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CD4$^+$ T Cell-derived Novel Peptide Thp5 Induces Interleukin-4 Production in CD4$^+$ T Cells to Direct T Helper 2 Cell Differentiation*[S]

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Background: CD4$^+$ T cells produce IL-4 that drives Th2 cell differentiation. Early production of IL-4 in naïve T cells leads to Th2 cell differentiation.
Results: Thp5, a novel peptide, regulates IL-4 production in early activated CD4$^+$ T cells.
Conclusion: Early activated CD4$^+$ T cells produce Thp5, which plays a critical role in the differentiation of Th2 cells.
Significance: Thp5 acts as an important determinant of Th2 cell differentiation during early T cell activation.

The differentiation of naïve CD4$^+$ T cells into T helper 2 (Th2) cells requires production of the cytokine IL-4 in the local microenvironment. It is evident that naïve/quiescently activated CD4$^+$ T cells produce the IL-4 that drives Th2 cell differentiation. Because early production of IL-4 in naïve T cells leads to preferential Th2 cell differentiation, this process needs to be tightly regulated so as to avoid catastrophic and misdirected Th2 cell differentiation. Here, we show that Thp5, a novel peptide with structural similarity to vasoactive intestinal peptide, regulates production of early IL-4 in newly activated CD4$^+$ T cells. Induction of IL-4 in CD4$^+$ T cells by Thp5 is independent of the transcription factor STAT6 but dependent on ERK1/2 signaling. Furthermore, cytokines (IL-12 and TGF-β) that promote the differentiation of Th1 or Th17 cells inhibit Thp5 induction, thus suppressing Th2 cell differentiation. We further showed that Thp5 enhances Th2 responses and exacerbates allergic airway inflammation in mice. Taken together, our findings reveal that early activated CD4$^+$ T cells produce Thp5, which plays a critical role as a molecular switch in the differentiation of Th cells, biasing the response toward the Th2 cell phenotype.

In addition to expressing peptide-bound major histocompatibility complex (MHC) and co-stimulatory molecules, professional antigen-presenting cells produce polarizing cytokines that drive the differentiation of T helper (Th)[8] cells. Antigen-presenting cell-derived interleukin (IL)-12 directs Th1 differentiation, whereas IL-6 and transforming growth factor (TGF)-β instruct Th17 differentiation (1). In contrast, differentiation of Th2 cells requires IL-4, which is not produced by professional antigen-presenting cells. It has been suggested that in some situations the initial IL-4 is produced by third-party cells, such as natural killer T cells, eosinophils, or basophils (2, 3). However, this hypothesis cannot explain how third-party cells become activated in response to specific antigens to induce Th2 cell differentiation, or how Th2 differentiation occurs in the absence of such cells. Alternatively, the early source of IL-4 might be the interacting CD4$^+$ T cells themselves. Indeed, it has been reported that freshly activated naïve CD4$^+$ T cells produce IL-4 that can drive Th2 differentiation in some settings (4–6). As this early IL-4 is the key determinant for the differentiation of Th2 cells, its production must be tightly regulated so as to avoid unwanted biased Th2 responses. Here, we show that T helper cell-induced peptide 5 (Thp5), a novel peptide with structural similarity to vasoactive intestinal peptide, regulates production of early IL-4 in newly activated

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[S] This article contains supplemental Figs. S1 and S2.
The atomic coordinates and structure factors (code 2LMA) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).
The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) 17584.
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[8] The abbreviations used are: Th cell, T helper cell; AAI, allergic airway inflammation; BAL, bronchoalveolar lavage; Thp5, T helper cell-induced peptide 5.

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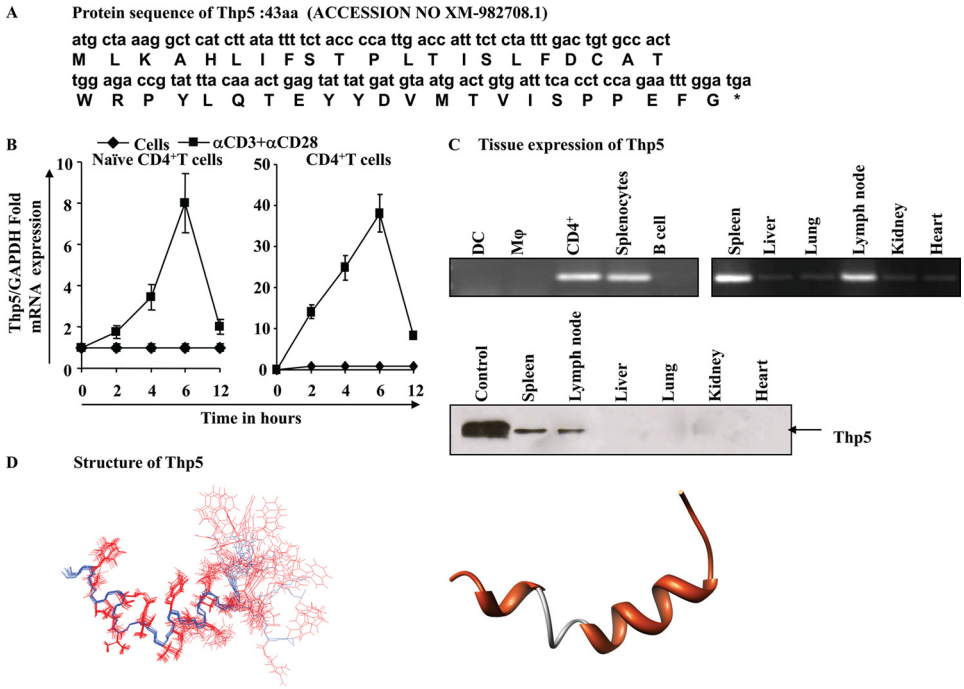

