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Cathelin-related antimicrobial peptide differentially regulates T- and B-cell function

Nicholas W. Kin[*,1], **Yao Chen**[*,1], **Emily K. Stefanov**[1], **Richard L. Gallo**[2,3], and **John F. Kearney**[1]

[1]Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL, USA

[2]Division of Dermatology, University of California San Diego, La Jolla, CA, USA

[3]VA Healthcare System, San Diego, CA, USA

Abstract

Mammalian antimicrobial peptides (AMPs) play an important role in host defense via direct antimicrobial activity as well as immune regulation. The mouse cathelin-related antimicrobial peptide (mCRAMP), produced from the mouse gene *Camp*, is the only mouse cathelicidin identified and the ortholog of the human gene encoding the peptide LL-37. This study tested the hypothesis that mouse B and T cells produce and respond to mCRAMP. We show that all mature mouse B-cell subsets, including follicular (FO), marginal zone (MZ), B1a, and B1b cells, as well as $CD4^+$ and $CD8^+$ T cells produce *Camp* mRNA and mCRAMP protein. $Camp^{-/-}$ B cells produced equivalent levels of IgM, IgG3, and IgG2c but less IgG1 and IgE, while $Camp^{-/-}$ $CD4^+$ T cells cultured in Th2-inducing conditions produced more IL-4-expressing cells when compared with WT cells, effects that were reversed upon addition of mCRAMP. In vivo, $Camp^{-/-}$ mice immunized with TNP-OVA absorbed in alum produced an enhanced TNP-specific IgG1 response when compared with WT mice. ELISpot analysis revealed increased numbers of TNP-specific IgG1-secreting splenic B cells and FACS analysis revealed increased $CD4^+$ T-cell IL-4 expression. Our results suggest that mCRAMP differentially regulates B- and T-cell function and implicate mCRAMP in the regulation of adaptive immune responses.

Keywords

Antibody; Cytokine; IgG1; mCRAMP

Introduction

Mammalian antimicrobial peptides (AMPs) include the gene families of defensins and cathelicidins. Defensins are characterized by six conserved cysteine residues and various disulfide bond configurations, while cathelicidins are characterized by the presence of a conserved cathelin-like domain, an N-terminal signal sequence, and a highly variable antimicrobial C-terminal domain [1, 2]. AMPs have direct lytic properties against a variety of organisms including bacteria, fungi, and viruses [1, 2]. In addition, it is becoming increasingly appreciated that AMPs are also immunomodulatory. For example, AMPs have

Full correspondence: Dr. John F. Kearney, Department of Microbiology, University of Alabama at Birmingham, 410 Shelby Biomedical Research Building, 1825 University Blvd., Birmingham, AL 35294, USA Fax: 11-205-996-9908 jfk@uab.edu.
*These authors contributed equally to this work.

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been shown to act as chemoattractants [3–5], protect skin and mucosal surfaces against bacterial infections [6–10], promote wound healing [11–13], and modulate changes in cellular function [14–18]. The mechanism by which AMPs modulate immune trafficking and function is not completely understood, although a number of potential receptors have been suggested for the human cathelicidin LL-37. These include EGFR [11, 13, 19], FPRL1 [3, 5], $P2X_7$ [20, 21], GAPDH [22], and CXCR2 [23].

The mouse cathelin-related antimicrobial peptide (mCRAMP) is encoded by the gene *Camp* and is the sole identified mouse cathelicidin. *Camp* is the mouse ortholog of the only human cathelicidin gene (*CAMP*), which encodes the peptide LL-37 [24]. mCRAMP forms a positively charged amphipathic α-helical structure [25, 26] and has direct antimicrobial properties through a number of proposed mechanisms [27]. While mCRAMP and other AMPs have been studied mainly for their role in regulating innate cell activation, their role in the adaptive immune response has been studied less extensively. LL-37 is expressed in human B and T cells [4, 28]; however, mCRAMP expression in mouse lymphocytes has not been investigated.

Mature B cells play an important role in the adaptive immune response through antigen presentation, T-cell-independent (TI) and -dependent (TD) antibody production, and regulatory functions [29, 30]. A TD antibody response is a tightly regulated process that needs T- and B-cell cooperation for an optimal antibody response. T-cell membrane-bound CD40L and secreted IL-4 interact with B-cell membrane-bound CD40 and IL-4R, respectively, to induce class switching to IgG1 [31, 32] and IgE [33], which are important antibody isotypes produced in a wide variety of immune responses. The ability of mouse B and T cells to produce and respond to mCRAMP and its role in an adaptive immune response is not fully known.

We hypothesized that mouse B and T cells express and respond to mCRAMP. In the current study, we show that all mature B-cell subsets tested, including marginal zone (MZ), follicular (FO), B1a, and B1b cells as well as all mature T-cell subsets tested express *CAMP* mRNA and mCRAMP protein directly ex vivo. *CAMP* mRNA was rapidly upregulated in mouse B and T cells following activation. Purified $CAMP^{-/-}$ B cells produced equivalent levels of IgM, IgG3, and IgG2c but less IgG1 and IgE, while purified $CAMP^{-/-}$ $CD4^+$ T cells cultured in Th2-inducing conditions produced more $IL\text{-}4^+$ cells when compared with WT B and T cells, effects that were reversed upon addition of exogenous mCRAMP. In addition, immunization of $CAMP^{-/-}$ mice with TNP-OVA, a TD antigen, showed an enhanced TNP-specific secondary IgG1 antibody response, increased IgG1 anti-body-secreting cells (ASCs), and increased IL-4-producing T cells. Overall, these results suggest an additional function of mCRAMP in the regulation of adaptive immune responses.

