Nuclear carbonic anhydrase 6B associates with PRMT5 to epigenetically promote IL-12 expression in innate response

Jia Xu[a,1], Xiaoqing Xu[a,1], Bingjing Wang[a], Yuanwu Ma[b], Lianfeng Zhang[b], Henan Xu[a], Ye Hu[a], Jiacheng Wu[a], and Xuetao Cao[a,c,2]

[a]Department of Immunology & Center for Immunotherapy, Institute of Basic Medical Sciences, Peking Union Medical College, Chinese Academy of Medical Sciences, Beijing 100005, China; [b]Key Laboratory of Human Disease Comparative Medicine, Ministry of Health, Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences, Beijing 100021, China; and [c]National Key Laboratory of Medical Immunology, Institute of Immunology, Second Military Medical University, Shanghai 200433, China

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Interleukin-12 (IL-12) is critical for induction of protective immunity against intracellular bacterial infection. However, the mechanisms for efficient induction of IL-12 in innate response remain poorly understood. Here we report that the B type of carbonic anhydrase 6 (*Car6-b*, which encoded CA-VI B) is essential for host defense against *Listeria monocytogenes* (LM) infection by epigenetically promoting IL-12 expression independent of its carbonic anhydrase activity. Deficiency of *Car6-b* attenuated IL-12 production upon LM infection both in vitro and in vivo. *Car6*$^{-/-}$ mice were more susceptible to LM infection with less production of IL-12. Mechanistically, the nuclear localized CA-VI B selectively promotes IL-12 expression by interaction with protein arginine *N*-methyltransferase 5 (PRMT5), which reduces symmetric dimethylation of histone H3 arginine 8 modification (H3R8me2s) at *Il12* promoters to facilitate chromatin accessibility, selectively enhancing c-Rel binding to the *Il12b* promoter. Our findings add insights to the epigenetic regulation of IL-12 induction in innate immunity.

Car6-b | IL-12 | PRMT5 | innate immunity | epigenetic modification

Innate immune response is critical for host defense against invading pathogens. Microbial and dangerous signals induce pattern-recognition receptor (PRR)-dependent activation of inflammatory signaling in innate immune cells such as macrophages and dendritic cells, leading to production of proinflammatory cytokines that display diversified immunological and inflammatory effects (1). Of these, IL-12 plays essential roles in inducing transcription factor (TF) STAT4-dependent production of IFN-γ in natural killer (NK) cells and T cells, contributing to development of Th1 cell-mediated protective immunity against intracellular pathogens such as *Mycobacterium tuberculosis* (TB) and *Listeria monocytogenes* (LM) (2). IL-12 expression is controlled by transcription activation of two separate genes, *Il12a* and *Il12b*, to form IL-12p70 with bioactivity. IL-12 deficiency results in defects in immune defense, causing increased susceptibility to infection by TB, *Salmonella*, or *Candida* both in humans and in mice (3).

IL-12 is also related to development of autoimmune and inflammatory disease, as demonstrated by resistance to experimental autoimmune disorders in mice lacking *Il12b* (4). Antagonists against IL-12p40 have been verified to be an effective treatment for a number of autoimmune diseases in clinical or preclinical trials (5). So the expression of IL-12 must be well balanced to achieve timely and effective protection against pathogenic infection. Further identification of new regulators of IL-12 production will contribute to a better understanding of the molecular basis for innate immunity against intracellular pathogens and also outline potential therapeutic strategies for control of infectious diseases.

Epigenetic mechanisms for gene expression during innate immunity and inflammation are attracting more and more attention now. Epigenetic regulations include DNA modifications, posttranslational modifications (PTMs) of histones, ATP-dependent chromatin remodeling, and noncoding RNA (ncRNA), which act in a coordinated manner to affect chromatin status and gene transcription and influence various immunological processes (6). Expression of IL-12 is mainly dependent on TLR-triggered nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways (2). C-Rel, which belongs to the NF-κB family, is a key TF in mediating TLR-stimulated expression of IL-12 (7). Histone methylation at the *Il12b* promoter is found to be important for IL-12 expression (8). LPS stimulation also induces rapid remodeling of a positioned nucleosome at the *Il12b* promoter, which might be important for accessibility of specific TFs to the promoter region (7). However, the detailed function of chromatin modifiers and transcription regulators in IL-12 expression and IL-12–mediated innate immunity in response to bacterial infections remains poorly understood.