FIGURE 1. **Thp5, a novel bioactive peptide expressed in activated CD4$^+$ T cells.** Microarray analysis revealed that XM_982708.1 gene is highly up-regulated in early activated CD4$^+$ T cells. *A*, nucleotide and amino acid sequence of Thp5. *B*, Thp5 mRNA expression in naïve (CD4$^+$ CD44^{low}CD62L^{high}) CD4$^+$ T or total CD4$^+$ T cells early after activation by α-CD3 and α-CD28 antibodies. *C*, tissue expression of Thp5 by RT-PCR (*top*) and Western blotting (*bottom*; as a positive control we used immunoprecipitated Thp5 from culture supernatant of CD4$^+$ T cells). *D*, solution-state structure of synthetic Thp5 peptide by NMR spectroscopy. *Left* figure shows the ensemble of 40 conformers of Thp5 after Cyana- simulated annealing calculation. Backbone bonds are shown in *blue*, and side chain bonds are shown in *red*. *Right* figure shows the secondary structure elements on the mean structure of Thp5. *Brown* segment shows helical region, and *gray* segment shows the loops and bends.

CD4$^+$ T cells. Induction of IL-4 in CD4$^+$ T cells by Thp5 is independent of the transcription factor signal transducer and activator of transcription (STAT)6 but dependent on extracellular signal-regulated kinases (ERK)1/2 signaling. Furthermore, cytokines (IL-12 and IL-6 plus TGF-β) that promote the differentiation of Th1 or Th17 cells inhibit Thp5 induction, thus suppressing Th2 cell differentiation. We further showed that Thp5 enhances Th2 responses and exacerbates allergic airway inflammation (AAI) in mice. Taken together, our findings reveal that early activated CD4$^+$ T cells produce Thp5, which plays a critical role as a molecular switch in the differentiation of Th cells, biasing the response toward the Th2 cell phenotype.

EXPERIMENTAL PROCEDURES

Animals, Bacterial Strains, Vectors, and Antibodies—BALB/c, C57BL/6, ERK1$^{-/-}$, STAT6$^{-/-}$, and OT-II T cell receptor transgenic mice were purchased from the Jackson Laboratories. CD1d$^{-/-}$ mice have been described (7). α-CD3 and α-CD28 antibodies were purchased from eBioscience. *Escherichia coli* (*E. coli*) host strain BL21 (DE3) and the plasmid vector pET23a were purchased from Novagen (Darmstadt, Germany). The pMD18-T vector was purchased from Takara (Dalian, China). Yeast extract and peptone for bacterial media were purchased from Oxoid (Hampshire, UK).

Construction of Recombinant Expression Vector pET23a-p5—Temperature gradient PCR was performed to obtain the Thp5 cDNA using a murine cDNA library as the template. Temperature gradient PCR revealed that 62 °C was the optimal temperature for renaturation. The amplified fragment was cloned into pMD18-T vector and confirmed by sequencing. cDNA for the murine Thp5 gene was then released from pMD18-T-Thp5 and inserted in-frame into the prokaryotic expression vector pET23a to obtain the expression vector pET23a-Thp5. The obtained recombinant clones were identified by NdeI and XhoI digestion. Both restriction enzyme digestion and DNA sequencing confirmed that the constructed pET23a-Thp5 vector had a correct sequence/structure.

Polyclonal Antibody Production—Male Wistar rats, aged 8–10 weeks, were injected intraperitoneally at 14-day intervals with 50 μg of immunogen (Thp5) dissolved in 200 μl of PBS buffer and then emulsified with 200 μl of Complete Freund's Adjuvant for the first immunization and with Incomplete Freund's Adjuvant for subsequent immunizations. Two booster intravenous injections were applied at 7-day intervals. Antisera from animals were collected after each booster injection to monitor the immune response (antibody titer) during immunization by Western blotting. 14 days after the last immunization, blood samples were collected, and the obtained antiserum was stored at −20 °C.

Solution Structure Analysis—An NMR sample was prepared in 50 mM sodium phosphate buffer containing 150 mM NaCl, pH 6.8. All NMR experiments were carried out at 298 K on Bruker Avance III spectrometers equipped with cryogenic triple-resonance probes, operating at field strengths of 500 and 700 MHz. 4,4-dimethyl-4-silapentane-1-sulfonic acid was used for ^{1}H chemical shift referencing.