Results

B and T cells express CAMP mRNA and mCRAMP protein

The human cathelicidin LL-37 is expressed in neutrophils, epithelial cells, mast cells, B cells, γΔ cells, and gd T cells (reviewed in [1, 28]), while the detailed expression of mCRAMP is less well described. To determine whether splenic B and T cells express mCRAMP, splenocytes from C57BL/6 mice were sort-purified to obtain MZ ($B220^+$, $CD21^{hi}$, $CD23^{low}$) B cells, FO ($B220^+$, $CD21^{int}$, $CD23^+$) B cells, $CD4^+$ and $CD8^+$ T cells. In addition, total peritoneal lavage cells were sort-purified to obtain B1a ($CD5^+$ $Mac\text{-}1^+$ $B220^{int}$), B1b ($CD5^+$ $Mac\text{-}1^+$ $B220^{int}$), B2 ($CD5^-$ $Mac\text{-}1^-$ $B220^{high}$), and T cells ($CD5^+$ $B220^-$). Post-sort analysis revealed greater than 95% purity for each B- and T-cell population (data not shown). Total RNA was isolated from each sort-purified cell population and RT-PCR was performed to detect *CAMP*, CD19, CD3e, and actin mRNA. All B and T

cells tested expressed *CAMP* mRNA directly ex vivo (Fig. 1A). To determine whether B and T cells express the mCRAMP protein, total protein was isolated from purified B and T cells and analyzed using Western blot. Figure 1B confirms the expression of the immature mCRAMP protein in the total resting B and T cells. To determine whether B and T cells regulate the expression of *CAMP* following cell activation, total $CD43^-$ splenic B cells were sort-purified and activated with CD40L and IL-4 or IFN-γ, while purified $CD4^+$ T cells were cultured in either Th1- or Th2-inducing conditions. Real-time PCR analysis for the relative expression level of *CAMP*, normalized to actin expression, revealed that both B and T cells increased *CAMP* expression following activation (Fig. 1C). Interestingly, B and T cells express less *CAMP* mRNA and mCRAMP protein relative to purified neutrophils (Fig. 1B and C). In addition, total numbers of B- and T-cell subsets as well as serum antibody levels were equivalent between C57BL/6 and $CAMP^{-/-}$ mice (data not shown). These data show that all B and T cells tested express *CAMP* mRNA and mCRAMP protein, suggesting that mCRAMP has the potential to regulate B- and T-cell functions.

CAMP-deficient T cells under Th2-inducing conditions produce more IL-4^+ cells in vitro

The ability of mCRAMP to directly regulate mouse T-cell cytokine production has not been fully investigated. WT and $CAMP^{-/-}$ naïve $CD4^+$ T cells were sort-purified and cultured in either Th1 (anti-CD3, -CD28, and rIFN-γ) or Th2 (anti-CD3, -CD28, -IL-12, and rIL-4) inducing conditions. Under Th1-inducing conditions, WT and $CAMP^{-/-}$ T cells expressed equivalent amounts of IFN-γ mRNA (Fig. 2A), equivalent numbers of IFN-γ^+ cells (Fig. 2B), and equivalent IFN-γ mean fluorescent intensity (MFI) (Fig. 2C). In contrast, $CAMP^{-/-}$ T cells cultured under Th2-inducing conditions expressed more IL-4 mRNA (Fig. 2D), more IL-4^+ cells (Fig. 2E), and equivalent IL-4 MFI (Fig. 2F). The addition of mCRAMP to the $CAMP^{-/-}$ T-cell cultures had no effect on the level of IFN-γ mRNA or number of IFN-γ^+ cells, while the level of IL-4 mRNA and IL-4^+ cells returned to that of WT. Overall, these results suggest that mCRAMP also functions in the regulation of Th2 IL-4-producing cell differentiation.

CAMP-deficient B cells produce less IgG1 and IgE antibody in vitro

The role of mCRAMP during an antibody response to TI and TD antigens has not been fully investigated. Since B cells express *CAMP*/mCRAMP and *CAMP* is rapidly upregulated following B-cell activation, the possibility exists that mCRAMP directly regulates B cells during an antibody response. Furthermore, since LPS induces class switching to IgG3 [34] and IL-4 induces class switch recombination (CSR) to IgG1 and IgE [31], and IFN-γ induces CSR to IgG2a/2c [35], respectively, we hypothesized that mCRAMP mRNA upregulation during activation with these factors might affect the levels of specific antibody isotypes produced. Resting splenic B cells were sort-purified from WT and $CAMP^{-/-}$ mice and activated in vitro in the presence of LPS, CD40L/IL-4, and CD40L/IFN-γ. WT and $CAMP^{-/-}$ B cells produce similar amounts of IgM (Fig. 3A) and IgG3 (Fig. 3B) in response to LPS stimulation, while CD40L/IFN-γ induced equivalent amounts of IgG2c (Fig. 3C). However, $CAMP^{-/-}$ B cells produced significantly less IgG1 (Fig. 3D) and IgE (Fig. 3E) in response to CD40L/IL-4 when compared with WT B cells. To determine whether mCRAMP directly mediated these effects in vitro and the optimal peptide concentration, mCRAMP peptide (1 ng/mL–1 μg/mL) was added to $CAMP^{-/-}$ B-cell cultures on day 0 with CD40L/IL-4 and the level of IgG1 was measured on day 5. The addition of mCRAMP resulted in a dose-dependent increase in IgG1 with an optimal concentration of 100 ng/mL (Fig. 3F). $CAMP^{-/-}$ B cells cultures were repeated with the addition of 100 ng/mL of mCRAMP and the level of IgG2c (Fig. 3C) was unchanged while IgG1 (Fig. 3D) and IgE (Fig. 3E) returned to WT levels. Overall, these results suggest that mCRAMP functions to positively regulate the level of antibody produced by B cells in an IL-4-dependent manner.

