mole-
cules are important for T cell activation by DCs. NAPDC also
had higher expression of MHC class II (HLA-DR) than MockDC
(Fig. 2D). The same mature phenotype as NAPDC was observed
when immDC was cultured with a maturation mixture established
by Lanzavecchia and colleagues (29) to become mDC (Fig. 2). Both
Ad5[i/ppt-sNAP] and Ad5[Mock] were replication-deficient vectors and
cannot lyse infected cells and be released in the supernatant, so
no activation of DCs due to the presence of virus particles was
expected. In fact, there was no difference in the expression profile
of surface markers between MockDC and immDC, implying that virus
particles were not present, or, even if small numbers of virus
particles remained in the supernatant, the DC maturation process
was not affected. Therefore, we conclude that the maturation of NAPDC
observed was due to the presence of secreted HP-NAP and that
NAPDCs have a similar phenotype to mDCs. We also investigated
monocyte-derived DCs obtained by differentiation of monocytes
isolated by adhesion to cell culture plate (Supplemental Fig. 1A–E)
and found a similar phenotype to monocyte-derived DCs obtained
by differentiation of monocytes isolated by CD14+ bead (Fig. 2).

**HP-NAP promotes DC migration in a CCR7-dependent manner**

CCR7, the chemokine receptor for chemokines CCL19 and
CCL21, is upregulated upon DC maturation and controls migration
of DCs to lymph nodes, where they can prime naive B and T cells.
The expression of CCR7 on the differently matured DCs was
analyzed by evaluating mRNA levels by quantitative PCR, and
the protein expression levels were confirmed by flow cytometry,
NAPDC and mDC had higher expression levels of CCR7 mRNA
(Fig. 3A) and protein (Fig. 3B) than MockDC or immDC. Next,
the different DCs were tested for the migratory ability toward
CCL19 in an in vitro Transwell migration system. NAPDC and
mDC had better migration efficiency toward CCL19 (Fig. 3C) than

**FIGURE 1. Illustration of adenoviral vectors used and Western blot showing secretion of HP-NAP. (A)** An adenovirus serotype 5 vector was modified by replacing the E1 region with the HP-NAP transgene placed under the control of the H19-insulator–shielded, shortened prostate cell–specific recombinant promoter i/ppt and named Ad5[i/ppt-sNAP]. The HP-NAP is His tagged at the N terminus and has an artificial leader sequence for efficient secretion. There are six copies of target sequence for the liver-specific microRNA miR122 in the 3’ untranslated region of the transgene. Ad5[Mock] was made by removing the E1 region. (B) Supernatants from LNCaP cells transduced with either Ad5[i/ppt-sNAP] or Ad5[Mock] were resolved by 10% SDS-PAGE gel electrophoresis, and secreted HP-NAP protein was detected by Western blot using an anti–HP-NAP Ab (clone 16F4, a gift of I. Iankov, Mayo Clinic, Rochester, MN).
Ag uptake, processing, and presentation to T cells are some of the main functions of DCs. We therefore verified that NAPDC could act as professional APCs and expand autologous Ag-specific T cells. T cells from CMV-seropositive, HLA-A2–positive blood donors can be specifically expanded with autologous mDCs that present the CMV pp65955-503 peptide (30). All the experimental DCs were therefore loaded with the pp65955-503 peptide and cocultured with autologous T cells. As assessed by HLA-A*0201/pp65955-503 tetramer, NAPDC induced a 13-fold increase in expansion of CMV pp65955-503–specific T cells compared with the initial numbers that were present (Fig. 5A). The expansion was similar to what was obtained with mixture-matured mDC (Fig. 5A). MockDC or immDC induced lower expansion of Ag-specific T cells. Representative plots of CMV pp65955-503–specific T cell expansion from one donor are shown (Fig. 5C–G).

