

# DNA-dependent protein kinase inhibition blocks asthma in mice and modulates human endothelial and CD4<sup>+</sup> T-cell function without causing severe combined immunodeficiency

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**Background:** We reported that DNA-dependent protein kinase (DNA-PK) is critical for the expression of nuclear factor  $\kappa$ B–dependent genes in TNF- $\alpha$ –treated glioblastoma cells, suggesting an involvement in inflammatory diseases.

**Objective:** We sought to investigate the role of DNA-PK in asthmatic patients.

**Methods:** Cell culture and ovalbumin (OVA)– or house dust mite–based murine asthma models were used in this study.

**Results:** DNA-PK was essential for monocyte adhesion to TNF- $\alpha$ –treated endothelial cells. Administration of the DNA-PK inhibitor NU7441 reduced airway eosinophilia, mucus hypersecretion, airway hyperresponsiveness, and OVA-specific IgE production in mice prechallenged with OVA. Such effects correlated with a marked reduction in lung vascular cell adhesion molecule 1 expression and production of several cytokines, including IL-4, IL-5, IL-13, eotaxin, IL-2, and IL-12 and the chemokines monocyte chemoattractant protein 1 and keratinocyte-derived chemokine, with a negligible effect on IL-10/IFN- $\gamma$  production. DNA-PK inhibition by gene heterozygosity of the 450-kDa catalytic subunit of the kinase (DNA-PKcs<sup>+/-</sup>) also prevented manifestation of asthma-like traits. These results were confirmed in a chronic model of asthma by using house dust mite, a human allergen. Remarkably, such protection occurred without causing severe combined immunodeficiency. Adoptive transfer of T<sub>H</sub>2-skewed OT-II wild-type CD4<sup>+</sup> T cells reversed IgE and T<sub>H</sub>2 cytokine

production but not airway hyperresponsiveness in OVA-challenged DNA-PKcs<sup>+/-</sup> mice. DNA-PK inhibition reduced IL-4, IL-5, IL-13, eotaxin, IL-8, and monocyte chemoattractant protein 1 production without affecting IL-2, IL-12, IFN- $\gamma$ , and interferon-inducible protein 10 production in CD3/CD28-stimulated human CD4<sup>+</sup> T cells, potentially by blocking expression of *Gata3*. These effects occurred without significant reductions in T-cell proliferation. In mouse CD4<sup>+</sup> T cells *in vitro* DNA-PK inhibition severely blocked CD3/CD28-induced *Gata3* and *T-bet* expression in CD4<sup>+</sup> T cells and prevented differentiation of T<sub>H</sub>1 and T<sub>H</sub>2 cells under respective T<sub>H</sub>1- and T<sub>H</sub>2-skewing conditions.

**Conclusion:** Our results suggest DNA-PK as a novel determinant of asthma and a potential target for the treatment of the disease. (J Allergy Clin Immunol 2014;■■■:■■■-■■■.)

**Key words:** DNA-dependent protein kinase, asthma, GATA-3, T<sub>H</sub>2 cytokines, adhesion molecules, eosinophilia, severe combined immunodeficiency disease, T-cell receptor

DNA-dependent protein kinase (DNA-PK) is important for repairing double-stranded DNA breaks that are caused by DNA-damaging agents through nonhomologous end-joining (NHEJ).<sup>1,2</sup> DNA-PK is composed of a 450-kDa catalytic subunit (DNA-PKcs) and 2 DNA-binding subunits (Ku70 and Ku80). DNA-PKcs phosphorylates a number of proteins, including p53, transcription factors, RNA polymerase, histones, and Ku70/Ku80.<sup>2</sup> An important question in the research on DNA-PK is why human subjects have high levels of the kinase, although only less than 1% is needed to repair DNA damage.<sup>1,3</sup> Recently, our laboratory reported in a pioneering study that DNA-PK is required for the expression of vascular cell adhesion molecule 1 (VCAM-1), as well as many other nuclear factor  $\kappa$ B (NF- $\kappa$ B)–dependent genes that are downstream of TNF- $\alpha$ .<sup>4</sup> We provided evidence that the mechanism by which DNA-PK regulates NF- $\kappa$ B–dependent genes in response to TNF- $\alpha$  or IL-1 $\beta$  might be dependent on its ability to phosphorylate the p50 subunit of NF- $\kappa$ B at serine residue 20. The phosphorylation of this site appears to greatly influence the interaction between the p50 and p65 subunits of NF- $\kappa$ B, and it subsequently exerts a dominant role on the ability of the transcription factor to bind its  $\kappa$ B sequence to the VCAM-1 gene promoter.<sup>4</sup> Collectively, our reported results elucidated a novel mechanism through which DNA-PK positively regulates the expression of NF- $\kappa$ B–dependent genes by regulating p50 NF- $\kappa$ B, which is a subunit that has largely been regarded as a p65-aiding or gene-suppressing

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**Abbreviations used**

|           |                                                   |
|-----------|---------------------------------------------------|
| AHR:      | Airway hyperresponsiveness                        |
| BAL:      | Bronchoalveolar lavage                            |
| DNA-PK:   | DNA-dependent protein kinase                      |
| DNA-PKcs: | 450-kDa Catalytic subunit of DNA-PK               |
| FACS:     | Fluorescence-activated cell sorting               |
| HDM:      | House dust mite                                   |
| HUVEC:    | Human umbilical vein endothelial cell             |
| LSMC:     | Lung smooth muscle cell                           |
| LSUHSC:   | Louisiana State University Health Sciences Center |
| MCP-1:    | Monocyte chemoattractant protein 1                |
| NF-κB:    | Nuclear factor κB                                 |
| NHEJ:     | Nonhomologous end-joining                         |
| OVA:      | Ovalbumin                                         |
| SCID:     | Severe combined immunodeficiency                  |
| TCR:      | T-cell receptor                                   |
| VCAM-1:   | Vascular cell adhesion molecule 1                 |
| WT:       | Wild-type                                         |

subunit. More importantly, these findings suggested a possible role for DNA-PK in patients with inflammatory diseases, such as asthma.

The pathophysiology of asthma involves many cellular and subcellular events.<sup>5</sup> Exposure to allergens and other environmental factors triggers various responses that ultimately lead to bronchial constriction and impaired airflow in the lungs.<sup>5</sup> Airway inflammation appears to be central to the pathogenesis of allergic asthma, and this inflammation is thought to partly result from an overproduction of inflammatory factors, such as adhesion molecules, cytokines, and chemokines, as well as a number of enzymes that are involved in altering tissue homeostasis, such as nitric oxide synthases.<sup>5</sup> Many of the aforementioned inflammatory factors are highly dependent on NF-κB-associated signal transduction.<sup>6</sup> The role of NF-κB in asthmatic patients is demonstrated by the observation that p50 NF-κB-deficient mice do not display the typical eosinophilic airway inflammation in an ovalbumin (OVA)-induced asthma model.<sup>7</sup> This defect is associated with the requirement of p50 NF-κB for the expression of *Gata3*, the product of which is essential for the expression of T<sub>H</sub>2 cytokines, including IL-4, IL-5, and IL-13.<sup>8</sup> Consequently, factors that regulate NF-κB, including its p50 subunit, such as DNA-PK, might influence disease onset.

In the current study we tested the hypothesis that DNA-PK plays a critical role in the onset of asthma through regulation of inflammatory factors. An *in vitro* system of inflammation, acute and chronic animal models of asthma, adoptive transfers, and anti-CD3- and anti-CD28-treated CD4<sup>+</sup> T cells, including those isolated from human subjects, were used to conduct the study. The results suggested that DNA-PK is an important participant in the pathogenesis of asthma independent of its role in T- and B-cell development. In addition, DNA-PK might be a viable therapeutic target for the treatment of the disease.

**METHODS****Animals**

Male C57BL/6J mice (6–8 weeks old) were obtained from Jackson Laboratories (Bar Harbor, Me). C57BL/6 DNA-PKcs<sup>−/−</sup> mice were generated

by backcrossing mice under the C57BL/6xSV129 mixed background with C57BL/6 wild-type (WT) mice for at least 9 generations. The last generation was interbred to generate C57BL/6 DNA-PKcs<sup>−/−</sup> mice. Mice were kept in a specific pathogen-free facility at Louisiana State University Health Sciences Center (LSUHSC) and allowed unlimited access to sterilized chow and water. Experimental protocols were approved by the LSUHSC Animal Care and Use Committee.

**OVA sensitization and challenge, house dust mite extract challenge, and airway hyperresponsiveness measurement**

Mice were sensitized to 100 μg of Grade V chicken OVA (Sigma-Aldrich, St Louis, Mo) mixed with 2 mg of aluminum hydroxide in saline by means of intraperitoneal injection once a week for 2 weeks, as previously described.<sup>9</sup> Mice were then challenged with aerosolized 3% OVA for 30 minutes once for the single-challenge protocol or once daily for 3 days for the multiple-challenge protocol. Isoflurane-anesthetized mice were challenged intranasally with 25 μL of saline or 1 mg/mL whole house dust mite (HDM; *Dermatophagoides pteronyssinus*) extract (Greer Laboratories, Lenoir, NC) 3 times per week for 5 weeks. Intraperitoneal administration of the DNA-PK inhibitor NU7441 (Tocris Bioscience, Bristol, United Kingdom) or vehicle (saline) was conducted 30 minutes after OVA or HDM challenge. The dosage and route of NU7441 administration were determined according to the study by Zhao et al,<sup>10</sup> which examined the plasma pharmacokinetics of the drug. Mice were killed 48 hours later for bronchoalveolar lavage (BAL) or lung fixation and processing. Some mice were subjected to airway hyperresponsiveness (AHR) measurements 24 hours after OVA challenge, as previously described.<sup>11</sup>

**Organ recovery, tissue processing, cell staining, T<sub>H</sub>2 cytokine and OVA-specific IgE assessments, fluorescence-activated cell sorting analysis, and immunohistochemistry**

Lungs from killed animals were fixed with formalin for histologic analysis or subjected to BAL for cell counting and cytokine assessments, as previously described.<sup>11</sup> Tissue sections were subjected to hematoxylin and eosin or periodic acid-Schiff staining by using standard protocols. Some sections were subjected to immunohistochemistry, as previously described,<sup>11</sup> with antibodies to VCAM-1 (Biocare Medical, Concord, Calif). BAL fluids were subjected to cytospin, and the cells were stained with Diff-Quik (IMEB, San Marcos, Calif) for inflammatory cell assessments. Cytokine assessments were conducted with the Bio-Rad Bio-Plex system (Bio-Rad Laboratories, Hercules, Calif) or the Millipore MILLIPLEX MAP Human Cytokine/Chemokine system (Millipore, Billerica, Mass) for mice or human subjects, according to the manufacturer's instructions. OVA-specific IgE levels were quantified by means of a sandwich ELISA (Serotec, Raleigh, NC). Spleen weights from different groups were recorded, and the total splenic cell count was assessed with a cell counter (Cellometer; Nexelcom, Lawrence, Mass). For the populations of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, splenocytes were stained with anti-mouse CD3e allophycocyanin (145-2c11-APC), anti-mouse CD8a fluorescein isothiocyanate (Ly-2-53-6.7-FITC), and anti-mouse CD4 phycoerythrin (GK1.5-PE), followed by fluorescence-activated cell sorting (FACS) analysis. B-cell populations were determined by staining with anti-mouse CD19 (1D3-fluorescein isothiocyanate) and anti-Hu/Mo CD45R (B220; RA3-6B2). Also, single-cell suspensions from spleen and lung draining lymph nodes were generated and stained with anti-mouse CD19 (1D3-allophycocyanin) and anti-mouse CD138 (281-2-phycoerythrin) for plasma B cells. All antibodies that were used for FACS analysis were from BD Biosciences (San Jose, Calif). The multiplex assay and FACS were conducted at the LSUHSC Comprehensive Alcohol Research Center Core. Two deidentified lung specimens from subjects who died of severe asthma and a normal lung from a subject who died from an asthma-unrelated condition were acquired from the LSUHSC Pathology

Department. Sections from these samples and from the different experimental groups were subjected to immunohistochemistry, as previously described,<sup>11</sup> with antibodies to VCAM-1 (Biocare Medical).