Sequence-specific backbone and side chain resonance assignment were obtained using standard two-dimensional experiments (^{15}N,^{1}H HSQC, ^{13}C,^{1}H HSQC, ^{1}H,^{1}H TOCSY,

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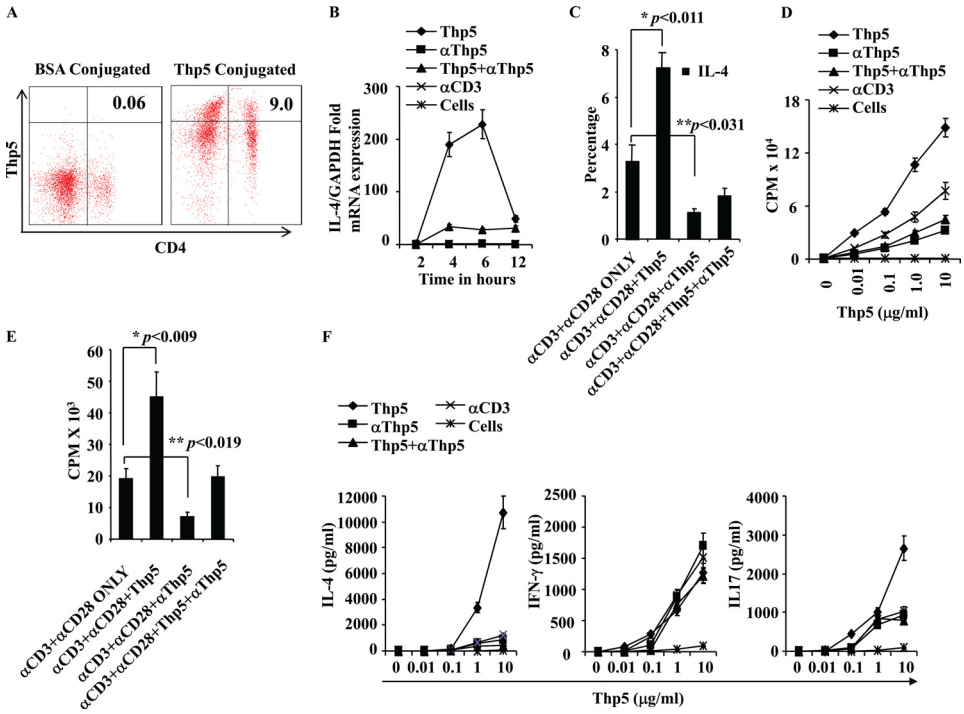

FIGURE 2. **Thp5 induces T cell proliferation and cytokine production.** *A*, FACS analysis showing that Thp5 binds with CD4$^+$ T cells. *B*, IL-4 mRNA expression in CD4$^+$ T cells at early time points after activation with Thp5. *C*, IL-4 production by sorted naïve (CD4$^+$ CD44^{low}CD62L^{high}) CD4$^+$ T cells activated with Thp5. *D*, proliferation of naïve (CD4$^+$ CD44^{low}CD62L^{high}) CD4$^+$ T cells in response to increasing concentrations of Thp5. *E*, proliferation of naïve CD4$^+$ T cells in response to Thp5 (1 μg/ml). *F*, IL-4, IFN-γ, and IL-17 production by purified CD4$^+$ T cells activated with increasing concentrations of Thp5.

^{1}H,^{1}H NOESY, NOESY mixing time, 100 ms). The sequence-specific assignments have been deposited in the BioMagResBank data base, accession number 17584. All spectra were processed with Topspin 2.1 (Bruker AG). All data were analyzed using Cara (8) Distance restraint for structure calculation was derived from the two-dimensional ^{1}H,^{1}H NOESY spectrum (9). Structure was calculated with the program Cyana using 200 randomized starting structures (10). The 20 conformers with the lowest target function values were selected to represent the NMR structure. The final energy minimization was done using AMBER. Figures were prepared with program MOLMOL (11).

Real Time PCR—RNA was isolated from whole splenocytes and CD4$^+$ T cells using a kit (Qiagen). 100 ng of total RNA was used to synthesize cDNA using oligo(dT)$_{16}$ primers and Omniscript RT kit (Qiagen). Thp5, IL-4, and GAPDH primers (ThP5 forward, 5′-TCTACCCCATTGACCATTTC-3′ and ThP5 reverse, 5′-TCATCCAAATTCTGGAGGTG-3′; IL-4 forward, 5′-AGATCACGGCATTTTGAACG-3′ and IL-4 reverse, 5′-TTTGGCACATCCATCTCCG-3′; GAPDH forward, 5′-CGTCCCGTAGACAAAATGGT-3′ and GAPDH reverse, 5′-TTGATGGCAACAATCTCCAC-3′) were used to quantitate mRNA transcripts using Bio-Rad CFX 100 with universal cycling conditions: 95 °C for 10 min and 40 cycles (denaturation at 95 °C for 15 s, annealing/extension at 62 °C for 1 min).