Migration of resident tissue DCs to draining lymph node after immunization with adenovirus expressing a secreted form of HP-NAP

After Ag uptake and maturation, DCs migrate from the periphery to draining lymph nodes to educate naive T and B cells. We analyzed this phenomenon in vivo by injecting Ad5PTDf35-[24-sNAP] or the control Ad5PTDf35-[24-sNAP] virus along with CellTracer dye CFSE i.d. in the right hind limb of mice. The rationale behind this experiment was that CFSE would label the cells at the site of injection and, upon maturation of DCs, by HP-NAP secreted from virus-infected cells; they would migrate to the draining lymph node. Injections without CFSE were used as gating controls for flow cytometry experiments. Brightly labeled CFSE+ cells present in the draining lymph node were CD11c+ CD11b+, consistent with MockDC or immDC. In wells without CCL19, no difference in migratory capacity was observed between the different DCs (Fig. 3C); hence, we confirmed that migration was dependent on CCL19. To further verify that migration was CCR7 dependent, DCs were incubated with CCR7-blocking Ab or control Ab. CCR7 blocking inhibited DC migration in all cases (Fig. 3D). NAPDC and mDC retained their migration capacity toward CCL19 when blocked with an isotype control Ab, whereas neither MockDC nor immDC migrated toward CCL19 in the presence of the isotype control Ab (Fig. 3D). We conclude that HP-NAP induced upregulation of CCR7, similar to the maturation mixture, and also promoted chemotaxis toward CCL19 under in vitro conditions.

Enhanced ex vivo expansion of Ag-specific T cells by HP-NAP–matured DCs

All the experimental DCs were either stimulated by coculturing with autologous T cells or left unstimulated (DC only) for 24 h before supernatants were collected and analyzed for different cytokines using a Th1/Th2 multiplex kit. NAPDC and mDC had similar proinflammatory cytokine secretion profile to each other, both with or without stimulation by autologous T cells (Fig. 4). Matured NAPDC and mDCs secreted higher levels of IL-6 than MockDC or immDC when cultured alone (Fig. 4A). However, when cocultured with autologous T cells, all DCs secreted almost equal amounts of IL-6 (Fig. 4A). High levels of IL-8 were measured in supernatants from NAPDC and mDC both when cultured alone and when cocultured with autologous T cells, but not in supernatants from MockDC or immDC (Fig. 4B). All types of DCs secreted equal levels of IL-10 (Fig. 4C). The maturation of DCs by HP-NAP induced higher secretion of IL-12 than MockDC and immDC (DC only) (Fig. 4D). Even higher levels of IL-12 were detected in supernatants from NAPDC and mDC when cocultured with autologous T cells, suggesting that activated T cells induce matured DCs to secrete higher levels of IL-12 (Fig. 4D), affirming a Th1-polarizing cytokine secretion profile. We observed that when NAPDC and mDC were cultured with autologous T cells, high levels of IL-2 and IFN-γ were detected, but not when DCs were left unstimulated (DC only) (Fig. 4E, 4F), indicating that these cytokines are secreted by the activated T cells. A similar cytokine profile, although at different amounts, was seen when DCs were obtained from monocytes isolated by adherence to the plastic (Supplemental Fig. 2A–E).

The ability of CMV pp65955-503–specific T cells to recognize its targets was tested by exposing T cells expanded by the different DCs to T2 cells pulsed with pp65955-503 peptide in a rechallenge assay. T cell activity was measured by its ability to secrete IFN-γ upon Ag recognition. CMV pp65955-503–specific T cells expanded by NAPDC and mDC efficiently recognized T2 cells presenting the peptide and secreted significantly higher levels of IFN-γ than MockDC or immDC (Fig. 5B, right panel). T cells exposed to unloaded T2 cells secreted very low levels of IFN-γ, confirming that T cell activity was Ag specific (Fig. 5B, left panel).

The fact that HP-NAP–activated DCs pulsed with a viral peptide expand Ag-specific T cells equally well as DCs matured with a maturation mixture is of great importance in a therapeutic setting or vaccination strategy because maturation with vector-encoded HP-NAP is far more applicable in vivo than is providing a maturation mixture.
The phenotype of migratory dermal DCs. For numerical comparison, migratory DCs were gated for either CD11b+ B220− CFSE+ cells or CD11chigh B220− CFSE+ cells. HP-NAP–encoding virus induced greater number of DC migration to draining lymph node (3.3% CFSE+ of CD11b+ B220− cells; 1.9% CFSE+ of CD11chigh B220− cells) than the control virus (1.4% CFSE+ of CD11b+ B220− cells; 0.7% CFSE+ of CD11chigh B220− cells) (Fig. 6a, 6b). No CFSE+ cells were detected in the nondraining lymph nodes, which were obtained from the left side of the same mice (Fig. 6a, 6b).