### Cell culture, treatments, immunoblot analysis, and cell adhesion assay

Human umbilical vein endothelial cells (HUVECs) and the monocytic cell line U937 were purchased from Life Technologies (Portland, Ore) and ATCC (Manassas, Va), respectively. Lung smooth muscle cells (LSMCs) were isolated from WT or DNA-PKcs<sup>+/-</sup> mice, as previously described.<sup>11</sup> Knockdown of DNA-PKcs in HUVECs was achieved with a lentiviral vector (sc-35200-v; Santa Cruz Biotechnology, Santa Cruz, Calif) encoding a DNA-PKcs-targeting small hairpin RNA. Treatments with TNF- $\alpha$ , preparation of protein extracts, and immunoblot analysis with antibodies to VCAM, actin, or glyceraldehyde-3-phosphate dehydrogenase (Santa Cruz Biotechnology) were conducted, as previously described.<sup>4</sup> For the cell adhesion assay, HUVECs were stimulated with TNF- $\alpha$  for 18 hours before addition of calcein-AM (Life Technologies)-labeled U937 cells. Nonadherent monocytes were removed, and fluorescence was measured.

### Human blood collection, mouse splenocyte preparation, CD4<sup>+</sup> T-cell purification, T-cell receptor stimulation, and RT-PCR

CD4<sup>+</sup> T-cell isolation, stimulation, measurement of proliferation, and RT-PCR were performed, as previously described.<sup>11,12</sup>

### T<sub>H</sub> cell skewing, T<sub>H</sub>1 and T<sub>H</sub>2 cytokine assessment, and adoptive transfer

CD4<sup>+</sup> T cells were purified from a single-cell suspension procured from spleens of OVA-sensitized WT or DNA-PKcs<sup>+/-</sup> mice by means of negative selection with the EasySep kit from Stem Cell Technologies (Vancouver, British Columbia, Canada). All CD4<sup>+</sup> T cells were stimulated on coated plates (1  $\mu$ g/mL anti-CD3 and 0.5  $\mu$ g/mL anti-CD28). CD4<sup>+</sup> subsets were generated by means of culture under the following conditions: T<sub>H</sub>1, IL-12 (10 ng/mL; R&D Systems, Minneapolis, Minn) and anti-IL-4 (10  $\mu$ g/mL; clone 11B11); T<sub>H</sub>2, IL-4 (100 U/mL, R&D Systems) and anti-IL-12 (10  $\mu$ g/mL; clone C17.8). Some groups of WT CD4<sup>+</sup> subsets were treated with NU7441. On day 3 after stimulation, cells were replated with IL-2 alone for an additional 2 days. The extracted total RNA from the cells was used for generation of cDNA by using Reverse Transcriptase III (Invitrogen, Carlsbad, Calif) and analyzed by means of real-time PCR with primers specific for mouse *Gata3*,<sup>11</sup> *Il4* (5'-CCCCAGCTAGTTGTCATCCTG-3' and 5'-CGCATCCGTGGATATGGCTC-3'), *Il5* (5'-GGGCTTCCTGCTCCTATCTA-3' and 5'-CAGTCATGGCA CAGTCTGAT-3'), *Il13* (5'-GGTCCACACAGGGCAACT-3' and 5'-AATAAGATCAAGAAGAAATGTGCTCAA-3'), *Ifng* (5'-TCAAGTGGCATAGATGTGGA-AGAA-3' and 5'-TGGCTCTGCAGGATTTTCATG-3'), and *Tbet* (5'-GCCAGGGAA-CCGCTTATATG-3' and 5'-GACGATCATCTGGGTACATTGT-3'). In addition, OT-II mice on a C57BL/6 background (Jackson Laboratory) were killed, and splenic CD4<sup>+</sup> T cells were skewed toward a T<sub>H</sub>2 population in the presence of OVA peptide (AnaSpec, Fremont, Calif). The produced T<sub>H</sub>2-like cells were injected intravenously into the tail veins of recipient mice (1  $\times$  10<sup>6</sup> cells per mouse). All mice were subjected to aerosolized OVA challenge daily for 4 days. AHR was assessed 24 hours later, and all mice were killed at 48 hours after the last challenge.

### Data analysis

All data are expressed as means  $\pm$  SDs of values from at least 6 mice per group, unless stated otherwise, or triplicate conditions when cells were used. Prism software (GraphPad Software, San Diego, Calif) was used to analyze differences between experimental groups by using 1-way ANOVA, followed

by Tukey multiple comparison tests. For some results, analysis was conducted with the unpaired Student *t* test.

## RESULTS

### DNA-PK protein level and function are critical for VCAM-1 expression and it is required for adhesion of inflammatory cells to endothelial cells on TNF- $\alpha$ treatment

Treatment of HUVECs with TNF- $\alpha$  induced robust VCAM-1 expression (Fig 1, A), which was significantly reduced in the presence of DNA-PK inhibitors, such as NU7441 or NU7026. In addition, partial knockdown of the catalytic subunit of DNA-PK (DNA-PKcs) achieved a similar effect as that of the drugs (Fig 1, B), thus demonstrating specificity of effect. The connection between DNA-PK and VCAM-1 expression was not limited to endothelial cells. In mouse LSMCs pharmacologic inhibition of DNA-PK markedly reduced VCAM-1 expression on TNF- $\alpha$  exposure (Fig 1, C). DNA-PKcs heterozygosity, which significantly reduced kinase expression (Fig 1, D),<sup>13</sup> decreased TNF- $\alpha$ -induced VCAM-1 expression in LSMCs (Fig 1, E). These results suggest that DNA-PK might play an important role in inflammatory cell recruitment. We next used an *in vitro* cell adhesion assay with U937, a human monocytic cell line, to test this hypothesis. TNF- $\alpha$  induced a high level of monocyte adhesion (Fig 1, F), whereas DNA-PKcs knockdown or inhibition with NU7441 dramatically reduced such adhesion. These results provide preliminary evidence of the involvement of DNA-PK in inflammatory diseases.

### DNA-PK is involved in allergic lung inflammation, and its inhibition reduces eosinophilia, mucus hypersecretion, AHR, and IgE production in a mouse model of asthma

Fig 2, A, shows robust expression of VCAM-1 primarily in endothelial cells of lung tissue from a patient who died from asthma. Challenge of OVA-sensitized C57BL/6 mice with aerosolized OVA induced substantial infiltration of eosinophils into the lungs (Fig 2, B and C). Moreover, this extent of eosinophilia was significantly reduced in mice that received a single intraperitoneal injection of NU7441 (5 mg/kg) 30 minutes after OVA challenge (Fig 2, B and C). NU7441 administration also reduced the total number of inflammatory cells, including lymphocytes, monocytes, macrophages, and neutrophils, that were recruited to the lungs of OVA-challenged mice (Fig 2, C). Such drug effects were also mirrored by a significant reduction in mucus hypersecretion in the lungs of OVA-challenged animals (Fig 2, D). Additionally, NU7441 administration completely blocked AHR to inhaled methacholine 24 hours after OVA challenge (Fig 2, E).

We next assessed whether the protective effects of NU7441 against eosinophilia and mucus production coincided with a blockade of IgE production, a major hallmark of human asthma. OVA sensitization was sufficient to induce substantial levels of IgE in sera of mice, which were significantly increased on OVA challenge (Fig 2, F). DNA-PK inhibition severely reduced the production of OVA-specific IgE in sera of treated animals (Fig 2, F). Remarkably, treatment with the drug caused a complete inhibition of BAL fluid OVA-specific IgE (Fig 2, G). OVA challenge induced a robust increase in VCAM-1 expression,