Isolation of Naïve CD4$^+$ T Cells—Spleens and lymph nodes were macerated using frosted glass slides to isolate T cells and whole splenocytes. CD4$^+$ T cells were enriched by passing through a nylon wool column. Cells were further isolated by positive selection using monoclonal antibodies to CD4 coupled with magnetic beads (MACS Miltenyi Biotec, Auburn, CA) using a MACS preparation column. For sorting naïve CD4$^+$ T cells, nylon wool-enriched cells were stained with anti-CD4, anti-CD44, and anti-CD62L antibodies. CD4$^+$ CD44^{low}CD62L^{high} naïve T cells were sorted by using FACS Aria (BD Biosciences).

T Cell Activation and Cytokine Detection—Purified CD4$^+$ T cells and whole splenocytes from different strains of mice were activated by plate-bound anti-CD3 antibodies (1 μg/ml) with either plate-bound anti-CD28 (2 μg/ml) antibodies or soluble anti-CD28 antibodies, respectively (eBiosocience). Cytokine levels in culture supernatants were determined by multiplexed bead array immunoassay using Luminex Technology (Bio-Plex; Bio-Rad Laboratories).

CD4$^+$ T Cell Proliferation—CD4$^+$ T cells were cultured in 24-well plates coated with anti-CD3 (1 μg/ml) and anti-CD28 (2 μg/ml) antibodies. Cells were cultured in the presence of various concentrations of Thp5 for 48 h and then pulsed with tritiated thymidine ([^{3}H]TdR, 1.0 μCi/well; Amersham Biosciences) before measuring incorporation of [^{3}H]TdR by means of a cell harvester and liquid scintillation counter (Wallac Trilux, PerkinElmer Life Sciences) 16 h later.

Induction of AAI—6–8-week-old male ERK1$^{-/-}$ and wild-type (C57BL/6) mice were sensitized by intraperitoneal injections of Thp5 (100 μg) or ovalbumin (100 μg) along with alum (1 mg) in 0.2 ml of PBS on days 0 and 7. On days 15–18, mice were challenged daily intranasally with Thp5 (0.5 mg/ml in 50 ml of PBS) or ovalbumin alone. Mice were killed after 1 day, and bronchoalveolar lavage (BAL) fluid was collected by intratracheal infusion of 2 ml of PBS, as described (12). Cells in the BAL fluid were analyzed on slides after hematoxylin & eosin staining.

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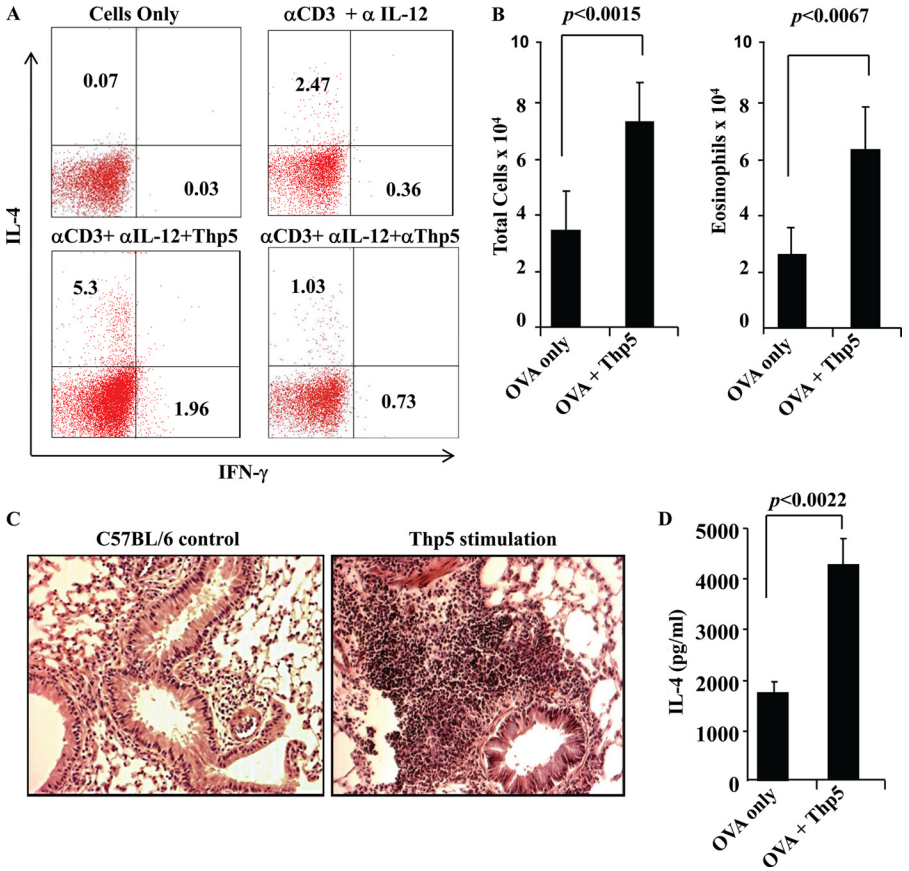

FIGURE 3. **Thp5 induces Th2 cell differentiation and AAI.** *A*, Th2 cell differentiation by Thp5 determined by intracellular IL-4 production in the presence of anti-IL12. *B*, inflammatory cells in the BAL fluid of mice with ovalbumin (*OVA*)-induced AAI. *C*, histological analysis of lungs of wild-type littermates (C57BL/6) induced for AAI and treated with or without Thp5. *D*, Th2 cytokines in the BAL fluid of mice induced for AAI and treated with Thp5. Results shown here are representative of three independent experiments.