Intracellular bacterial infection is one of the major health concerns today. For example, TB results in severe lung injury (9). Also, the widely existed Gram-positive intracellular pathogen LM causes severe listeriosis and even death. LM has been used as a model organism for the study of intracellular pathogens (10). LM mainly infects and induces IL-12 production in macrophages in vivo (11). E-selectin is a key adhesion molecule encoded by *Sele*-mediating recruitment of Th1 cells into inflamed tissues (12). To study LM-triggered innate response, we

Significance

The immune system is important for host defense against invading pathogens by producing proinflammatory cytokines and IFNs. IL-12 is a vital proinflammatory cytokine that combines innate immunity with adaptive immunity. In our study, we find that CA-VI B preferentially expressed in macrophages can interact with PRMT5, consequently suppressing H3R8me2s modification in *Il12* promoters to promote IL-12 production to trigger an antibacterial immune response. Our study adds insight about the function of CA family members in innate immune response by selectively inducing cytokine IL-12 production through regulating histone arginine modification, which is independent of its carbonic anhydrase activity.

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The authors declare no conflict of interest.

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Data deposition: The cDNA microarray data are available at Gene Expression Omnibus (GEO) database, https://www.ncbi.nlm.nih.gov/geo/ (accession no. GSE90808). All original RNA-sequencing and ChIP-sequencing datasets have been submitted to the NCBI Sequence Read Archive (SRA) (accession nos. SRP108814 and SRP108823, respectively).

[1]J.X and X.X. contributed equally to this work.

[2]To whom correspondence should be addressed. Email: caoxt@immunol.org.

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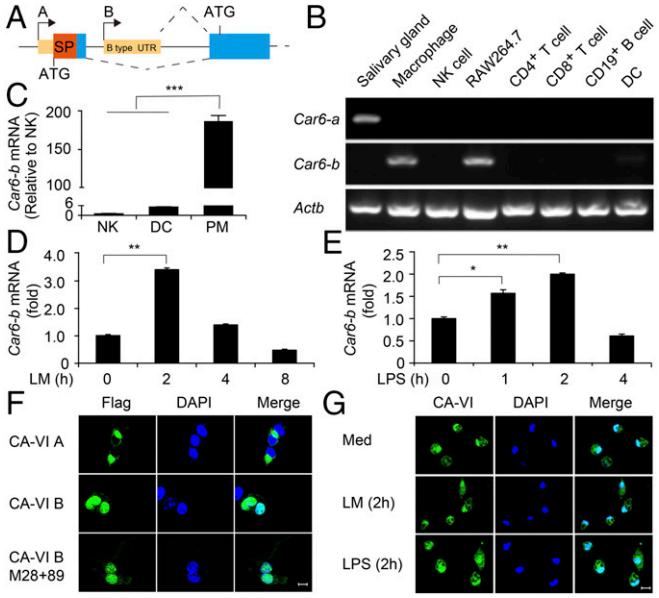

Fig. 1. *Car6-b* is preferentially expressed in macrophages. (*A*) The structure of the A and B type of *Car6* in genome. SP, signal peptide; UTR, untranslated region. (*B*) RT-PCR analysis of *Car6-a* and *Car6-b* in immune cells; *Actb* serves as a loading control. *Car6-a* expressed in the salivary gland serves as a positive control. (*C*) qPCR analysis of *Car6-b* in mouse immune cells; results were normalized to those of *Actb*. (*D* and *E*) *Car6-b* expression in PMs after stimulation by LM (*D*) or LPS (*E*) for indicated times. Data were shown as mean ± SD ($n = 3$). $^*P < 0.05$, $^{**}P < 0.01$ $^{***}P < 0.001$. (*F* and *G*) Immunofluorescence assay of the location of CA-VI in 293T cells (*F*) and PMs (*G*) stimulated with LM or LPS for 2 h or left unstimulated (Med). (Scale bar for *F* and *G*, 10 μm.)

compared mRNA expression in macrophages from $Sele^{+/+}$ and $Sele^{-/-}$ mice infected with LM. Interestingly, *Car6*, which encoded CA-VI, was significantly decreased in $Sele^{-/-}$ macrophages. *Car6* has two transcripts: One is the secreted A type of CA-VI A (encoded by *Car6-a*), and the other is intracellular CA-VI B. CA-VI belongs to the carbonic anhydrase (CA) family (13). CA includes 16 members catalyzing the reversible hydration of CO_2. CAs can be grouped into four kinds according to their different subcellular locations: CA-I, -II, -III, -VII, and -XIII are localized in cytoplasm; CA-IV, -IX, -XII, and -XIV are membrane-bound; CA-V is localized in mitochondria; and CA-VI is the only secreted CA (14). *Car6* might be involved in cellular oxidative stress in the submandibular gland and tissue homeostasis in the respiratory system and brain (15). However, the roles of CAs in innate immunity remain unknown. Here, we demonstrated that *Car6-b* selectively promotes IL-12 expression by interaction with PRMT5 and consequently facilitates innate response against intracellular bacterial infection.