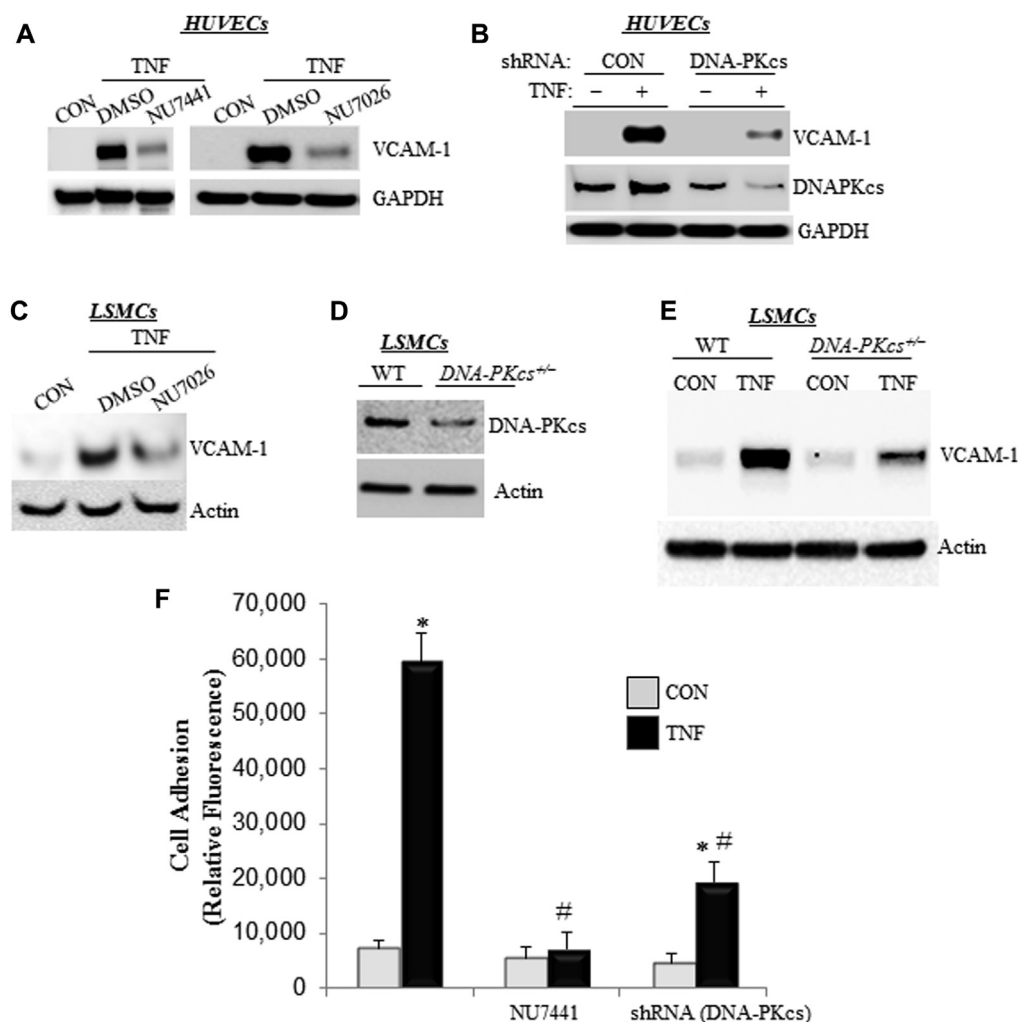


**FIG 1.** DNA-PK protein level and function are required for VCAM-1 expression and adhesion of inflammatory cells to endothelial cells on TNF- $\alpha$  treatment. **A**, HUVECs were pretreated with 1  $\mu$ M/L NU7026 or NU7441 or 0.1% dimethyl sulfoxide (DMSO) for 30 minutes before treatment with TNF- $\alpha$  for 24 hours in the continued presence of the drugs. Protein extracts were subjected to immunoblot analysis with antibodies to VCAM-1 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). **B**, HUVECs were transduced with a lentiviral vector encoding control or small hairpin RNA targeting DNA-PKcs. Cells were then treated with TNF- $\alpha$  for 24 hours, after which protein extracts were subjected to immunoblot analysis with antibodies to VCAM-1, DNA-PKcs, or GAPDH. **C**, LSMCs were pretreated with NU7026 or 0.1% DMSO for 30 minutes before treatment with TNF- $\alpha$  for 24 hours in the continued presence of the drugs. Protein extracts were subjected to immunoblot analysis with antibodies to VCAM-1 or actin. **D**, Immunoblot analysis of protein extracts from WT or DNA-PKcs<sup>-/-</sup> LSMCs with antibodies to DNA-PKcs or actin. **E**, WT or DNA-PKcs<sup>-/-</sup> LSMCs were treated with TNF- $\alpha$  for 24 hours. VCAM-1 protein expression was assessed by means of immunoblot analysis. **F**, HUVECs subjected to DNA-PKcs knockdown or treated with NU7441 were stimulated with TNF- $\alpha$  for 18 hours before addition of calcein-AM-labeled U937 cells. Nonadherent monocytes were removed, and fluorescence was measured. Fluorescence is expressed in arbitrary units. \*Difference from untreated cells and #difference from TNF- $\alpha$ -treated cells ( $P < .01$ ).

primarily in endothelial cells and, to a lesser extent, in inflammatory and smooth muscle cells (Fig 2, H). This expression was markedly reduced in the lungs of mice treated with NU7441 after OVA challenge.

We next examined whether DNA-PK inhibition blocks AHR and IgE production on repeated challenge with OVA. Fig 2, I, shows that a single administration of NU7441 was sufficient to block AHR manifestation after multiple OVA challenges. NU7441 treatment also caused a significant reduction in OVA-specific IgE levels both in the sera and BAL fluid of treated animals (Fig 2, J).

### DNA-PK inhibition blocks OVA-induced asthma-like traits by reducing production of T<sub>H</sub>2 cytokines in a mouse model of asthma

OVA exposure induced the production of IL-2, IL-4, IL-5, IL-13, and eotaxin (Fig 3, A). Treatment of mice with NU7441 after OVA challenge significantly reduced or blocked production of the aforementioned cytokines and chemokines, with little to no effect on IL-10 (Fig 3, B) and IFN- $\gamma$  (Fig 3, C) levels. However, the OVA-induced increase in IL-12 levels was completely abrogated by treatment with NU7441 (Fig 3, D). Treatment with NU7441 also blocked the production

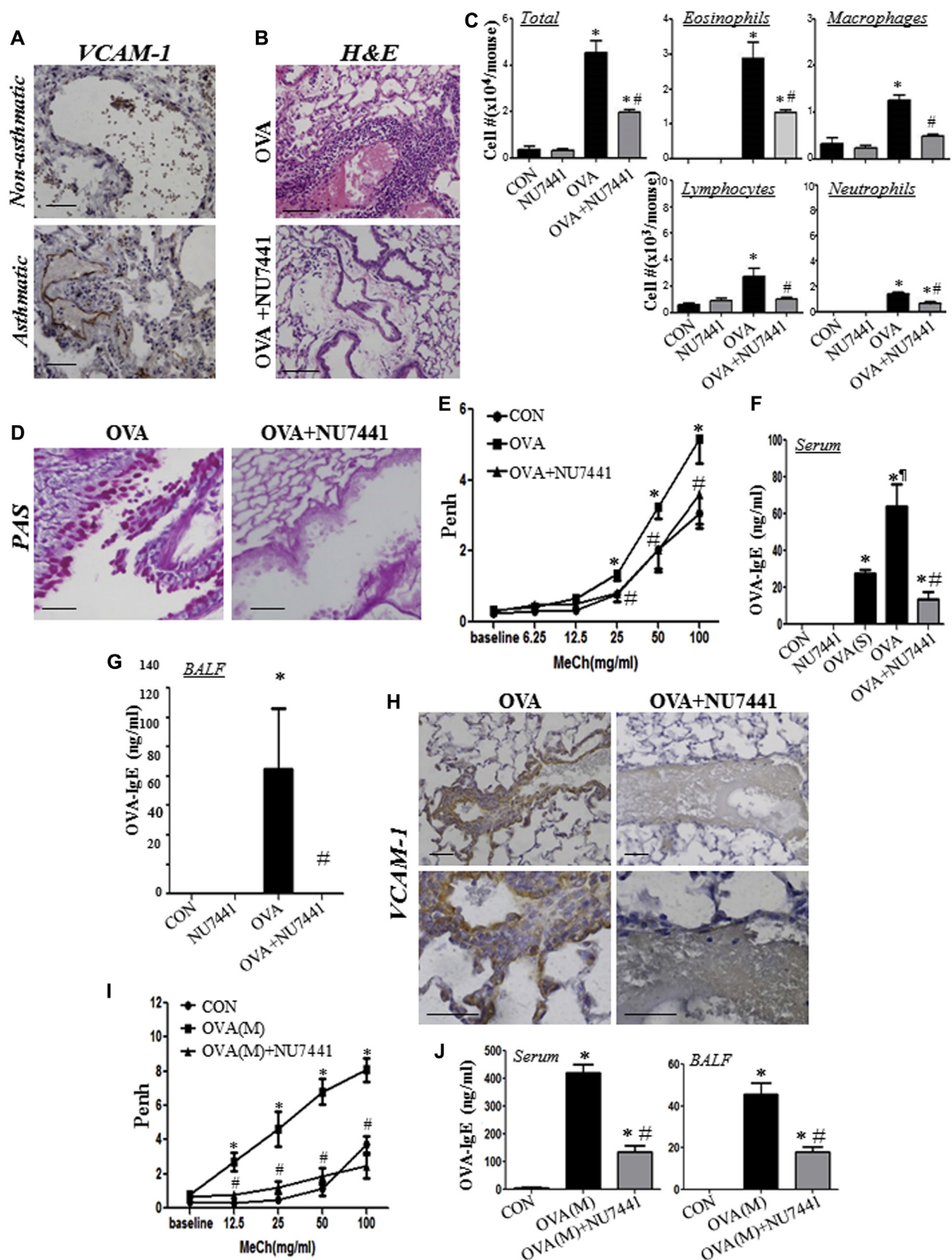


**FIG 2.** DNA-PK is involved in allergic lung inflammation, and its inhibition reduces eosinophilia, mucus hypersecretion, AHR, and IgE production in a mouse model of asthma. **A**, Lung tissue sections from an asthmatic patient and nonasthmatic subject were subjected to immunohistochemistry staining with antibodies to human VCAM-1. WT mice were subjected to OVA sensitization followed by challenge or left

of keratinocyte-derived chemokines (Fig 3, E) in the lungs of OVA-challenged mice. Monocyte chemoattractant protein 1 (MCP-1) levels barely increased above baseline in the lungs of OVA-challenged mice. Any increase that occurred was completely reduced by NU7441 treatment (Fig 3, F). In sera of OVA-challenged mice, however, MCP-1 levels were more pronounced, and treatment with NU7441 completely blocked such increased production (Fig 3, G).

### DNA-PKcs gene heterozygosity prevents eosinophilia, mucus hypersecretion, production of $T_H2$ cytokines, increase in OVA-specific IgE levels, and AHR in an animal model of asthma

Similar to cells in culture, DNA-PKcs gene heterozygosity reduces expression of the kinase in whole tissue (Fig 4, A). Fig 4, B, shows that DNA-PKcs heterozygosity markedly blocked OVA challenge-mediated eosinophilia. Concomitantly, mucus production was reduced in the DNA-PKcs<sup>+/-</sup> mice (Fig 4, C). The blockade of airway eosinophilia and mucus production in OVA-challenged DNA-PKcs<sup>+/-</sup> mice was associated with a complete blockade of OVA-specific IgE production in the lungs (Fig 4, D), as well as its significant reduction in the sera of treated animals (Fig 4, E). VCAM-1 expression was also reduced in the lungs of OVA-challenged DNA-PKcs<sup>+/-</sup> mice (Fig 4, F). This was associated with a marked reduction in the production of IL-4, IL-5, IL-2, and eotaxin (Fig 4, G). However, DNA-PKcs heterozygosity exerted no effect on IFN- $\gamma$  levels in control or OVA-challenged mice (Fig 4, H). Additionally, DNA-PKcs heterozygosity completely blocked AHR to increasing doses of methacholine in OVA-challenged mice (Fig 4, I). These results confirm those attained by using the kinase inhibitor and provide support to the notion that the effect of DNA-PK inhibition on asthma is specific.