Structure and morphology of the CFSE-labeled cells within the lymph nodes after i.d. injection with virus were visualized with two-photon microscopy. In HP-NAP virus–injected groups, CFSE+ cells were large and had characteristic structure and morphology expected for typical DCs (Fig. 6d). The mock virus–injected group had very few CFSE+ cells with morphology characteristic of DCs, whereas some other green cell-like spots that did not have characteristic morphology of DCs were observed and determined as background (Fig. 6c). The collagen structures in the lymph node were visualized by detecting the second harmonic signals using the nondescanned detectors with barrier filters in the 400- to 487-nm range (blue-colored fibers in Fig. 6c, 6d). Thus, we confirmed that i.d. injection of a vector encoding HP-NAP matured endogenous DCs and induced migration of DCs to the draining lymph node.

Activation status of the green (CFSE+) migrating DCs in the lymph nodes was analyzed by staining for CD86. For numerical comparison of CD86 expression on migratory DCs, cells were gated on CD11b+ CD11chigh B220−, and the number of CFSE+ cells expressing CD86 was enumerated. The gating strategy is represented in Supplemental Fig. 3. The percentage of CFSE+ CD86+ cells (from CD11b+ CD11chigh B220− gated cells) in the draining lymph node of the mice injected with HP-NAP–encoding virus (2.2%) was higher compared with the control virus–injected mice (1.0%) (Fig. 7A). No or very few CFSE+ cells were detected in the nondraining lymph nodes, which were obtained from the opposite side of the same mice (Fig. 7A). In the HP-NAP–encoding virus–injected mice, migratory DCs (CFSE+) expressed higher levels of CD86 when compared with resident DCs in the lymph node (CFSE−) (Fig. 7B), pointing out that the majority of the HP-NAP–induced migrating DCs have an activated phenotype expressing CD86 on their surface. This analysis was not performed on the control virus–injected mice because there were few CFSE+ CD86+ cells to obtain any numerical conclusion. Contour plots of CFSE+ CD86+ expression from all cells in the lymph node of a mouse injected with HP-NAP–encoding virus (lower panel) or control virus (upper panel) are represented in Fig. 7C.

Discussion
In this work, we aimed to study the effect of adenovirus-encoded HP-NAP on DC functions, including maturation status, cytokine profile, migration, and the ability to stimulate specific T cell response. HP-NAP has been proposed to be a potential therapeutic protein in different therapeutic settings ranging from vaccination to cancer therapy (6–9), and its effects seem to involve both the adaptive and innate immune system. It has well-described effects on innate immune cells, being a chemoattractant for neutrophils and monocytes and activating them to produce reactive oxygen species, chemokines (IL-8, MIP-1α, and MIP-1β), and Th1 cytokines IL-12 and IL-23 (5, 12, 13). DCs play a major role in bridging the innate and adaptive immune system, and HP-NAP, being a TLR-2 agonist, is a potential candidate for directly stimulating DCs to induce Ag-specific immune response. Also, DC activation via TLR-2 is known to turn on the NF-κB pathway,
which plays an important role during its maturation process (31). Previous studies report that the whole *H. pylori* bacteria matures DCs in vitro but has a tolerogenic effect with secretion of IL-10 (26) and DC-induced regulatory T cell skewing (32). Besides HP-NAP, *H. pylori* has many other virulence factors, such as CagA and VacA, which confer tolerogenic and immunosuppressive