Camp-deficient B cells produce less IgG1 mRNA per cell

The mechanism by which *Camp*$^{-/-}$ B cells produce less IgG1 in comparison to WT B cells could be explained by a number of factors including differences in proliferation, survival, and CSR. To determine the mechanism by which *Camp*$^{-/-}$ B cells produce less IgG1, resting B cells were sort-purified and activated with CD40L/IL-4 or LPS/IL-4. The total live B-cell number (Fig. 4A), the percentage of surface IgG1^{+} B cells (Fig. 4B), and the cell cycle analysis (data not shown) were determined, showing no difference between WT and *Camp*$^{-/-}$ B cells. ELISpot experiments were performed on day 5 B-cell cultures and spots were enumerated to determine the number of IgG1-secreting B cells. Total spot counts were equivalent between WT and *Camp*$^{-/-}$ B cells (Fig. 4C), suggesting that CSR is not affected. However, visual inspection of the spot size of WT B cells appeared larger than that of *Camp*$^{-/-}$ B cell spots. Total ASC spots were dissolved with DMSO and the absorbance was measured at 650 nm (Fig. 4D), showing a significant decrease in absorbance in the *Camp*$^{-/-}$ B cells. Overall, these results suggest that the differences in IgG1 production between WT and *Camp*$^{-/-}$ B cells are not due to defects in proliferation, cell cycle progression, survival, or CSR, but are due to decreased IgG1 production per *Camp*$^{-/-}$ B cell.

Differences in IgG1 production between WT and *Camp*$^{-/-}$ B cells could be explained if there was a change in CSR to IgG1 and a linear relationship has been shown between the amount of B-cell sterile Iγ1 transcript and CSR [36]. Alternatively, the amount of IgG1 mRNA production could be increased in the WT cells compared with *Camp*$^{-/-}$ cells. Therefore, to determine the amount of Iγ1 and IgG1 mRNA in WT and *Camp*$^{-/-}$ cells, B cells were sort-purified and activated as described earlier and total RNA was isolated on days 2–4. Semi-quantitative RT-PCR showed no significant difference in the levels of Iγ1 transcript over the time course analyzed (Fig. 4E), suggesting no change in CSR. However, the level of IgG1 mRNA was significantly higher in the WT compared with *Camp*$^{-/-}$ B cells (Fig. 4F), suggesting that mCRAMP was increasing either the rate or stability of the IgG1 mRNA. To determine the stability of the IgG1 mRNA, actinomycin D was added to the B-cell cultures on day 5 and total RNA was collected every 2 h for a total of 12 h. The stability of the IgG1 mRNA did not differ significantly between the WT and *Camp*$^{-/-}$ B cells (Fig. 4G). Thus, it appears that mCRAMP production by B cells increases the amount of IgG1 produced per cell by increasing the rate of IgG1 mRNA trancription, without affecting CSR or the stability of the IgG1 mRNA.

Camp-deficient mice produce more IgG1 in response to a TD antigen

Our data presented in Fig. 2 show that mCRAMP negatively regulates the level of T-cell IL-4 production in vitro, while our data presented in Fig. 3 show that mCRAMP positively regulates the level of B-cell IgG1 production in vitro. However, the antibody responses to TI-1, TI-2, and TD antigens have not been investigated extensively in *Camp*$^{-/-}$ mice to date. To investigate the antibody response in vivo to these three groups of antigens, WT and *Camp*$^{-/-}$ mice were immunized with either TNP-LPS (TI-1), *S. pneumoniae* (TI-2), or TNP-OVA absorbed to Alum (TD). The levels of IgM and IgG3 antibodies against TNP and phosphorylcholine(PC) were determined by ELISA and showed no significant difference between WT and *Camp*$^{-/-}$ mice (Fig. 5A–D), similar to our findings with LPS-activated B cells in vitro. Mice were also immunized i.p. and s.c. with TNP-OVA absorbed in alum on days 0 and 21 and the level of serum IgG1 antibody was measured. TNP-specific IgG1 was significantly higher in the *Camp*$^{-/-}$ mice following the second i.p. immunization (Fig. 5E) and first s.c. immunization (Fig. 5F). TNP-specific IgG2b and IgG2c were also determined and no differences were detected between WT and *Camp*$^{-/-}$ mice (data not shown). Overall, these results suggest that mCRAMP negatively regulates the TD antibody response in vivo, although the specific cell type responding to and affected by mCRAMP remains unknown.