### DNA-PK inhibition pharmacologically or by gene heterozygosity blocks asthma-like manifestation in an HDM asthma model

Despite the success of the OVA asthma model in mechanistic studies, it still has many limitations, including the development of tolerance during chronic exposure and the requirement for systemic priming with adjuvant. A better model that more closely

resembles human asthma is inhaled delivery of HDM. This model involves mucosal sensitization within the lungs with a true allergen to induce  $T_H2$ -driven inflammation.<sup>14</sup> Fig 5, A, shows that NU7441 administration concomitantly with HDM exposure markedly reduced AHR to inhaled methacholine. Interestingly, a single administration of the DNA-PK inhibitor at the end of the HDM exposure protocol provided significant protection against AHR. Similarly, DNA-PKcs heterozygosity provided protection against AHR in HDM-exposed mice, suggesting specificity of the effect (Fig 5, B). Protection against AHR by NU7441 administration, either concomitantly with HDM exposure or at the end of allergen exposure, or by gene heterozygosity was accompanied by a substantial reduction in eosinophilia and overall lung cellularity (Fig 5, C). These effects were also mirrored by a major decrease in levels of the  $T_H2$  cytokines eotaxin, IL-4, and IL-5 but not IL-10 (Fig 5, D). The moderate HDM-mediated increase in IFN- $\gamma$  levels was also blocked by DNA-PK inhibition.

### Adoptive transfer of *in vitro* $T_H2$ -skewed CD4<sup>+</sup> T cells partially reverses IgE and $T_H2$ cytokine production, but not AHR, in OVA-exposed DNA-PKcs<sup>+/-</sup> mice

To begin understanding the role of DNA-PK in immune versus structural cells during an allergic response, we examined whether adoptive transfer of  $T_H2$ -skewed CD4<sup>+</sup> T cells isolated from OT-II mice reverses asthma-like traits in naive DNA-PKcs<sup>+/-</sup> mice on exposure to OVA. The results shows that the adoptive transfer was sufficient to partially reverse IgE production in DNA-PKcs<sup>+/-</sup> mice (Fig 6, A) and to induce production of  $T_H2$  cytokines, such as IL-2, IL-4, and IL-5 (Fig 6, B), on exposure to aerosolized OVA to levels equivalent or close to those observed in WT counterparts. Interestingly, however, although the cell transfer induced AHR in OVA-treated WT mice, it was not sufficient to reverse such a response in DNA-PKcs<sup>+/-</sup> mice (Fig 6, C). Eosinophil numbers were minimal in the lungs of WT mice on OVA exposure, and as such, it was difficult to determine whether the adoptive transfer was sufficient to restore eosinophilia in DNA-PKcs<sup>+/-</sup> mice. Nevertheless, these results suggest an important role for DNA-PK in T-cell function, as well as the function of nonimmune cells, such as smooth muscle cells, which are necessary for AHR manifestation.

untreated. Mice were administered intraperitoneally 5 mg/kg NU7441 or saline 30 minutes after OVA challenge. Control mice received either 5 mg/kg NU7441 or saline. Mice were killed 48 hours later. **B**, Hematoxylin and eosin (H&E) staining of lung sections. **C**, Assessment of differentially stained cells in BAL fluid. Data are expressed as the total number of cells per mouse. Data are means  $\pm$  SDs of values from at least 6 mice per group. \*Difference from unchallenged mice and #difference from OVA-challenged mice ( $P < .01$ ). **D**, Periodic acid-Schiff (PAS) staining of lung sections. **E**, Assessment of enhanced pause (Penh) value recorded 24 hours after a single OVA challenge by using whole-body plethysmography on exposure to the indicated concentrations of aerosolized methacholine (MeCh). Results are plotted as maximal fold increase of Penh relative to baseline and expressed as means  $\pm$  SEMs ( $n = 6$  mice per group). \*Difference from control mice and #difference from OVA-challenged mice ( $P < .01$ ). **F** and **G**, Assessment of sera (Fig 2, F) or bronchoalveolar lavage fluid (BALF; Fig 2, G) collected from the different experimental groups for OVA-specific IgE. \*Difference from control mice, #difference from OVA-challenged mice, and ¶difference from OVA-sensitized but not challenged (OVA/S) mice ( $P < .01$ ). **H**, Immunohistochemistry staining of lung sections with antibodies to mouse VCAM-1. Bars = 5  $\mu$ m. **I**, Assessment of Penh after multiple OVA challenges (M) and a single NU7441 administration 30 minutes after the last challenge. Measurements were conducted 24 hours after the last OVA challenge. Results are plotted and analyzed as in Fig 2, E. **J**, Assessment of OVA-specific IgE in sera or bronchoalveolar lavage fluid (BALF) collected from the experimental groups described in Fig 2, I. \*Difference from control mice ( $P < .01$ ). #Difference from OVA-challenged mice and ¶difference from OVA-sensitized but not challenged mice ( $P < .01$ ).

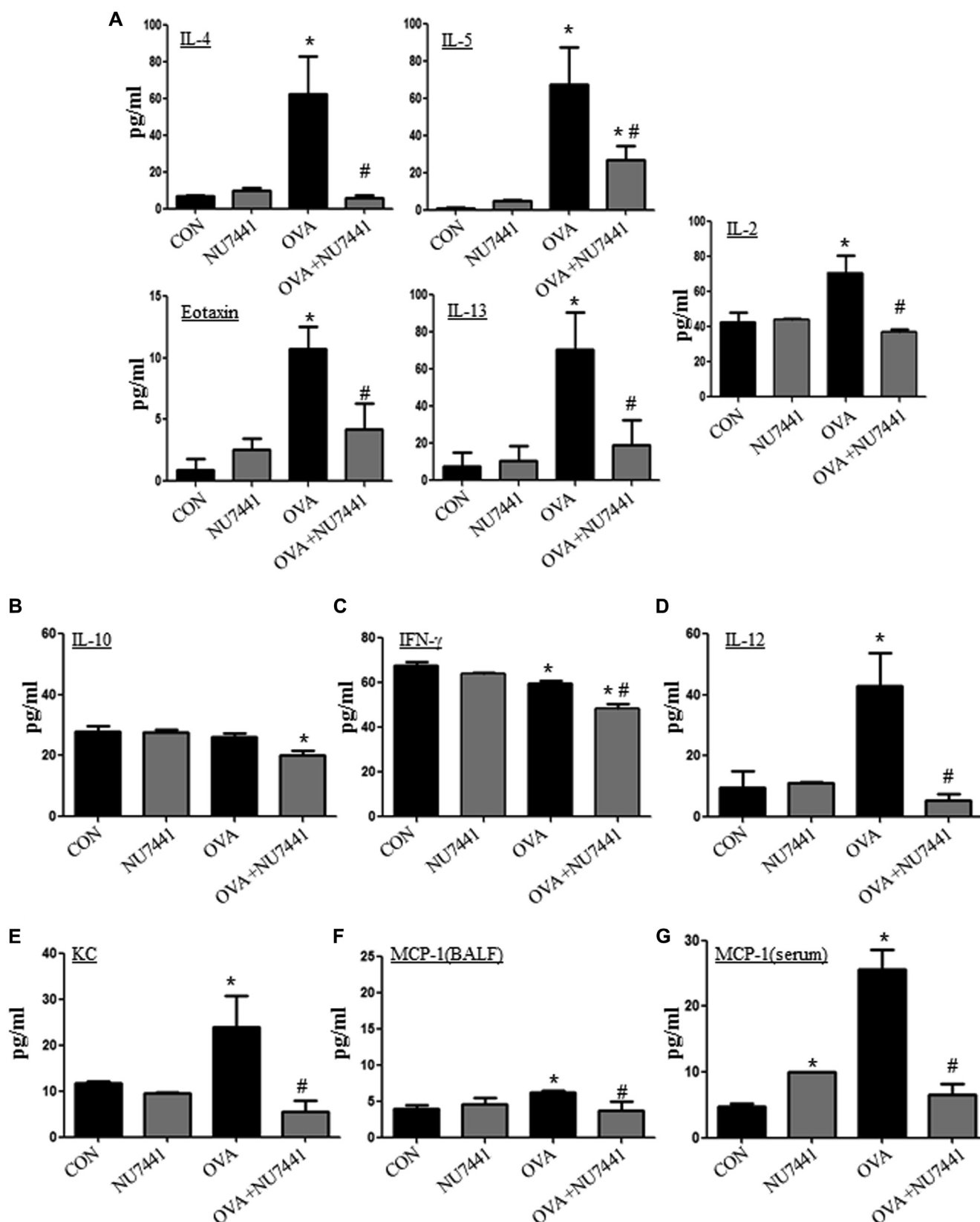


**FIG 3.** Effect of DNA-PK inhibition on OVA-induced production of  $T_H1$  and  $T_H2$  cytokines. Assessment of BAL fluid for IL-4, IL-5, eotaxin, IL-13, or IL-2 (**A**); IL-10 (**B**); IFN- $\gamma$  (**C**); IL-12 (**D**); keratinocyte-derived chemokine (KC, **E**); or MCP-1 (**F**) is shown, as is assessment of sera for MCP-1 (**G**). Data are means  $\pm$  SDs of values from at least 6 mice per group. \*Difference from unchallenged mice and #difference from OVA-challenged mice ( $P < .01$ ). BALF, Bronchoalveolar lavage fluid.