FIGURE 4. HP-NAP–matured DCs release Th1 cytokine by themselves and even more so upon stimulation with autologous T cells. CD14+ magnetic bead−isolated monocytes were differentiated into DCs and matured under different culture conditions into MockDCs, NAPDCs, and mDCs or left undifferentiated as immDCs. They were then cultured with or without autologous T cells at a 1:1 ratio for 24 h before supernatants were harvested for cytokine analysis. Secretion of (A) IL-6, (B) IL-8, (C) IL-10, (D) IL-12p70, (E) IFN-γ, and (G) TNF-α was detected from supernatants harvested from five individual donors (in duplicates for each donor) using Th1/Th2 cytokine multiplex assay. DCs matured with maturation mixture (mDC) served as positive control, and untreated DCs (immDCs) served as negative control. Statistical comparison of means within each group (DC:T cell culture or only DCs) was assessed using two-way ANOVA with Bonferroni posttest correction for multiple comparisons. Data represent mean + SD (n.s, *p > 0.05; *p < 0.05, **p < 0.01, ***p < 0.01, n = 5). n.s, No significance.

FIGURE 5. Ag-specific proliferation of autologous T cells induced by peptide-pulsed HP-NAP–matured DCs. Adherence-isolated monocytes from CMV-seropositive, HLA-A2–positive blood donors were differentiated to DCs and matured under different conditions. They were then pulsed with the CMV pp65$_{495-503}$ peptide and used to stimulate autologous T cells for 2 wk in the presence of IL-2. (A) CMV-pp65–specific T cell expansion was detected and quantified by flow cytometry using PE-conjugated HLA-A*0201/pp65$_{495-503}$ tetramer. Data represent mean + SD of percentage of tetramer-positive cells among the CD3+ T cell population from six individual donors. (C–G) Representative FACS plots of T cells stimulated with CMV pp65$_{495-503}$ peptide−loaded DCs and stained with HLA-A*0201/pp65$_{495-503}$ tetramer from one donor. (B) The DC-expanded T cells were restimulated by exposure of T2 cells loaded either with CMV pp65$_{495-503}$ peptide (+Target) or without peptide (−Target). IFN-γ secretion was measured by ELISA from supernatants harvested 24 h postrestimulation from six individual donors (in triplicates for each donor). Statistical comparison of means was assessed using one-way ANOVA with Bonferroni posttest for multiple comparisons. Data represent mean + SD (**p < 0.01, ***p < 0.01, n = 6).
effects on DCs and T cells (33). Therefore, it is important to study the effect of HP-NAP alone to validate its promising therapeutic effects observed in various preclinical settings.

HP-NAP–matured DCs were phenotypically similar to Th1 mixture-matured mDCs and expressed high levels of costimulatory molecules CD80 and CD86 and MHC class II molecules on their surface. However, mature tolerogenic DCs with immune-suppressive characteristics also express these costimulatory molecules at comparable levels to mature immunogenic DCs but have low surface expression of CD40, an important molecule needed for efficient immunostimulatory DC function (20). Crosstalk between CD40 on DCs with CD40L on Th cells is essential for activation of DCs, IL-12 production, and subsequent activation of effector T cells, resulting in an inflammatory response (34). Low CD40 expression has also been observed on H. pylori–infected DCs from gastric cancer patients, suggesting that these DCs have an impaired function to induce autologous T cell activation (35). We observed increased expression of CD40 on HP-NAP–matured DCs, similar to mixture-matured mDCs (Fig. 2E), which is essential for the efficient functioning of DCs as activators of effector T cells. To note, DCs obtained from CD14 bead-isolated monocytes had a more mature and activated phenotype (Fig. 4A–E) than those DCs obtained from monocytes isolated by adherence to plastic (Supplemental Fig. 1A–E). This can be explained by the presence of other blood cells such as granulocytes and leukocytes during isolation of monocytes by adherence to plastic. These cells might interfere with the differentiation process, giving a more heterogeneous population of cells, which is not the case when monocytes are isolated by anti-CD14 Ab-conjugated magnetic beads.