CD4$^+$ T cells produce more IL-4 and induce more IgG1$^+$ASCs

The results of our in vivo immunization experiments in Fig. 5 suggest that mCRAMP is negatively regulating the antibody response to a TD antigen, TNP-OVA/Alum. Since our in vitro data suggest a differential regulation of B and T cells, we sought to determine the mechanism by which more TNP-specific IgG1 is made by *Camp*$^{-/-}$ mice compared with WT mice. ELISpot analysis of the spleens at 4 days after the second immunization with TNP-OVA/Alum shows that *Camp*$^{-/-}$ mice have more TNP-specific IgG1$^+$ ASCs than WT (Fig. 6A). Since our in vitro data in Fig. 4 suggested that mCRAMP had no effect on isotype switching to IgG1, one potential explanation could be that the production of IL-4 was increased, similar to our findings in Fig. 2 with purified T cells in vitro. RT-PCR was performed to determine the level of total IL-4 mRNA in total spleen. Figure 6B shows that *Camp*$^{-/-}$ spleens contain more IL-4 mRNA than WT spleens. In addition, intracellular staining for IL-4 showed that the numbers of CD4$^+$ IL-4$^+$ T cells were significantly increased in the *Camp*$^{-/-}$ mice (Fig. 6C). Overall, these results suggest that mCRAMP negatively regulates TD antibody responses by regulation of T-cell IL-4 production.

Discussion

Analysis of AMPs has shown that their cellular expression is widespread and their functions are diverse. *Camp*$^{-/-}$ mouse are more susceptible to, and fail to clear, numerous infections [1], supporting a role for AMPs in host defense and immune regulation. Our data showing that mouse B and T cells are capable of expressing and responding to mCRAMP further add to this complexity. Importantly, while the use of *Camp*$^{-/-}$ mice has aided in the study of AMP biology, it is not definitive in differentiating the direct antimicrobial activity from the immune regulation. In addition, our data show that mCRAMP has the ability to regulate B and T cells in vivo, although there is still no clarity as to the exact source of mCRAMP and the mechanism by which it regulates B- and T-cell function.

Using the *Camp*$^{-/-}$ mouse [24], we investigated the role of mCRAMP in regulating adaptive immune responses. Our data show that *Camp*$^{-/-}$ mice immunized with TNP-OVA/Alum produced more TNP-specific IgG1 antibody when compared with WT mice. In contrast, Kurosaka et al. showed that mCRAMP acted as an immune adjuvant and enhanced TD antibody production in WT mice [3]. The most obvious difference in the experiment design, which may contribute to the opposing findings, is that we studied effects of endogenously produced mCRAMP by comparing antibody responses in WT versus *Camp*$^{-/-}$ mice, while Kurosaka et al. [3] added additional exogenous mCRAMP to WT mice. The administration of exogenous mCRAMP to a WT mouse that is also making mCRAMP in response to the immunization may or may not accurately model the role of mCRAMP during an antibody response. In support of this possibility, previous studies have demonstrated that exogenous and endogenous mCRAMP function differently in macrophage activation [15]. An alternative explanation is the specific identity of the cathelicidin peptides produced by the *Camp* gene. It has been shown previously that alternative proteolytic processing is possible for endogenously expressed cathelicidin peptides, which may lead to different physiological effects in vivo [37]. Therefore, it is likely that the immunological response under investigation will be altered depending on the concentration, location, cell types, and the form of mCRAMP released during the response.

The role of AMPs in regulating the magnitude of the adaptive immune antibody responses has not been investigated extensively and the results to date are contradictory. LL-37 (20 μg/mL) was shown to decrease IgM and IgG2a production from mouse splenic B cells activated with LPS and IFN-γ, primarily through inhibition of cell activation and proliferation [16]. In contrast, another study demonstrated that LL-37 (6 μg/mL) increased the sensitivity of human peripheral B cells to CpG, enhancing B-cell activation and

increasing IgM and IgG production [14]. Our data using mCRAMP (100 ng/mL) and purified mouse B cells agree with the latter study [14] and show that mCRAMP increases the amount of IgG1 and IgE antibody production in *Camp*$^{-/-}$ B cells. Of course, two obvious differences that may account for the discrepancies seen are the use of LL-37 versus mCRAMP peptides and mouse versus human B cells. In addition, another very important variable to consider is AMP concentration. Since it is nearly impossible to measure the physiological concentration within the splenic microenvironment where these responses are occurring, we titrated the mCRAMP concentration within our culture system ranging from 1 ng/mL to 10 μg/mL. Consistent with previous findings [38], our data showed that mCRAMP at the highest concentration tested induced cell apoptosis, while moderate concentrations increased IgG1 production, and the lowest concentration showed no effect on IgG1 production. These observations suggest that the AMP concentration within the microenviroment of an immune response may partially dictate the positive or negative effect on antibody production.

Our in vitro and in vivo data show that T cells exposed to mCRAMP produce less IL-4. However, the possibility exists that other cell types are affected by mCRAMP and secondarily affecting the T cells. LL-37 has been shown to drive mouse DC differentiation and enhance IL-6 and IL-12 production, while inhibiting IL-4 production. In addition, LL-37-exposed DCs increased IFN-γ production from T cells and polarize them to Th1 cells [39]. Our in vitro data clearly show that mCRAMP is capable of acting directly on purified T cells that were polarized to Th2 cells and decrease their IL-4 production. Similarly, our in vivo data show that T cells produced more IL-4 in the absence of mCRAMP expression. IL-4 is the critical cytokine for the IgG1 class switch, and its elevated expression in the *Camp*$^{-/-}$ spleen after secondary i.p. immunization is associated with an increased number of antigen-specific IgG1-secreting cells. These results suggest that endogenous mCRAMP regulates antigen-specific IgG1 production in vivo by suppressing CD4^{+} T-cell IL-4 expression, although whether this is a direct effect or indirect through another cell type is yet to be determined.

mCRAMP is an AMP that is beginning to be appreciated as a potent and important immunomodulatory molecule. While our data begin to elucidate the role of mCRAMP in the adaptive immune response, more information is needed to fully understand its role in the different microenvironments within the host. It is clear that the cell type producing and/or responding to mCRAMP will partially determine the effect observed. Additional studies are needed to fully understand the role of mCRAMP and other AMPs in the adaptive immune response.