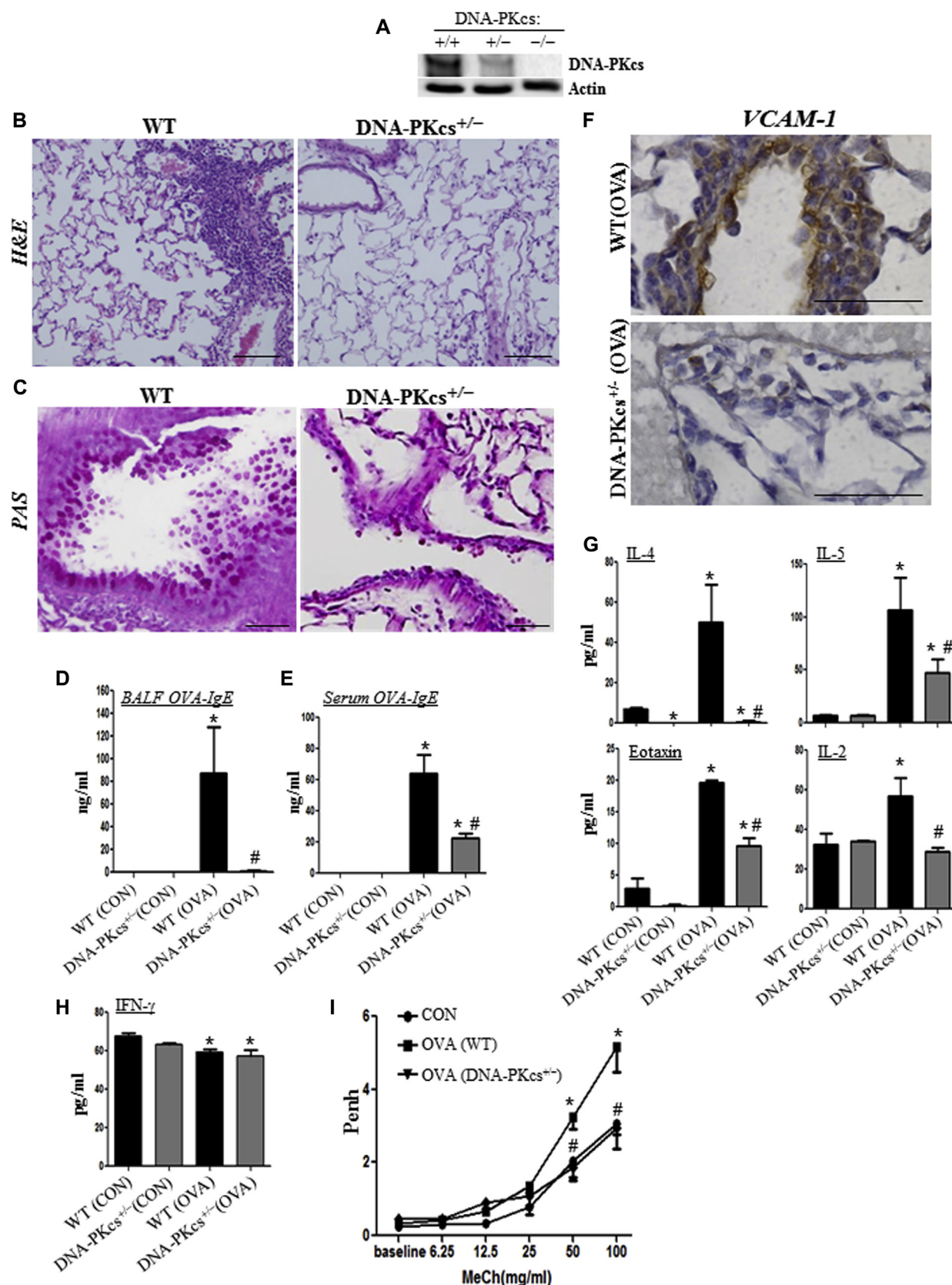


**FIG 4.** DNA-PKcs heterozygosity is sufficient to reduce asthma-associated traits. WT and DNA-PKcs<sup>+/-</sup> mice were subjected to OVA sensitization followed by challenge or left untreated, as described above. **A**, Immunoblot analysis of lung tissue with antibodies to DNA-PKcs or actin. **B** and **C**, Hematoxylin and eosin (H&E; Fig 4, **B**) and periodic acid-Schiff (PAS; Fig 4, **C**) staining of lung sections. **D** and **E**, Assessment of

## DNA-PK inhibition by gene heterozygosity or pharmacologic drugs does not cause a severe combined immunodeficiency phenotype, alter the fate of plasma B cells, or change overall spleen weight

DNA-PK deficiency or mutations have been associated with defective NHEJ and thus development of a severe combined immunodeficiency (SCID) phenotype in mice.<sup>3,15</sup> Fig 7, A, confirms this phenotype and shows that the spleens of DNA-PKcs<sup>-/-</sup> mice were almost completely devoid of B (B220<sup>+</sup>CD19<sup>+</sup>) and T (CD4<sup>+</sup> and CD8<sup>+</sup>) cells. Interestingly, DNA-PKcs heterozygosity did not cause this defect because DNA-PKcs<sup>+/-</sup> mice exhibited B- and T-cell populations comparable with those in WT mice (Fig 7, A), which is consistent with published reports.<sup>15</sup> Similarly, inhibition of DNA-PK by NU7441 did not affect T- and B-cell numbers (Fig 7, B). These minimal effects (or lack of effect) on T- and B-cell numbers by DNA-PK inhibition were mirrored by a lack of effect on total numbers of spleen cells (Fig 7, D) and of the overall weight of spleens (Fig 7, E) in treated animals. DNA-PK inhibition by NU7441 or gene heterozygosity did not affect the fate of CD4<sup>+</sup> T cells in spleens of OVA-challenged mice (data not shown). NU7441 treatment caused no effect on mouse CD19/CD138 plasma B cells in spleens or lung draining lymph nodes of OVA-challenged animals (Fig 7, E). These results suggest that the role of DNA-PK in inflammation might be different from that in the generation of B and T cells and that the protective effects conferred by its inhibition are not associated with a deficiency in B and T cells or the development of a SCID phenotype.

## Differential roles of DNA-PK in the production of T<sub>H</sub>2 and T<sub>H</sub>1 cytokines on T-cell receptor stimulation in human CD4<sup>+</sup> T cells without a prominent effect on their proliferation

We next examined the effect of DNA-PK inhibition on the proliferation of CD4<sup>+</sup> T cells isolated from peripheral blood of healthy human subjects. As shown in Fig 8, A, DNA-PK inhibition with a low concentration (0.25  $\mu$ mol/L) of NU7441 exerted no effect on T-cell proliferation, whereas medium concentrations of the drug exerted low to moderate effects. At 0.5 and 1  $\mu$ mol/L, NU7441 treatment reduced cell proliferation by less than 15% and 25%, respectively. However, at 5  $\mu$ mol/L, the drug almost completely blocked the proliferation of CD4<sup>+</sup> T cells.

Next, we determined whether the reduction in cytokine production observed on DNA-PK inhibition through drugs or gene heterozygosity in the animal model was strictly associated with an effect on T-cell function. NU7441 treatment caused a severe reduction in production of the T<sub>H</sub>2 cytokines IL-4, IL-5, IL-13, and eotaxin and the chemokines IL-8 and MCP-1 in response to T-cell receptor (TCR) stimulation (Fig 8, B). Interestingly, production of IL-4, IL-5, IL-8, and MCP-1 was

significantly reduced at a concentration of NU7441 that exerted no inhibitory effect on CD4<sup>+</sup> T-cell proliferation. Most prominently, IL-4 production was almost completely blocked at a drug concentration (0.5  $\mu$ mol/L) that minimally affected TCR-stimulated T-cell proliferation (<15%).

The effect of DNA-PK inhibition on cytokine production was not indiscriminate. Indeed, as shown in Fig 8, C, NU7441 treatment did not affect the production of IL-2, IL-12(p40), and interferon-inducible protein 10, with a minimal effect on IFN- $\gamma$  at its highest concentration. These findings show that DNA-PK might have a specific role in allergen-mediated inflammation, partly through its control of CD4<sup>+</sup> T-cell function, without affecting their proliferation.

## DNA-PK inhibition severely blocks CD3/CD28-induced *Gata3* expression in naive mouse or human CD4<sup>+</sup> T cells and prevents *in vitro* differentiation of mouse T<sub>H</sub>1 and T<sub>H</sub>2 cells under respective T<sub>H</sub>1- and T<sub>H</sub>2-skewing conditions

The transcription factor GATA-3 is considered the master regulator for the development of T<sub>H</sub>2 cells<sup>16</sup> because it binds and promotes the activation of the *Il4/Il5/Il13* cytokine locus. Interestingly, GATA-3 expression was shown to be highly dependent on p50 NF- $\kappa$ B.<sup>8</sup> As shown by us, DNA-PK regulates NF- $\kappa$ B-dependent genes by phosphorylating p50 NF- $\kappa$ B.<sup>4</sup> Thus we speculated that DNA-PK might be critical for the expression of GATA-3. Indeed, as shown in Fig 9, A and B, DNA-PK function was critical for the efficient expression of *Gata3* on CD3/CD28 stimulation of mouse or human CD4<sup>+</sup> T cells. It is noteworthy that a complete inhibition of *Gata3* expression was achieved with the lowest concentration (0.25  $\mu$ mol/L) of NU7441 tested, which caused no inhibition of T-cell proliferation. These results might explain the modulatory effect of DNA-PK inhibition on IL-4, IL-5, and IL-13 production and the overall protective effect against the manifestation of asthma traits in our animal model. We next examined whether DNA-PK inhibition differentially affects mouse CD4<sup>+</sup> T-cell differentiation into T<sub>H</sub>2 and T<sub>H</sub>1 cells when grown under respective T<sub>H</sub>2- and T<sub>H</sub>1-skewing conditions. The results show that DNA-PK inhibition, pharmacologically or by gene heterozygosity, completely blocked the differentiation of CD4<sup>+</sup> T cells into T<sub>H</sub>2 cells, as assessed by the expression of *Gata3* and *Il4* mRNA (Fig 9, C), as well as *Il5* and *Il13* (data not shown). Surprisingly, however, DNA-PK inhibition was equally efficient at reducing T<sub>H</sub>1 cell differentiation, as assessed based on expression of *T-bet* and *Ifng*, suggesting a broader role for the kinase in T-cell function and differentiation.

## DISCUSSION

Asthma manifestations require the involvement of many cell types, including immune, inflammatory, and structural cells, as well as a large number of mediators. The role of endothelial cells

bronchoalveolar lavage fluid (BALF; Fig 4, D) or sera (Fig 4, E) collected from the different experimental groups for OVA-specific IgE. \*Difference from unchallenged mice and #difference from OVA-challenged WT mice ( $P < .01$ ). F, Immunohistochemistry staining of lung sections with antibodies to mouse VCAM-1. Bars = 5  $\mu$ m. G and H, BAL fluid from the different experimental groups was assessed for IL-4, IL-5, eotaxin, IL-2 (Fig 4, G) or IFN- $\gamma$  (Fig 4, H). I, Assessment of enhanced pause (Penh) by using whole-body plethysmography. Results are plotted as maximal fold increase of Penh relative to baseline and expressed as means  $\pm$  SEMs ( $n = 6$  mice per group). MeCh, Methacholine. \*Difference from control WT mice and #difference from OVA-challenged WT mice ( $P < .01$ ).