Ag capture and presentation to T cells is one of the main functions of DCs, and some secreted components of H. pylori bacteria, like cagA, can reduce the phagocytic ability of monocytes (36), which in turn can lead to impaired Ag presentation and DC function. Also, the type of maturation stimuli DCs receive plays an important role in Ag presentation to T cells and subsequent T cell expansion, polarization, and function. DC activation via TLR signaling results in effective priming of IFN-γ–producing effector T cell expansion and promotes Th1 or Th2 effector differentiation. This is not the case when DCs are indirectly activated with inflammatory mediators (37). HP-NAP being a TLR-2 agonist (5) was able to activate DCs, which when loaded with CMV pp65495–503 peptide induced Ag-specific T cell proliferation (Fig. 5A). This was not the case for MockDC and immDC. Furthermore, CMV pp65495–503–specific T cells expanded from exposure to peptide-pulsed NAPDC secreted high levels of IFN-γ, when re-exposed to CMV pp65495–503–presenting target cells (Fig. 5B), which is a signature of activation of functional effector T cells in response to Ag exposure (37).

Compared with immDCs and mockDCs, NAPDC secreted higher levels of IL-8 and IL-12, but not IL-6 or IL-10, upon stimulation with autologous T cells (Fig. 4). This is different to stimulation with the whole H. pylori bacteria, where also IL-6 and IL-10 secretion is present (26). The presence of IL-12 in tumor stroma restores the function of dysfunctional myeloid-derived cells such as macrophages, DCs, and myeloid-derived suppressor cells.
within mouse tumors and also enhances antitumor activity of adoptively transferred CD8+ T cells (38). Furthermore, IL-12 has well-known antitumor effects, which are mediated by improving the functions of effector cells like CD4+ T cells, CD8+ T cells, NK cells, and NKT cells (39–41). IL-2 and IFN-γ were also detected in cultures with DCs and autologous T cells but not when DCs were left unstimulated (DCs only) (Fig. 4E, 4F), implying that T cells secrete these cytokines after interaction with matured DCs. Secretion of IL-2 and IFN-γ is characteristic of Th1-polarized T cells (42). These results, together with the comparison with a previously described method to mature type-1 DCs with a mixture consisting of R848, polyI:C, and IFN-γ (mDCs) (29), emphasize that HP-NAP, besides maturing DCs, predominantly induces Th1-type immune responses. Usually, the tumor microenvironment is immunosuppressive, with presence of IL-10 and TGF-β, which induces regulatory T cell responses and reduces the prevalence of Th1 CD4+ T cells. This shifts the Th1/Th2 immune balance, and the immunosuppressive nature of these cells can lead to tolerance against tumor Ags or pathogens (43, 44). IL-10−/− mice, compared with wild-type mice, develop severe gastritis when infected with H. pylori bacteria; therefore, IL-10 may have a potential role in downregulating H. pylori–induced inflammation at the site of infection (45). However, we did not use H. pylori bacteria to stimulate DCs, but only HP-NAP, and did not observe IL-10 secretion from NAPDC, when cultured in the presence of tumor cell supernatant during the maturation process. Effective priming of naive or memory T cells requires productive interaction with DCs, which have captured Ags at the site of inflammation or tissue damage, matured and migrated to draining lymph nodes. DC chemokine receptor profile is very important for trafficking of DCs in the body, and it changes with their maturation state. CCR1, CCR2, CCR5, and CCR6 are usually expressed on immDCs, and they direct DC migration toward the inflammatory sites. Upon receiving maturation signals, CCR7 and CCR4 are upregulated, which redirects them toward the CCL21- and CCL19-rich lymphatic system (46). However, it is claimed that CCR7 expression is necessary for efficient homing of Ag-bearing mDCs specifically to the T cell zones in the lymphatic system, even though other receptors are involved in unspecific guidance of DCs through the lymphatics (47). HP-NAP activation of DCs induced high expression of CCR7 and CCR7-dependent migration of DCs toward CCL19 similar to mixture-matured mDCs (Fig. 3). The i.d. injection with Ad5PTDf35-[Δ24-sNAP] in vivo induced higher percentage of DCs migrating to draining lymph nodes than Ad5PTDf35-[Δ24] (control virus)-injected mice (Fig. 6). Activated/Matured endogenous mouse DCs are characterized by high surface expression of costimulatory molecules such as CD40, CD80, and CD86 (48). In this study, we choose to show expression of CD86 as an example to postulate that HP-NAP–induced migratory DCs have an activated/mature phenotype (49). HP-NAP–encoding adenovirus induced higher number of migrating DCs than Ad5PTDf35-[Δ24] (24-sNAP) (control virus)-injected mice (Fig. 6). NAP–induced migratory DCs have an activated/mature phenotype. It would be of interest to investigate whether HP-NAP could induce DC migration to T cell zones in vivo, but we have not found an optimal method to detect T cells in an intact lymph node. An accurate i.d. injection is required to monitor migratory DCs because, if the injection was given s.c., there is a risk of leakage of CSFE into the draining lymph node, which could possibly stain DCs residing in the lymph node, giving false-positive results. This was observed in one of the PBS-injected mice (data not shown). In conclusion, we have observed that vector-encoded HP-NAP can promote maturation and migration of DCs in vivo. DCs matured by vector-encoded HP-NAP have the capability to induce Ag-specific T cell expansion and induce predominantly a Th1-polarizing cytokine secretion profile. These data indicate that one of the mechanisms by which HP-NAP mediates its Ag-specific responses in the ther-