Materials and methods

Animals

C57BL/6 mice were purchased from the Jackson Laboratory. Camp-deficient 129/SVJ mice (Camp$^{-/-}$, KO) were backcrossed to B6 mice for ten generations and identified by PCR analysis as described previously [8]. All mice were maintained under pathogen-free conditions and under approved animal protocols from the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham.

Peptide preparation and storage

The 38 amino acid mCRAMP peptide (ISRLAGLLRKGGEKIGEKLKKIGQKIKNFFQKLVPQPE) was synthesized by Alpha Diagnostic Int. (San Antonio, TX, USA) and the lyophilized peptides were resuspended in

0.01% acetic acid to generate 100 μM working stocks, which were stored at −80°C until time of use.

B-cell purification and activation

B-cell purification and activation was performed as described previously [40]. Purified splenic B cells were obtained using a CD43 magnetic bead depletion strategy (Miltenyi Biotec). B cells (5×10^4) were cultured in 96-well flat-bottom plates in 200μL of complete medium (cRPMI). B cells were stimulated with 20 μg/mL LPS (Sigma-Aldrich), 1 ng/mL recombinant mouse IL-4 (eBioscience), 10 ng/mL recombinant mouse IFN-γ (eBioscience), and/or CD40L-expressing Sf9 cells (a gift from Dr. Virginia Sanders, The Ohio State University) at a B cell-to-Sf9 ratio of 10:1. Culture supernatants were collected and stored at −80°C until further analysis.

Flow cytometry and cell sorting

Flow cytometry and cell sorting was performed as described previously [41]. Intracellular staining was performed using the Cytofix/Cytoperm kit (BD Biosciences). FITC-labeled anti-γ1, anti-CD23, anti-Mac-1; PE-labeled anti-CD5, anti-Mac 1, anti-IL-4; APC-labeled anti-B220, PE-Cy7-anti-CD4, PB-anti-B220, PE-anti-IL-4, and PE-rIgG1 isotype antibodies were purchased from BD Pharmingen. Anti-CD21 (clone 7G6) antibody was purified and labeled with PE in our laboratory. Cy5-labeled goat anti-mouse IgM antibody was purchased from Jackson ImmunoResearch. FcR blocker Ab93 was generated in our laboratory [42]. Experiments were performed on a FACSCalibur (BD Biosciences), cell sorting using a FACSAria (BD Biosciences), and analysis using FlowJo software (Tree Star).

Immunization

Seven- to nine-wk-old female mice were immunized i.v. with 1×10^8 heat-killed *Streptococcus pneumonia* (R36A) or i.p. with 100 μg TNP-LPS (Biosearch Technologies). Sera were collected on day 0 prior to immunization and days 3, 7, 14 after immunization. Mice were also immunized i.p. or s.c. with 100 μg TNP-OVA (Biosearch Technologies) absorbed in 4 mg alum (Sigma-Aldrich) on days 0 and 21. Sera were collected on day 0 prior to immunization and days 7, 14, 21, 28, and 35 after immunization.

ELISA

Total immunoglobulin levels were determined by ELISA, as described previously [43]. Briefly, total IgM, IgG3, IgG2c, IgG1, and IgE were captured by plate-bound goat anti-mouse IgM, IgG, or IgE and detected with alkaline phosphatase-conjugated goat anti-mouse IgM, IgG3, IgG2c, IgG1, and IgE (Southern Biotechnology Associates), respectively. A standard curve was prepared using known quantities of BH8 (anti-PC IgM, generated in our laboratory) or anti-TNP Ab (IgG1, eBioscience). To measure specific anti-PC or anti-TNP Abs concentration, plates were coated with PC-BSA or TNP-BSA. *p*-Nitrophenyl phosphate (Sigma-Aldrich) was added, and color development was determined on a Titertek Multiskan Plus reader (Labsystems, ICN Biomedicals) at 405 nm.

ELISPOT

The 96-well high-binding plates were coated with goat anti-mouse IgG or TNP-OVA and single-cell splenic suspensions were prepared 7 days after primary or secondary TNP-OVA/Alum immunization. In addition, 1×10^6 total splenocytes were seeded in each well containing 100 μL cRPMI followed by a 1:3 serial dilution. Cells were incubated at 37°C for 24 h before being lysed with PBS containing 0.05% Tween 20. Alkaline phosphatase-conjugated goat anti-mouse IgG1 was added and spots visualized by 5-bromo-4-chloro-3-indolyl phosphate (Sigma-Aldrich) and counted under a dissection microscope. Spots were

then dissolved in 50 μL DMSO and absorbance of each well was measure with a spectrophotometer at 650 nm.