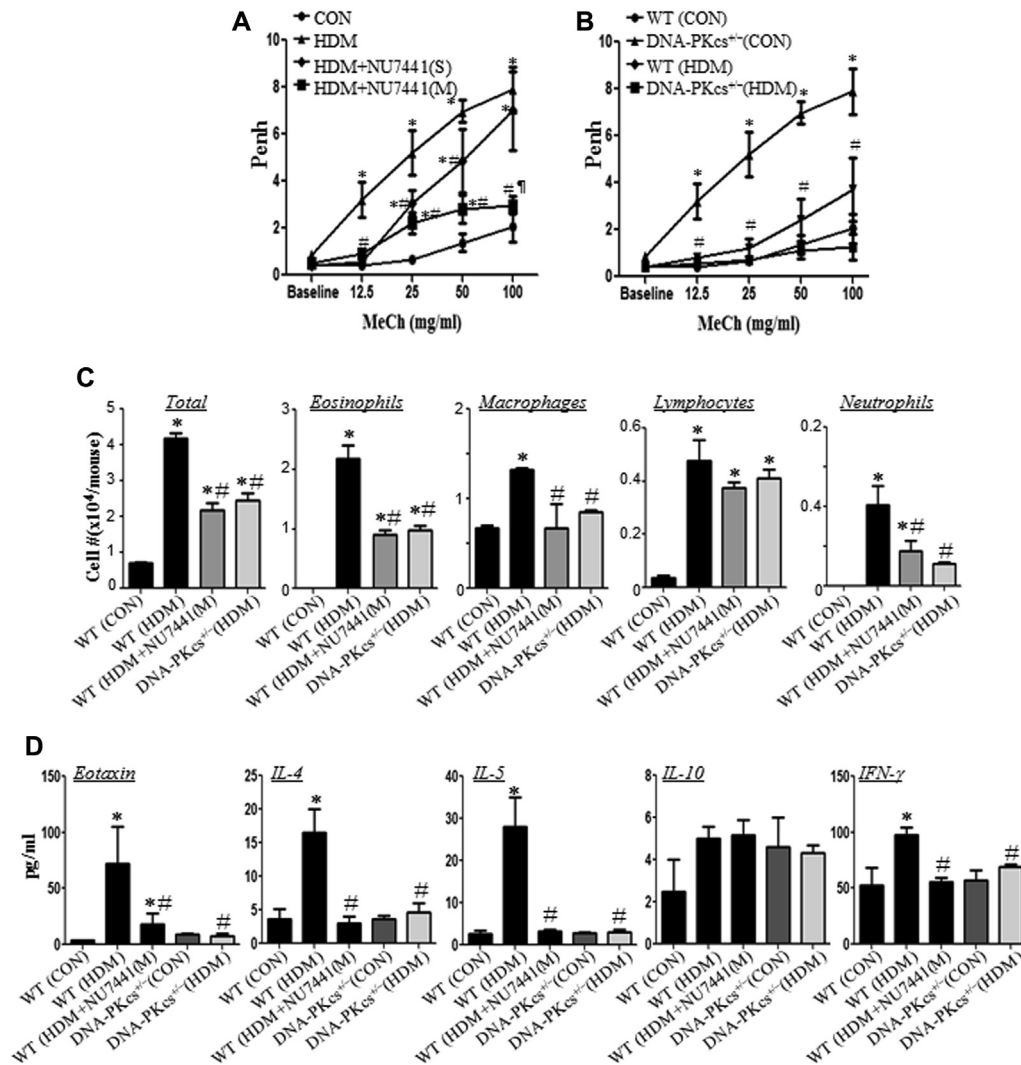


**FIG 5.** DNA-PK inhibition, pharmacologically or by gene heterozygosity, blocks asthma-like manifestations in a chronic HDM asthma model. WT or DNA-PKcs<sup>+/−</sup> mice were subjected to a chronic intranasal challenge with HDM. WT mice were administered NU7441 (5 mg/kg) either 30 minutes after each HDM exposure (M) or once 30 minutes after the last HDM exposure (S). Twenty-four hours after the last HDM exposure, mice were subjected to enhanced pause (Penh) measurements and then killed 24 hours later. **A**, Penh of HDM-challenged WT mice that received NU7441 with HDM exposure. **B**, Penh of HDM-challenged WT or DNA-PKcs<sup>+/−</sup> mice. Note that measurements for HDM-exposed and control mice are the same as those depicted in Fig 5, A. Results are plotted as the maximal fold increase of Penh relative to baseline and expressed as means  $\pm$  SEMs (n = 6 mice per group). **C**, BAL fluid cells were differentially stained, and total cells, eosinophils, macrophages, lymphocytes, and neutrophils were counted. Data are expressed as the total number of cells per mouse. Data are means  $\pm$  SDs of values from at least 6 mice per group. **D**, BAL fluids from the different experimental groups were assessed for eotaxin, IL-4, IL-5, IL-10, and IFN- $\gamma$ . \*Difference from WT control mice, #difference from WT HDM-challenged mice, and ¶difference from WT HDM-challenged mice treated with single administration of NU7441 (HDM+NU7441[S],  $P < .01$ ). MeCh, Methacholine.

in different stages of asthma is undoubtedly very important because they regulate the trafficking and transmigration of inflammatory cells, including eosinophils, lymphocytes, monocytes, and neutrophils.<sup>17</sup> VCAM-1 expression is critical for the recruitment of eosinophils and other inflammatory and immune cells to the lung.<sup>17</sup> In the current study we confirmed that DNA-PK plays an active role in inflammatory diseases. The effect of the DNA-PK inhibitor on VCAM-1 expression was clearly evident in the lungs of OVA-treated mice and in cultured endothelial cells. The reducing effect of DNA-PK inhibition on the

expression of cytokines (eg, IL-5 and eotaxin) that are essential for the differentiation, recruitment, and survival of eosinophils in OVA-exposed lungs might be an important cause of the observed marked reduction in eosinophilia. Interestingly, a single administration of the kinase inhibitor was sufficient to achieve such protection, even after repeated challenges with OVA. These results suggest that DNA-PK inhibition might reduce already established disease. Given that drugs can exert nonspecific effects, we demonstrated the specificity of the connection between DNA-PK and the inflammatory response by using a genetic approach.

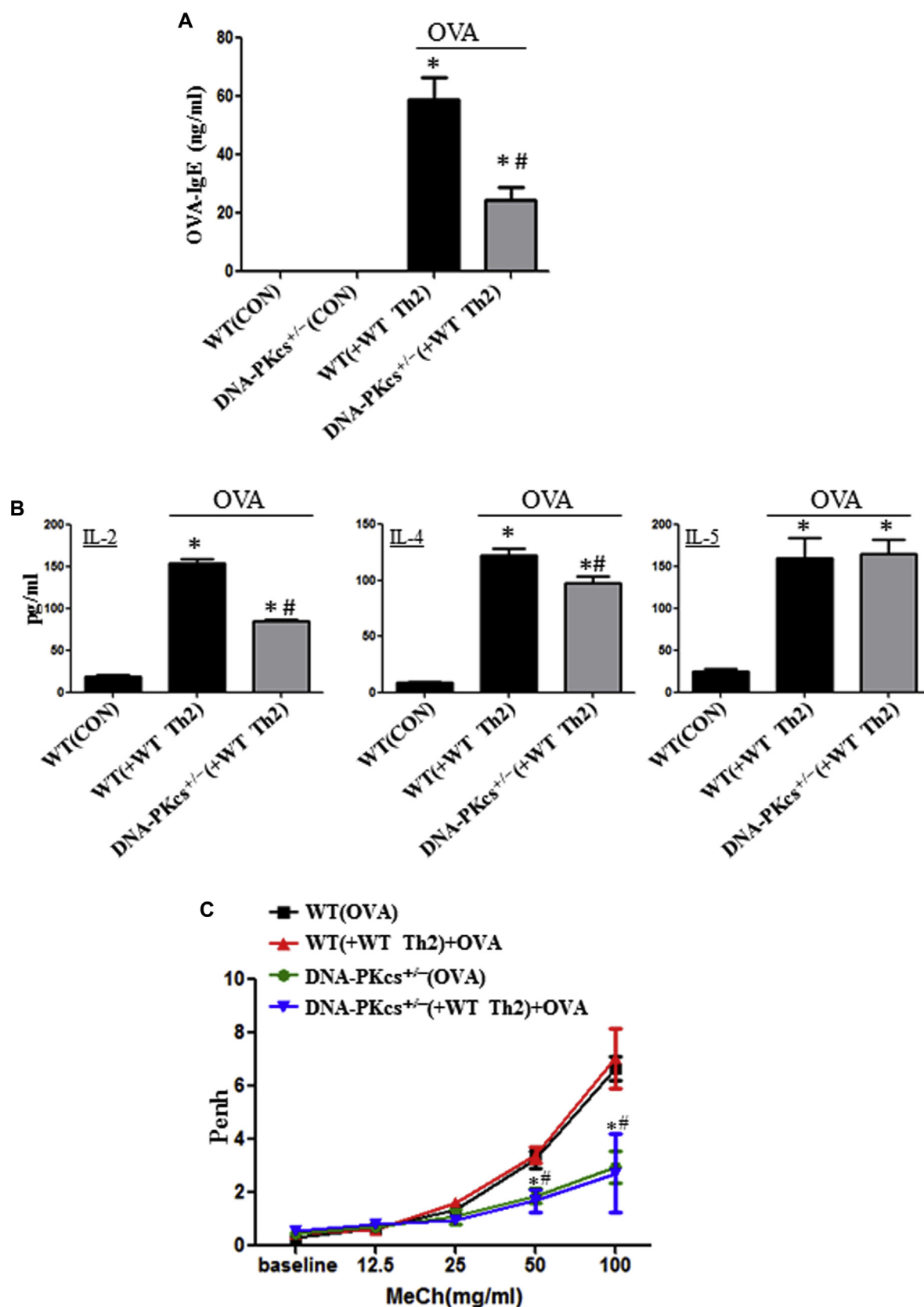


**FIG 6.** Adoptive transfer of *in vitro* T<sub>H</sub>2-skewed CD4<sup>+</sup> T cells partially reverses IgE and T<sub>H</sub>2 cytokine production but not AHR in OVA-exposed DNA-PKcs<sup>+/-</sup> mice. Splenic CD4<sup>+</sup> T cells from killed OT-II mice were skewed toward the T<sub>H</sub>2 population in the presence of OT-II peptide. Cells ( $1 \times 10^6$ ) per mouse were injected intravenously into the tail vein of each recipient mouse. All mice were subjected to aerosolized

We re-examined the connection between DNA-PK and asthma using inhaled delivery of HDM, a model that more closely resembles human asthma. Such a model involves mucosal sensitization within the lungs with a true allergen to induce  $T_H2$ -driven inflammation.<sup>14</sup> DNA-PK inhibition, pharmacologically or by gene heterozygosity, provided excellent protection against many of the asthma-like traits observed in this model. It is noteworthy that a single injection of the DNA-PK inhibitor was sufficient to significantly reduce OVA or HDM-induced AHR. The fact that eosinophilia and IL-4 levels were markedly affected by DNA-PK inhibition suggests a potential reduction of IgE levels. However, allergen-specific IgE levels were minimal in HDM-exposed WT mice. This is consistent with the study by De Alba et al,<sup>18</sup> in which they reported a requirement for an adjuvant (eg, alum) to induce detectable levels of HDM-specific IgE in mice. Accordingly, it is difficult to establish whether DNA-PK inhibition would reduce production of the immunoglobulin.