![FIGURE 7](http://www.jimmunol.org/)
apeutic setting is via activation of DCs. However, further studies are required to confirm that activation of DC by HP-NAP is via TLR-2 and subsequent NF-κB pathway activation. We believe that our study further strengthens the notion that HP-NAP is an important therapeutic protein, and it can be used as an adjuvant for DC-based cancer vaccines and cancer immunotherapy.

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Disclosures

The authors have no financial conflicts of interest.

References


Corrections


The Western blot in Fig. 1B of the published article is not for the detection of Helicobacter pylori neutrophil-activating protein (HP-NAP) secretion by the Ad5[i/ppt-sNAP] virus, as stated in the figure and figure legend, but for the detection of Ad5PTDf35[Δ24-sNAP] virus, which is used in experiments for Figs. 6 and 7.


The correct Western blot for detection of HP-NAP expression by the Ad5[i/ppt-sNAP] virus is shown below. The band appears at the same expected molecular mass (15 kDa). There is no change in the figure legend or in the Materials and Methods. The figure legend published previously appears below for reference.

![Western blot](image-url)
Supplementary Figure S1: Phenotype of HP-NAP-matured DCs. PBMCs were obtained from healthy blood donors and monocytes were isolated by adherence to plastic. They were then differentiated to immature DCs by GM-CSF and IL-4 for 7 days and matured under different culture conditions for 24 hours. The phenotype was determined by staining DCs with fluorescence-conjugated antibodies. Scatter plots of mean fluorescence intensity of the cell surface markers (a) CD83, (b) CD80, (c) CD86, (d) HLA-DR, and (e) CD40 on DCs from 14 individual donors are shown. DCs matured with maturation cocktail (mDC) served as positive control and untreated DCs (immDC) served as negative control. Statistical comparison of means was assessed using Kruskal-Wallis test with Dunns’s post-test for multiple comparisons. Data represents mean MFI and error bars represent SD (*p<0.05, **p<0.01, ***p<0.001 n=15)
Supplementary Figure S2: HP-NAP-matured DCs release Th1 cytokine upon stimulation with or without autologous T-cells: PBMCs were obtained from healthy blood donors and monocytes were isolated by adherence to plastic. They were then differentiated to immature DCs by GM-CSF and IL-4 for 7 days matured under different culture conditions into Mock DCs, NAP DCs and mDCs or left undifferentiated as immDC. They were then cultured with or without autologous T cells at a 1:1 ratio for 24 hours before supernatants were harvested for cytokine analysis. Secretion of (a) IL-6, (b) IL-8, (c) IL-10, (d) IL-12p70, (e) IL-2, (f) IFN-γ and (g) TNF-α were detected from supernatants harvested from 5 individual donors (in duplicates for each donor) using Th1/Th2 cytokine multiplex assay. DCs matured with maturation cocktail (mDC) served as positive control and untreated DCs (immDC) served as negative control. Statistical comparison of means within each group (DC: T cell culture or only DCs) was assessed using Two-way ANOVA with Bonferroni post-test correction for multiple comparisons. Data represents mean ± SD (n.s p>0.05, *p<0.05, **p<0.01, ***p<0.01 n=5). n.s: no significance
Supplementary Figure S3: Gating strategy for analysis of CD86 expression of migrating endogenous DCs