RT-PCR

RT-PCR was performed as described previously [41]. Briefly, total RNA was isolated using TRIzol (Invitrogen), cDNA was generated using the Omniscript RT-PCR kit (Qiagen), and PCR was performed using GoGreen Taq master mix (Promega) or SYBER green master mix (Invitrogen) at an annealing temperature of 60°C for 30–35 cycles. The following primer pairs were used: β-actin: 5′-TACAGCTTCACCACCACAGC-3′ and 5′-AAGGAAGGCTGGAAAAGAGC-3′; Camp: 5′-CGAGCTGTGGATGACTTCAA-3′ and 5′-CAGGCTCGTTACAGCTGATG-3′; CD19: 5′- GGAGGCAATGTTGTGCTGC-3′ and 5′- ACAATCACTAGCAAGATGCCC-3′; CD3e: 5′-ATGCGGTGGAACACTTTCTGG-3′ and 5′-GCACGTCAACTCTACACTGGT-3′; IL-4: 5′-ACCACAGAGAGTGAGCTCG-3′ and 5′-ATGGTGGCTCAGTACTACG-3′.

In vitro activation and differentiation of CD4$^+$ T cells

Purified splenic naï CD4$^+$ T cells (0.5×10^6 cells/mL) were obtained using negative selection followed by a CD62L$^+$ magnetic bead selection (Miltenyi Biotec) and stimulated with 2 μg/mL plate-bound anti-CD3 and 2 μg/mL anti-CD28 (eBioscience). Cells were cultured in 96-well flat-bottom plates in 200μL of cRPMI with 1 ng/mL recombinant mouse IL-4, 10 ng/mL recombinant mouse IFN-γ, 5 μg/mL anti-IL-12 antibody (eBioscience), in the presence or absence of 100–1000 ng/mL mCRAMP peptide. Cells were incubated at 37°C for 4–7 days before analysis. For determination of in vivo IL-4 production, total splenocytes were isolated on days 7 and 4 following the primary and secondary immunizations. In total, 10^6 splenocytes were cultured in cRPMI in the presence or absence of 2.5 μg/mL ConA for 24 h. Brefeldin A was added after 19 h of stimulation, 5 h prior to analysis, and cells were collected and analyzed using flow cytometry.

Western blot

Western blot analysis was performed as described previously [43]. Briefly, protein samples (5–20 μg) were isolated and resolved by electrophoresis on a 4–20% gradient Tris-HCl gel, transferred to Immobilon-P polyvinylidene difluoride membrances (Millipore), probed with either anti-CRAMP (Santa Cruz) at a 1:200 dilution or anti-actin at a 1:10 000 dilution, detected with HRP-labeled secondary Ab at a 1:1000–1:10 000 dilution, and developed with the SuperSignal West Pico kit (Thermo Scientific).

Statistical analysis

Data with three or more groups were analyzed by a one-way ANOVA followed by post hoc analysis, while data with two groups were analyzed by a two-tailed unpaired *t* test. Statistically significant results were determined by a *p* value of *<0.05, **<0.01, ***<0.001.

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Abbreviations

AMP	antimicrobial peptide
ASC	antibody-secreting cell
B6	C57BL/6
Camp	cathelicidin gene
CSR	class switch recombination
FO	follicular
LL-37	human cathelicidin
mCRAMP	mouse cathelin-related antimicrobial peptide
MZ	marginal zone
TD	T-cell dependent
TI	T-cell independent

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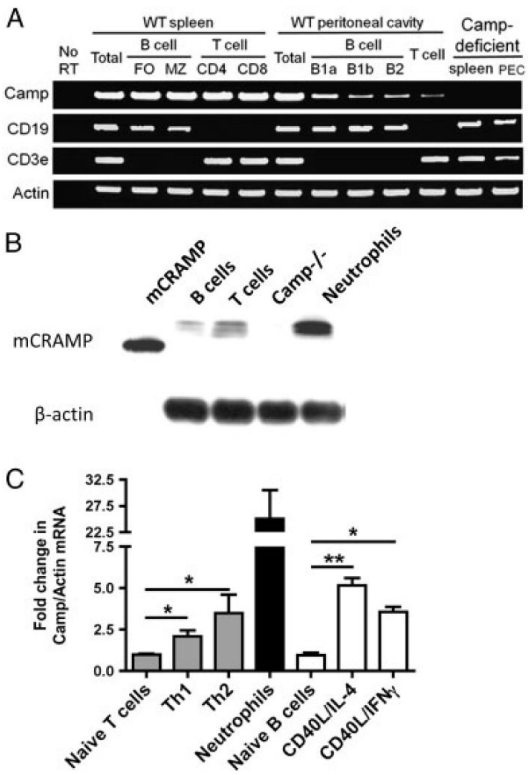

Figure 1.

B and T cells express Camp and mCRAMP. MZ B cells, FO B cells, $CD4^+$ and $CD8^+$ T cells were purified from C57BL/6 spleens while B1a, B1b, B2, and total T cells were purified from peritoneal cavity washes. Total spleens and peritoneal cavity washes were also collected from *Camp*$^{-/-}$ (KO) mice. (A) Total RNA was isolated and analyzed using RT-PCR analysis for the mRNA levels of *Camp*, CD19, CD3e, and actin. (B) Total protein was isolated from synthetic mature mCRAMP, sort-purified $CD43^-$ B cells, $CD4^+CD62L^+$ naïve T cells, *Camp*$^{-/-}$ spleen, and neutrophils and analyzed by Western blot for the expression of mCRAMP and β-actin. (C) B and T cells were activated in vitro with the indicated stimuli and the level of *Camp* and actin mRNA was measured by real-time PCR and presented as fold change in Camp expression normalized to actin. Data represent the mean1SEM from three independent experiments. One representative gel and blot are shown. Data were analyzed by a one-way ANOVA followed by post hoc analysis. *p<0.05, *p<0.01.