The DNA-PK complex is critical for NHEJ, which in turn is necessary for V(D)J recombination, a process that ensures normal B- and T-cell differentiation.<sup>2</sup> The gene deletion of DNA-PKcs,<sup>15</sup> Ku70,<sup>19</sup> or Ku80<sup>20</sup> causes a deficiency in NHEJ, and mice harboring the homozygous deletion of one of these genes exhibit major defects in T- and B-cell populations, thus having an SCID phenotype. Interestingly, DNA-PKcs heterozygosity, which reduces the expression of the kinase by approximately 50% (Figs 1, D, and 4, A)<sup>13</sup> and cellular enzymatic activity by a similar level, does not cause this defect. These mice have T- and B-cell populations that are comparable with those in WT mice, as shown in Fig 7 and originally reported by Gao et al.<sup>15</sup> These findings demonstrate that DNA-PKcs heterozygosity is sufficient for NHEJ and the V(D)J recombination necessary to produce normal T- and B-cell populations. The remarkable aspect of our findings is that the ability of DNA-PK to play a key role in inflammation might be independent of its role in T- and B-cell development. This conclusion is supported by our finding that even repeated administration of the kinase inhibitor did not cause a major reduction in T- or B-cell populations. However, our results suggest an important role for the kinase in T- and B-cell function. Adoptive transfer of  $T_H2$ -skewed  $CD4^+$  T cells was sufficient to reverse  $T_H2$  cytokine production but was only able to partially reduce production of OVA-specific IgE in DNA-PKcs<sup>+/-</sup> mice. The role of DNA-PK might not be restricted to immune or inflammatory cells. Indeed, adoptive transfer of activated T cells did not reverse AHR in DNA-PKcs<sup>+/-</sup> mice, which suggests an important role for DNA-PK in cells that participate in AHR manifestation, such as smooth muscle cells.

In our *in vitro* studies with human  $CD4^+$  T cells, DNA-PK inhibition at concentrations of NU7441 that exerted no effect on cell proliferation (0.25  $\mu$ mol/L) markedly and, in some instances, severely reduced levels of a number of cytokines and chemokines, such as IL-4, IL-5, IL-8, IL-13, and MCP-1. Conversely, treatments with concentrations that severely reduced

$CD4^+$  T-cell proliferation had no effect on IL-12 levels. The significant effect of NU441 on IFN- $\gamma$  was observed only at the highest concentration (5  $\mu$ mol/L) tested and might stem from the actual decrease in cell proliferation. Overall, the effect on IFN- $\gamma$  is consistent with the effect of DNA-PK inhibition, either by drugs or gene heterozygosity, on cytokine production in OVA-challenged mice. The lack of an effect of DNA-PK inhibition on IL-12 in TCR-stimulated  $CD4^+$  T cells is in sharp contrast to the decrease observed in the animal model. Although speculative, the decrease in IL-12 levels seen in the animal model might be associated with the severe reduction in lymphocytes in the lungs of treated mice, and DNA-PK enzymatic activity might not be necessary for IL-12 production. Despite these results attained by using  $CD4^+$  T cells from healthy human subjects, our experiments with mouse  $CD4^+$  T cells showed that DNA-PK inhibition, pharmacologically or through gene heterozygosity, completely reduced the expression of factors necessary for  $T_H2$  and  $T_H1$  differentiation of cells. The reduction in IFN- $\gamma$  levels in  $T_H1$ -skewed  $CD4^+$  T cells might not be surprising given our observation that DNA-PK inhibition completely blocked production of the cytokine in the HDM model. However, these results are in contrast with those attained by using human  $CD4^+$  T cells (Fig 9). We believe that this difference might be because human cells express more than 50 times the level of the kinase observed in rodent cells.<sup>21</sup> This result is very intriguing given that although IFN- $\gamma$  expression is affected by DNA-PK inhibition in mouse cells, it is not affected in human  $CD4^+$  T cells; it appears that specificity of DNA-PK to the  $T_H2$  response might be a human trait. When we consider the results of the *in vivo* model, DNA-PK inhibition, pharmacologically or through gene heterozygosity, reduced but did not completely abolish IL-5 production, suggesting that  $T_H2$  cells were generated on OVA exposure. Accordingly, a great deal of work is required to reconcile such a discrepancy.

In  $CD4^+$  T cells DNA-PK appeared to be critical for GATA-3 expression on TCR stimulation. This requirement was observed in both human and mouse cells. GATA-3 is a major regulator of the *Il4/Il5/Il13* cytokine locus.<sup>16</sup> The inhibition of the expression of this transcription factor might explain the modulatory effect of DNA-PK inhibition of IL-4, IL-5, and IL-13 production and the protective effect against the manifestation of asthma traits in the animal model. Interestingly, GATA-3 expression is highly dependent on p50 NF- $\kappa$ B,<sup>8</sup> and the efficient function of p50 NF- $\kappa$ B, in turn, is mediated by its phosphorylation by DNA-PK.<sup>4</sup> Accordingly, it is highly likely that the modulation of GATA-3 gene expression by DNA-PK inhibition is associated with a blockade in p50 NF- $\kappa$ B phosphorylation. This notion is supported by the fact that the effects of p50 NF- $\kappa$ B gene deletion on OVA challenge-induced asthma is rather similar to that achieved on DNA-PK inhibition.<sup>8</sup> Indeed, although IL-4, IL-5, IL-13, eotaxin, and MCP-1 levels were markedly reduced in OVA-challenged p50 NF- $\kappa$ B<sup>-/-</sup> mice, IL-2, IFN- $\gamma$ , and interferon-inducible protein 10 levels were not.<sup>6,7</sup> It is important

OVA challenge daily for 4 days. Twenty-four hours after the last OVA exposure, mice were subjected to enhanced pause (Penh) measurements and then killed 24 hours later. **A**, Assessment of sera for OVA-specific IgE. **B**, Assessment of sera for IL-2, IL-4, or IL-5. \*Difference from control WT mice and #difference from WT mice receiving adoptive transfer (WT+WT Th2),  $P < .01$ . **C**, Assessment of Penh in the different experimental groups 24 hours after the last OVA challenge. Results are plotted as the maximal fold increase of Penh relative to baseline and expressed as means  $\pm$  SEMs ( $n = 6$  mice per group). \*Difference from OVA-sensitized and challenged WT mice (WT[OVA]) and #difference from OVA-challenged WT- $T_H2$  recipient mice (WT+WT Th2)+OVA,  $P < .01$ . MeCh, Methacholine.

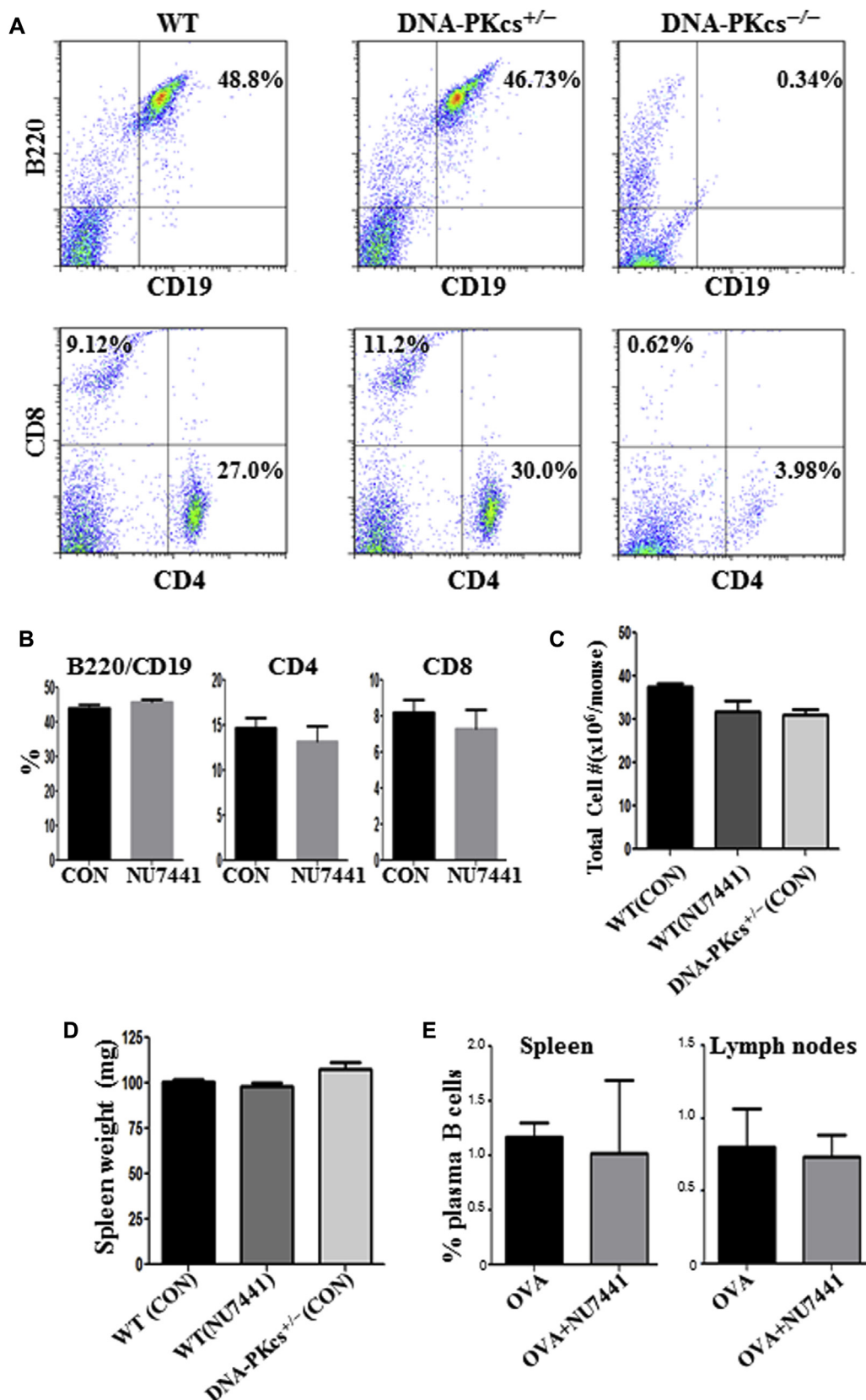


**FIG 7.** DNA-PK inhibition, pharmacologically or by gene heterozygosity, does not cause an SCID phenotype, alter the fate of plasma B cells, or change spleen weight. **A**, Single-cell suspensions from spleens of WT, DNA-PKcs<sup>+/-</sup>, or DNA-PKcs<sup>-/-</sup> mice were stained with fluorescently labeled antibodies to CD19 or B220. Cells were also stained with antibodies to CD4 or CD8, followed by FACS analysis. **B**, WT mice received either saline or 5 mg/kg NU7441 and were killed 48 hours later. Single-cell suspensions from spleens were subjected to FACS analysis with antibodies to B220, CD19, CD4, or CD8. **C**, Comparison of total cell counts in spleens of DNA-PKcs<sup>+/-</sup> and WT mice that received saline or NU7441. **D**, Comparison of spleen weights of the different groups. **E**, Assessment of CD19/CD138<sup>+</sup> plasma B cells in single-cell suspensions from spleens or lung draining lymph nodes from OVA-challenged mice that received saline or NU7441.