FACS Analysis—Spleen cells (1×10^6) were taken from C57BL/6 mice and stained with Alexa Fluor-conjugated (Alexa-Fluor 647 protein labeling kit A20173; Invitrogen) Thp5 along with Alexa Fluor 647-conjugated BSA as a control. Cells were acquired with a BD FACS Canto^{TM} II (BD Biosciences) and analyzed by FlowJo (Tree star) software.

Statistical Analysis—Data were analyzed in Excel 2007 and subjected to Student's *t* test for statistical significance. Statistical significance was considered when $p \leq 0.05$.

RESULTS AND DISCUSSION

We investigated the molecular changes in CD4$^+$ T cells during their early activation by microarray analysis. We repeatedly found expression of a small protein/peptide (accession number XM-982708.1; Fig. 1*A* and supplemental Fig. S1*A*) designated Thp5, that is dramatically up-regulated in CD4$^+$ T cells early after activation (Fig. 1*B*). We further tested whether Thp5 is produced by newly activated, naïve CD4$^+$ T cells. We sorted CD4$^+$ CD44^{low}CD62L^{high} naïve T cells and activated these cells with plate-bound anti-CD3 and anti-CD28 antibodies. We found that naïve CD4$^+$ T cells also produced Thp5 and with kinetics similar that of the total pool of CD4$^+$ T cells (Fig. 1*B*). Next, we tested the distribution of expression in different organs and immune cell types and found that Thp5 is expressed predominantly in lymphoid organs with the highest expression in CD4$^+$ T cells (Fig. 1*C*). We cloned and expressed Thp5 as a polyhistidine-tagged (His tag) fusion peptide in *E. coli* (supplemental Fig. S1*B*) and used the purified fusion peptide to generate Thp5-specific, polyclonal antisera (supplemental Fig. S1*C*). To determine whether Thp5 is expressed alone or as part of a complex, we immunoprecipitated culture supernatants from anti-CD3 and anti-CD28 antibody-activated spleen cells with anti-Thp5 sera, which did not reveal any interacting proteins (supplemental Fig. S1*D*), suggesting that it is not a part of any protein complex. Amino acid sequence analysis followed by sequence homology analysis was unable to identify significant homology with any known cytokines, chemokines, or bioactive peptides. However, solution structural analysis by NMR spectroscopy revealed a helical structure with a bend in the middle, resembling vasoactive intestinal peptide (Fig. 1*D*) (13). The coordinates of all 40 conformers have been deposited with the Protein Data Bank. The corresponding Protein Data Bank code is 2LMA. The sequence-specific NMR resonance assignments have been deposited in the BioMagResBank data base, accession number 17584.

Vasoactive intestinal peptide assists in the differentiation of Th2 cells and Treg cells (14–16). Therefore, we tested the kinetics of Thp5 expression in CD4$^+$ T cells upon activation by plate-bound anti-CD3 and anti-CD28 antibodies. Interestingly, we found that Thp5 is expressed very early after activation, within 3–6 h (Fig. 1*B*). Next, we investigated the expression of

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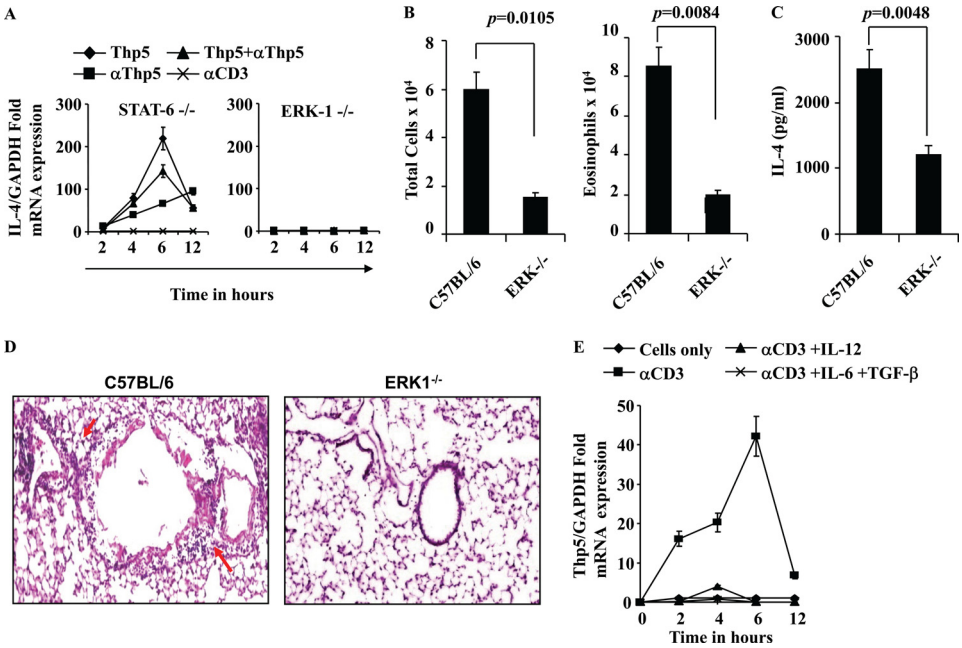