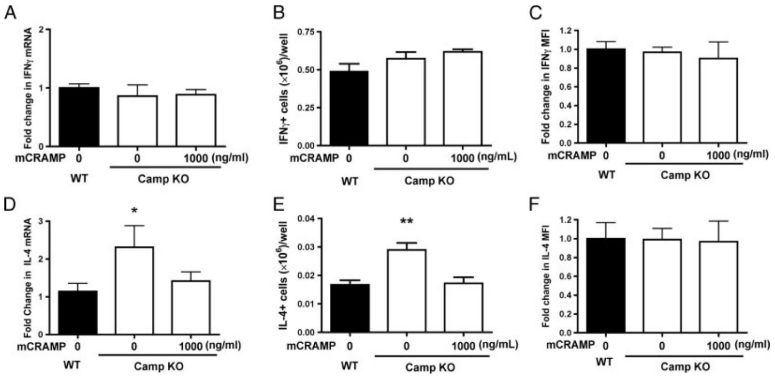

Figure 2.

Camp-deficient T cells produce more IL-4 in vitro. Purified naïve $CD4^+CD62L^+$ T cells were cultured under Th1 (anti-CD3, -CD28, rIFN-γ)or Th2 (anti-CD3, -CD28, -IL-12, and rIL-4) driving conditions, in the presence or absence of mCRAMP. Total RNA was collected from WT (black bars) and *Camp* KO (white bars) cultures on day 4 and analyzed by RT-PCR for (A) IFN-γ and (D) IL-4. T cells were also collected and stained for intracellular (B and C) IFN-γ and (E and F) IL-4. (B and E) The total number and (C and F) fold change in MFI of IFN-γ and IL-4 are shown. Data represent the mean+ SEM from three independent experiments. Data were analyzed by a one-way ANOVA followed by post hoc analysis. *p<0.05, *p<0.01.

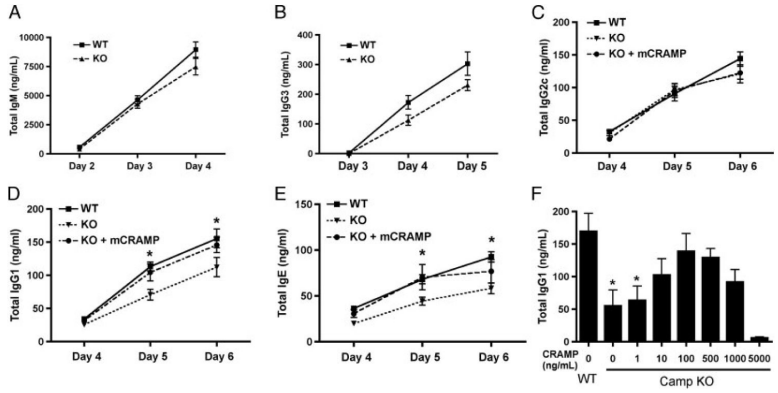

Figure 3.

Camp-deficient B cells produce less IgG1 and IgE antibody in vitro. Resting $CD43^{-}$ WT and $Camp^{-/-}$ (KO) B cells were purified and cultured in the presence of LPS for the indicated number of days and cell culture supernatants were collected and analyzed by ELISA for the level of (A) IgM and (B) IgG3 antibody. B cells were also cultured with CD40L/IFN-γ or CD40L/IL-4, in the presence or absence of 100 ng/mL mCRAMP, and cell culture supernatant was collected and analyzed on days 4–6 for (C) IgG2c, (D) IgG1, and (E) IgE antibody, respectively. (F) CD40L/IL-4-activated *Camp* KO B cells were cultured in the presence of increasing concentrations of mCRAMP and total IgG1 was measured in culture supernatant on day 6. Data represent the mean+SEM from three independent experiments. Data with three or more groups were analyzed by a one-way ANOVA followed by post hoc analysis, while data with two groups were analyzed by a two-tailed unpaired *t* test. $*p<0.05$.

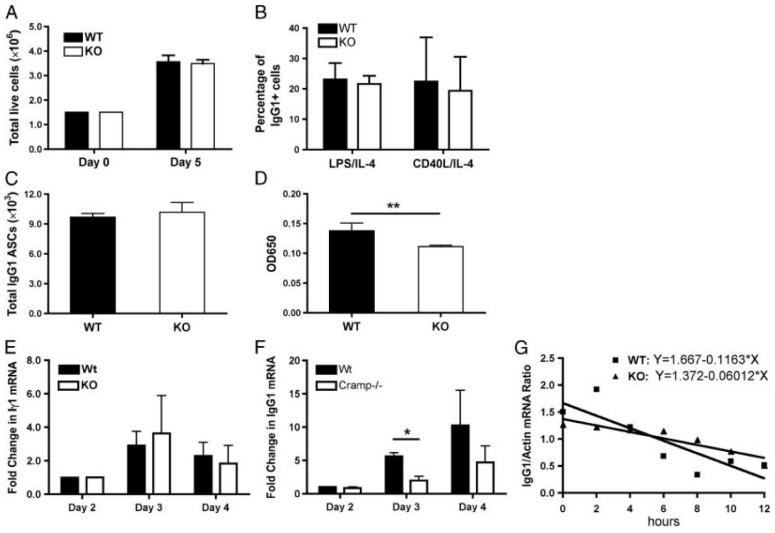

Figure 4.