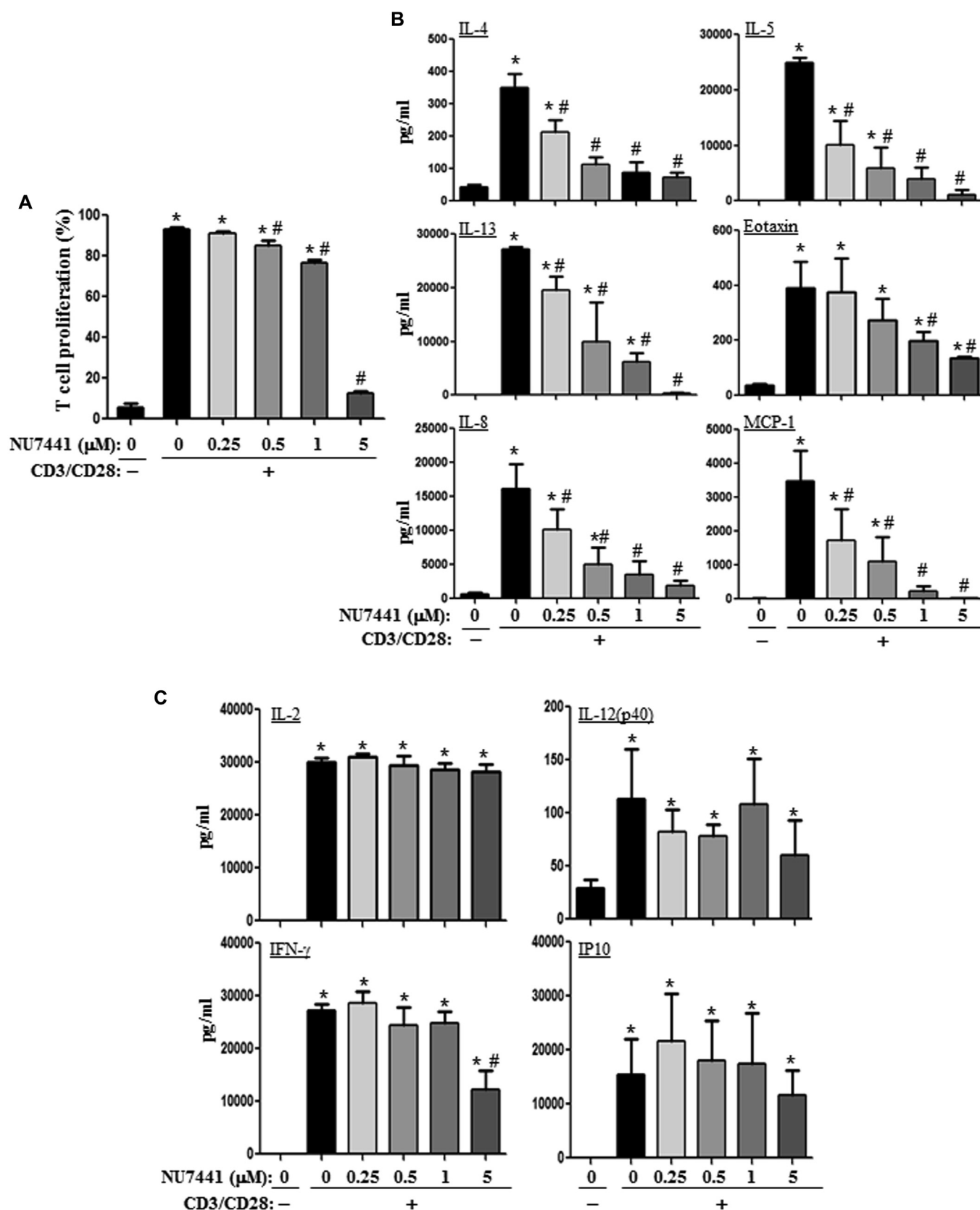


**FIG 8.** Effects of pharmacologic inhibition of DNA-PK on CD4<sup>+</sup> T-cell proliferation and production of T<sub>H</sub>1 and T<sub>H</sub>2 cytokines on TCR stimulation. **A**, Negatively selected human CD4<sup>+</sup> T cells from healthy donors were stimulated in triplicates with antibodies to CD3/CD28 in the absence or presence of different concentrations of NU7441. Cell proliferation was assessed by using carboxyfluorescein succinimidyl ester staining. **B** and **C**, CD4<sup>+</sup> T cells were stimulated as in Fig 8, A, and cytokines were assessed in culture medium 72 hours later. Data are presented as means  $\pm$  SDs of values obtained from duplicates of the triplicates. *IP10*, Interferon-inducible protein 10. \*Difference from nonstimulated cells and #difference from CD3/CD28-stimulated cells ( $P < .01$ ).

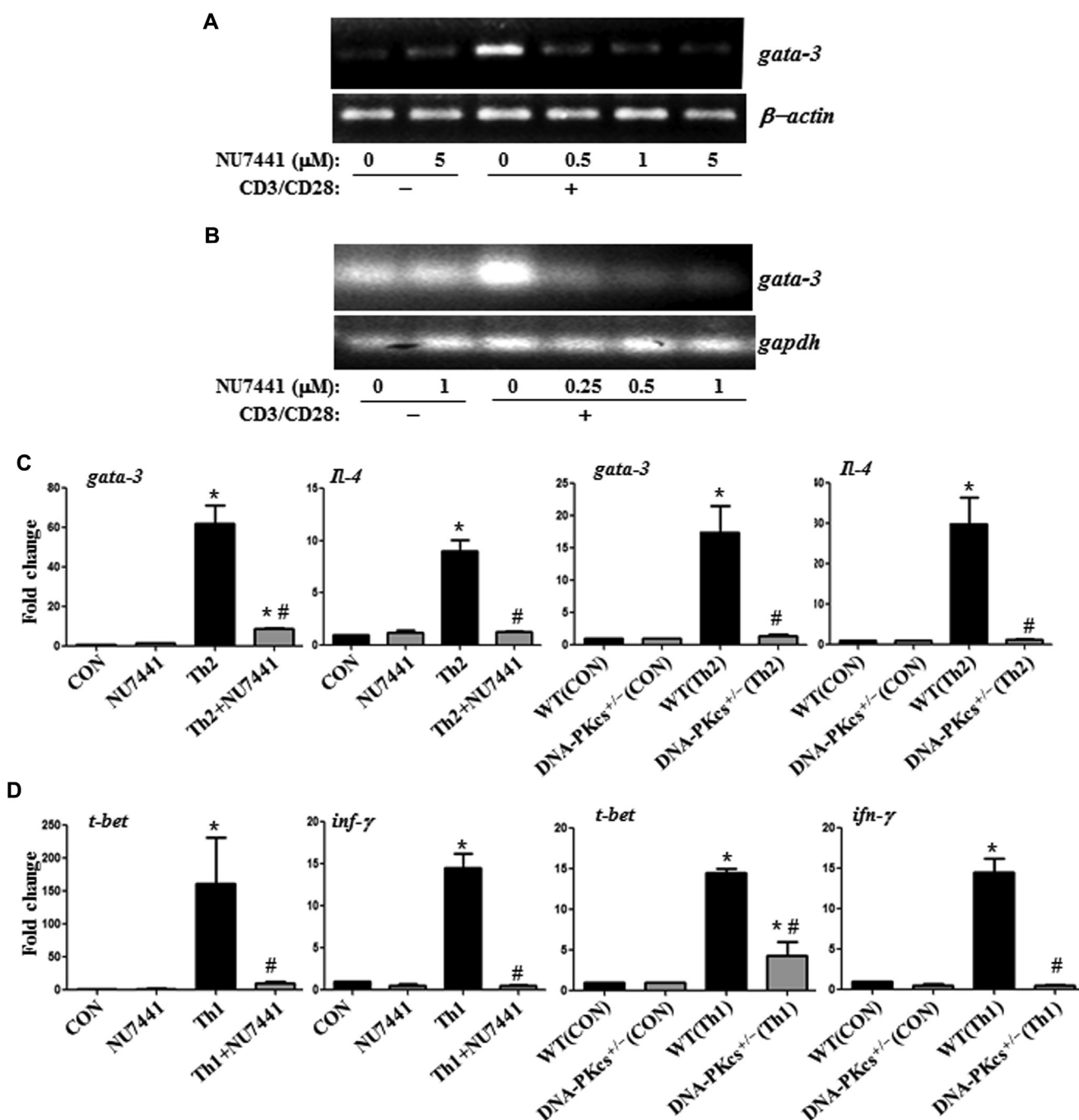


**FIG 9.** DNA-PK inhibition blocks CD3/CD28-induced *Gata3* expression in naive mouse or human CD4<sup>+</sup> T cells and prevents *in vitro* differentiation of mouse T<sub>H</sub>1 and T<sub>H</sub>2 cells under respective T<sub>H</sub>1- and T<sub>H</sub>2-skewing conditions. **A** and **B**, CD4<sup>+</sup> T cells isolated from spleens of WT mice (Fig 9, A) or PBMCs collected from healthy donors (Fig 9, B) were stimulated with antibodies to CD3/CD28 for 24 hours in the absence or presence of different concentrations of NU7441. Isolated RNA was reverse transcribed, and the resulting cDNA was subjected to conventional PCR with primer sets specific to human or mouse *Gata3*. CD4<sup>+</sup> T cells isolated from spleens of OVA-sensitized WT or DNA-PKcs<sup>+/-</sup> mice were skewed toward a T<sub>H</sub>1 phenotype with IL-12 and anti-IL-4 antibodies or toward a T<sub>H</sub>2 phenotype with IL-4 and anti-IL-12 antibodies. Subsets of WT CD4<sup>+</sup> T cells were skewed toward T<sub>H</sub>1 and T<sub>H</sub>2 in the presence of 1  $\mu$ mol/L NU7441. **C** and **D**, Isolated RNA was reverse transcribed, and the resulting cDNA was subjected to real-time PCR with primer sets specific to mouse *Gata3* or *Il4* (Fig 9, C) or with primer sets specific to *T-bet* or *Ifng* (Fig 9, D). \*Difference from nonstimulated cells and #difference from T<sub>H</sub>2-skewed (Fig 9, C) or T<sub>H</sub>1-skewed (Fig 9, D) cells ( $P < .01$ ).

to acknowledge that phosphorylation of p50 NF- $\kappa$ B by DNA-PK might not be the sole reason for the connection between the kinase and the expression of  $T_H2$  cytokines. Other factors might contribute to the protective effect of DNA-PK inhibition. The expression of *T-bet* is not regulated by p50 NF- $\kappa$ B because its expression is not altered by p50 NF- $\kappa$ B gene deletion, as reported by Das et al.<sup>8</sup> Accordingly, it is quite possible that DNA-PK is involved in additional signaling processes beside that of NF- $\kappa$ B. Clearly, a great deal of experimentation is required to fully understand the mechanism through which DNA-PK influences allergic inflammation.

Overall, the present study only begins to unravel a novel facet of DNA-PK for its participation in the complex process of allergic inflammation and AHR. Our findings suggest that the kinase might represent a novel therapeutic target for the treatment of asthma.

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**Clinical implications: Our results suggest DNA-PK as a novel determinant of asthma and that its potential as a therapeutic target for the treatment of the disease merits further examination.**

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