FIGURE 4. **Thp5 induces IL-4 production in a STAT6-independent and ERK-dependent manner.** *A*, early burst of IL-4 mRNA expression in the presence of Thp5 in STAT6$^{-/-}$ and ERK$^{-/-}$ knock-out mice. *B*, inflammatory cells in the BAL fluids of wild-type littermates (C57BL/6) and ERK$^{-/-}$ mice induced for AAI. *C*, IL-4 concentration in the BAL fluid of wild-type littermates (C57BL/6) and ERK$^{-/-}$ mice induced for AAI. *D*, histology of lungs of wild-type littermates (C57BL/6) and ERK$^{-/-}$ mice induced for AAI. *E*, Thp5 mRNA expression analysis in CD4$^+$ T cells in the presence of Th1-differentiating cytokine (IL-12) or Th17-differentiating cytokines (IL-6+TGF-β) at different time points.

the putative receptor of Thp5 on splenocytes. We conjugated recombinant Thp5 or ovalbumin with green fluorescent protein (GFP) and stained splenocytes. We found that Thp5 bound with CD4$^+$ T cells and most other cell types in the spleen (Fig. 2*A*). To determine the functional effects of Thp5 on CD4$^+$ T cells, we cultured purified CD4$^+$ T cells with plate-bound anti-CD3 and anti-CD28 antibodies in the presence or absence of Thp5. We found an early burst of IL-4 production by CD4$^+$ T cells (Fig. 2*B*). To confirm the role of Thp5 in the production of this early burst of IL-4, we neutralized Thp5 with Thp5-specific rat antiserum (supplemental Fig. S2*A*) and observed a strong suppression of early IL-4 production (Fig. 2*B*). We also tested whether Thp5 assists in the differentiation of CD4$^+$ T cells with an IL-4-producing phenotype. For this purpose, we activated CD4$^+$ CD44^{low}CD62L^{high} naïve T cells with plate-bound anti-CD3 and anti-CD28 antibodies in the presence or absence of Thp5 or anti-Thp5 antibodies. Cells were stained for intracellular IL-4. We found that inhibition of Thp5 dramatically inhibits, whereas supplementation of Thp5 significantly up-regulates, the differentiation of IL-4-producing cells (Fig. 2*C*). To confirm further that activated/memory T cells are not the source of IL-4, we performed similar experiments with splenocytes from OT-II T cell receptor transgenic mice, which produced IL-4 to a similar extent as splenocytes from wild-type animals (supplemental Fig. S2*C*). Because CD1d-restricted natural killer T cells produce copious amounts of IL-4 early after activation (17), we tested Thp5-induced IL-4 production in splenocytes from CD1d-deficient (CD1d$^{-/-}$) animals. We found no difference in IL-4 production compared with wild-type splenocytes, suggesting that natural killer T cells are not the major source of Thp5-induced IL-4 (supplemental Fig. S2*B*). Next, we evaluated whether Thp5 assists in the proliferation and activation of CD4$^+$ T cells. For this purpose, we activated purified CD4$^+$ T cells with a suboptimal dose of plate-bound anti-CD3 and anti-CD28 antibodies, in the presence of increasing concentrations of Thp5, and measured proliferation and cytokine production 3 days later. We found that Thp5 augments proliferation of CD4$^+$ T cells in a dose-dependent manner (Fig. 2*D*). We also observed that similar to the total pool of CD4$^+$ T cells, proliferation of naïve CD4$^+$ T cells was augmented by the presence of Thp5 (Fig. 2*E*). To assess whether Thp5 induces a biased differentiation of Th cells, we determined cytokine production in the culture supernatants. We found that Thp5 dramatically induces IL-4 production in both spleen cells and purified CD4$^+$ T cells, but has only modest effects on the production of IFN-γ and IL-17, suggesting that this peptide assists in the differentiation of IL-4-producing Th cells (Fig. 2*F*). As expected, addition of anti-Thp5 sera abrogated IL-4 production in both spleen cells and purified CD4$^+$ T cells.