Camp-deficient B cells produce less IgG1 per cell, with no effect on proliferation, survival, or isotype switching. (A) Resting CD43^{-} WT and *Camp*$^{-/-}$ (KO) B cells were sort-purified and total live CD40L/IL-4-activated B cells were enumerated on days 0 and 5. (B) The percentage of surface IgG1^{+} B cells in LPS/IL-4- and CD40L/IL-4-activated cultures was determined using flow cytometry. (C) The number of IgG1-secreting B cells was determined using ELISpot analysis on CD40L/IL-4-activated cells. (D) The IgG1 spots developed from the ELISpot procedure were dissolved using DMSO and the optical density was determined at 650 nm. (E and F) RT-PCR analysis was performed on days 2–4 after activation for the level of (E) Iγ1 and (F) IgG1 mRNA. (G) Actinomycin D was added to day 5 WT and *Camp*$^{-/-}$ B-cell cultures and total RNA was collected every 2 h for 12 h. RT-PCR was performed for the level of IgG1 mRNA at each time point and regression analysis was applied to estimate the stability of the mRNA transcript. Data represent the mean1SEM from three independent experiments. Data were analyzed by a two-tailed unpaired *t* test. *p<0.05, **p<0.01.

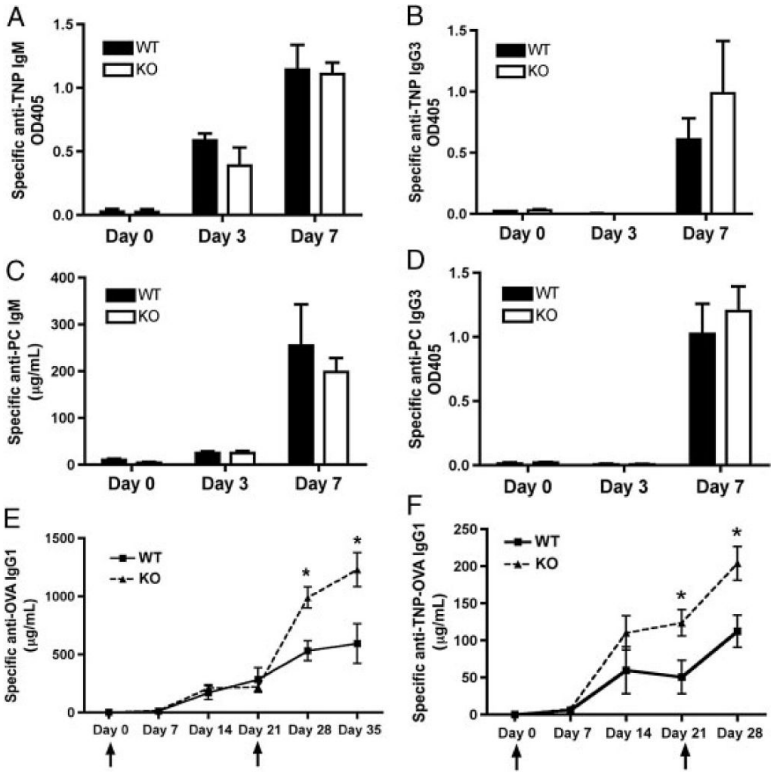

Figure 5.

Camp-deficient mice produce more IgG1 in response to a T-cell-dependent antigen. (A–D) WT and *Camp*$^{-/-}$ (KO) mice were immunized with TNP-LPS or S. pneumoniae on day 0 and the level of serum antibody was measured using ELISA. The level of TNP-specific (A) IgM and (B) IgG3 as well as PC-specific (C) IgM and (D) IgG3 were measured on days 0, 3, and 7. (E and F) WT and *Camp*$^{-/-}$ (KO) mice were also immunized with TNPOVA/alum on days 0 and 21. The amount of TNP-OVA-specific IgG1 was measured following (E) i.p. or (F) s.c. immunizations. Data represent the mean1SEM from three independent experiments, five mice per group. ↑ injection. Data were analyzed by a two-tailed unpaired *t* test. *p<0.05.

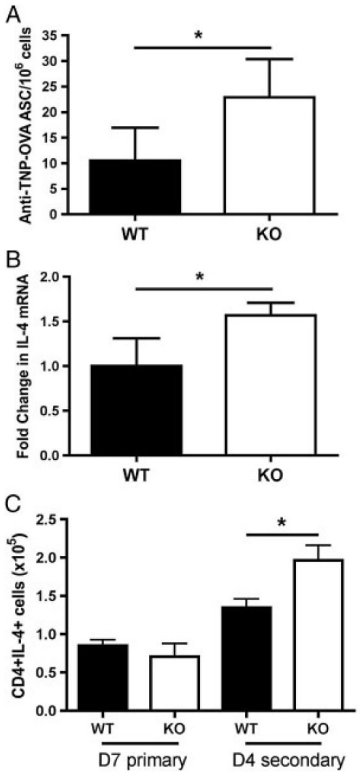

Figure 6.

Camp-deficient mice produce more IL-4 and induce more antigen-specific IgG1[1] ASCs. Total splenic cells were isolated on days 7 and 4 following primary and secondary immunization with TNP-OVA/alum, respectively. (A) ELISpot analysis was performed on the splenic cells for anti-TNP-OVA IgG1 ASCs. (B) Total RNA was isolated and RTPCR analysis was performed for the level of IL-4 and actin mRNA, shown as fold change in IL-4 expression normalized to actin. (C) Flow cytometric analysis was performed and total CD4[1]IL-4[1] T cells were determined by intracellular cytokine staining. Data represent the mean 1SEM from three independent experiments, five mice per group. Data were analyzed by a two-tailed unpaired *t* test. *$p<0.05$.