To assess whether Thp5 alone, in the absence of exogenous IL-4, can induce Th2 differentiation, we activated CD4$^+$ T cells with plate-bound anti-CD3 and anti-CD28 antibodies in the presence of Thp5 and anti-IFN-γ for 4 days and determined intracellular levels of IL-4. Interestingly, we found that Thp5 alone was sufficient to drive Th2 cell differentiation (Fig. 3*A*). To evaluate the role of Thp5 *in vivo*, we induced AAI in mice treated with or without Thp5. Animals that received Thp5 exhibited significantly higher lung inflammation as evidenced by the increased infiltration of inflammatory cells in the bronchoalveolar lavage fluid (Fig. 3*B*) and by histological analysis of lung tissue (Fig. 3*C*). Consistent with these findings, we observed a dramatic increase in Th2 cytokines in the BAL fluid (Fig. 3*D*). Collectively, these observations suggested that Thp5

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rapidly induces IL-4 in naïve CD4$^+$ T cells, which assists in the differentiation of Th2 cells.

Differentiation of Th2 cells is dependent on the availability of IL-4 in the microenvironment, with a critical role for the transcription factor STAT6 (18). Therefore, we examined whether early IL-4 production induced by Thp5 is dependent on STAT6. For this purpose, we activated spleen cells derived from STAT6$^{-/-}$ mice in the presence of Thp5. We found that the early burst of IL-4 is independent of STAT6 (Fig. 4*A*), which is consistent with prior studies showing that STAT6 induction in CD4$^+$ T cells requires at least 48 h of activation. Previous reports have suggested that ERK1/2 is involved in the differentiation of Th2 cells (6, 19, 20) and that ERK1/2 are induced very early after activation. Thus, we tested whether Thp5-induced IL-4 is dependent on ERK1/2. We activated spleen cells from ERK1$^{-/-}$ mice in the presence of Thp5, which revealed that Thp5 was unable to induce IL-4 in splenic CD4$^+$ T cells from ERK1$^{-/-}$ mice (Fig. 4*A*). This finding was further strengthened by our observation that ERK1$^{-/-}$ mice have dramatically reduced susceptibility to AAI than their wild-type littermates, as demonstrated by the reduced infiltration of inflammatory cells (Fig. 4*B*) and lower levels of Th2 cytokines (Fig. 4*C*) in the BAL fluid. Histological analysis also revealed dramatically reduced inflammation in the lungs of ERK1$^{-/-}$ mice (Fig. 4*D*).

Our findings suggest that the early production of IL-4 by activated CD4$^+$ T cells is regulated by the presence of Thp5 in the microenvironment. However, because Thp5 is also produced by early activated T cells, its expression must also be regulated to permit differentiation of other Th cell subsets, namely Th1 and Th17 cells. Thus, we tested the regulation of Thp5 expression in the presence of the Th1- and Th17-differentiating cytokines IL-12 and IL-6 plus TGF-β, respectively. Interestingly, we found that the presence of either of these cytokines in the culture medium dramatically inhibited Thp5 expression (Fig. 4*E*).

Taken together, our findings indicate that Thp5 is a novel bioactive peptide that is produced by early activated CD4$^+$ T cells in a STAT6-independent but ERK1/2-dependent manner. Thp5 induces IL-4 production in CD4$^+$ T cells in an autocrine fashion, which plays an important role in the differentiation of Th2 cells. Furthermore, the availability of Th1- and/or Th17-differentiating cytokines in the local microenvironment dramatically inhibits Thp5 production. Thus, Thp5 acts as an important determinant of Th2 cell differentiation during early T cell activation.

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FIGURE S1: Expression and purification of Thp5. (A) Gene structure of Thp5. **(B)** SDS PAGE analysis showing recombinant Thp5 expression in *E. coli* strain Bl21 (DE3) and Western Blot of Thp5. **(C)** Western Blot to show the presence of polyclonal antibodies (α-Thp5) in rat sera. **(D)** SDS PAGE analysis showing immunoprecipitation of Thp5 from culture supernatant of purified $CD4^+$ T cells.

FIGURE S2: Thp5 induces IL-4 production in a CD1d-independent manner. Production of IL-4 by $CD4^+$ T cells from in **(A)** C57BL/6, **(B)** $CD1d^{-/-}$ and **(C)** OT-II TCR Tg mice treated with plate-bound anti-CD3 and anti-CD28 antibodies in the presence or absence of Thp5 and anti-Thp5 antibodies.

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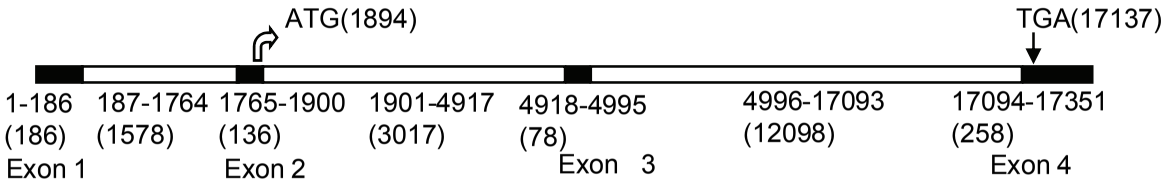
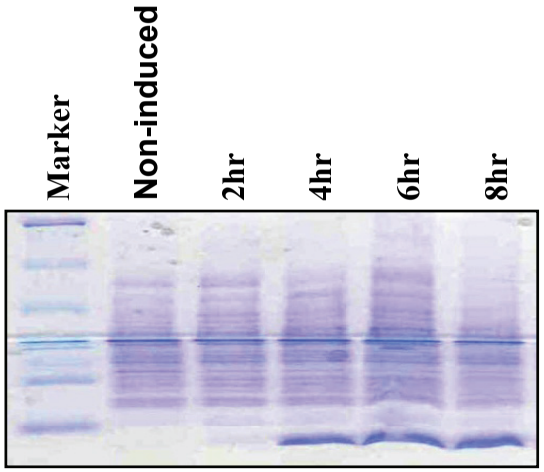
A **Gene structure** Gene= XM_982708.1 : chromosome="X", mol_wt=4866

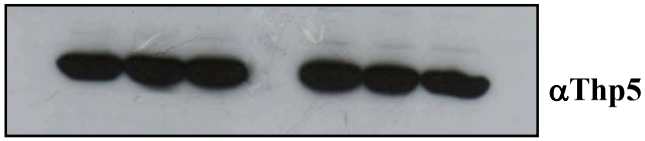


B **Expression in E. *coli***



His-tag Thp5 protein expression Western-blot

C **Polyclonal antibody raised in rat against Thp5 Western-blot**



D **Immunoprecipitation of Thp5**

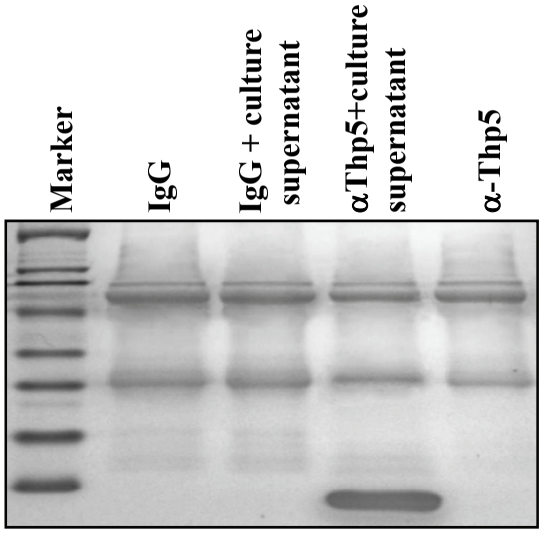


Fig.S1; Khan et al.

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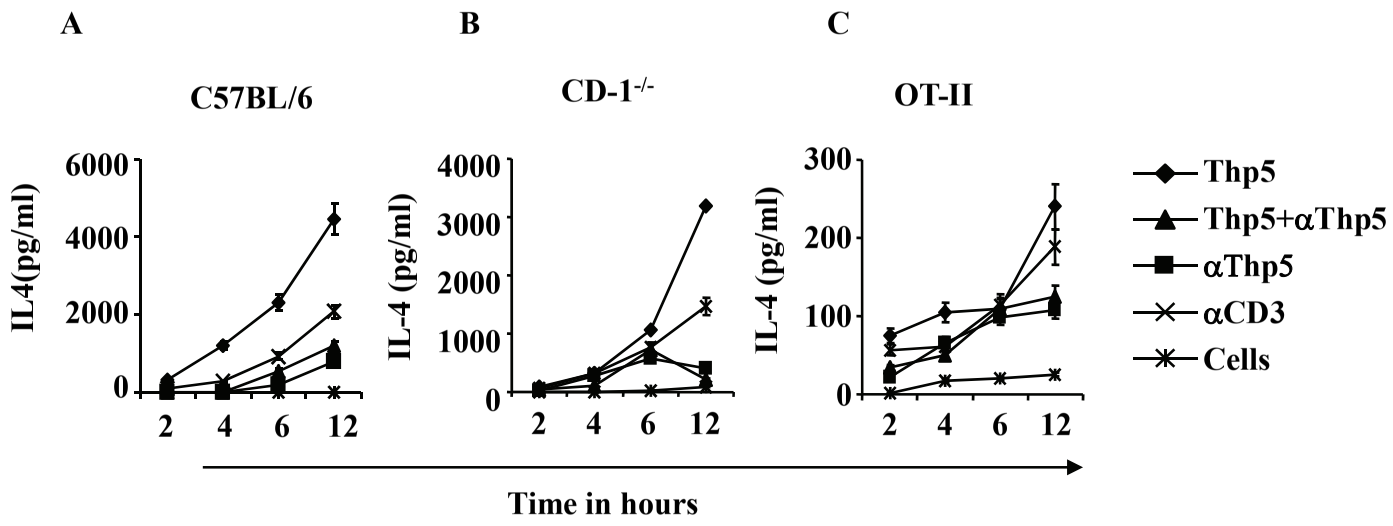

Fig.S2; Khan et al.

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