Results

***Car6-b* Is Preferentially Expressed in Macrophages and Inducible upon Innate Stimuli.** By cDNA microarray analysis of LM-infected peritoneal macrophages (PMs) from $Sele^{-/-}$ and $Sele^{+/+}$ mice, we found mRNA expression of *Car6* was significantly decreased in $Sele^{-/-}$ PMs (Fig. S1 *A* and *B*). We further analyzed the molecular mechanism of the impaired induction of *Car6* mRNA in the absence of E-selectin. Given that E-selectin binds its ligands to activate the MAPK signaling pathway, to investigate the molecular mechanism behind how E-selectin engagement could up-regulate *Car6* expression, we used ERK-specific inhibitor FR180204 to pretreat PMs before infection of LM. We found that *Car6* expression was reduced in the presence of the ERK inhibitor (Fig. S1*C*), indicating that E-selectin engagement up-regulates *Car6* expression via the ERK pathway.

As shown in Fig. 1*A*, the two types of *Car6* have different transcription start sites (TSSs) and use disparate start codon ATG. Therefore, CA-VI A has an additional 57-aa peptide on the N terminus than CA-VI B does (13). We next measured the mRNA expression profile of *Car6-a* and *Car6-b* in various immune cells, including NK cells, DCs, $CD4^+$ T cells, $CD8^+$ T cells, $CD19^+$ B cells, PMs, and cell line RAW264.7. Although *Car6-a* was undetectable in all of the immune cells, except for the salivary gland, which served as a positive control, *Car6-b* was predominantly expressed in macrophages including PMs and RAW264.7 cells (Fig. 1*B*). We further confirmed that *Car6-b* expression in macrophages was higher than that in NKs and DCs by quantitative real-time PCR (qPCR) (Fig. 1*C*). Furthermore, *Car6-b* was significantly induced by LM and LPS stimulation (Fig. 1 *D* and *E*). Confocal analysis indicated that CA-VI B was located in the nucleus of 293T cells (Fig. 1*F*) and PMs (Fig. 1*G*) with or without innate stimuli, which was consistent with the observation that CA-VI B is located in the nucleus of mouse embryo fibroblast cells in response to stress (13). In addition, the enzyme catalytic mutant of *Car6-b* (M28+89) was also localized in the nucleus (Fig. 1*F*). Taken together, *Car6-b* is preferentially expressed in macrophages and inducible upon innate stimuli.

***Car6-b* Selectively Promotes IL-12 Expression in Macrophages upon Innate Stimuli.** We next investigated the function of *Car6-b* in innate immune response. Given that only *Car6-b* but not *Car6-a* is expressed in macrophages, we silenced *Car6-b* in PMs (Fig. S2*A*) and found that LM- or LPS-induced *Il12b*, *Il12a* mRNA expression, and IL-12p70 production were dramatically reduced in *Car6-b*–silenced PMs (Fig. 2 *A*–*C*). IL-23 belongs to the IL-12 family and is composed of the IL-12p40 and IL-23p19 subunits. LM- and LPS-induced expression of *Il23a* (encoding IL-23p19) was also reduced by *Car6-b* knockdown (Fig. 2*D*). However, silencing of *Car6-b* did not affect the production of IL-6 and TNF-α by PMs stimulated with LM or LPS (Fig. S2 *B*–*E*).

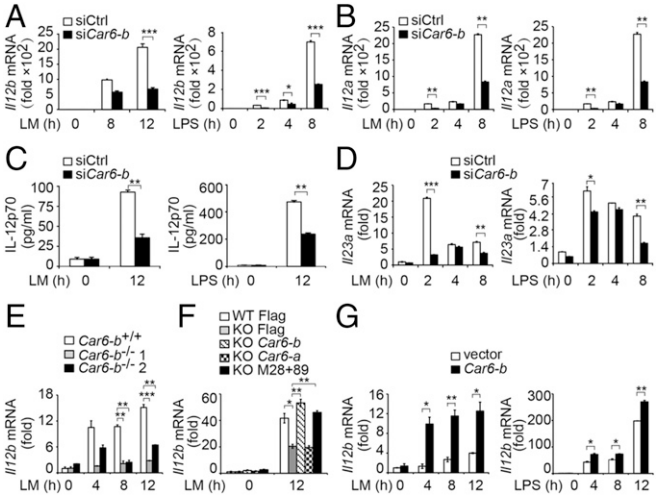

Fig. 2. *Car6-b* promotes IL-12 expression in macrophages upon innate stimuli. (*A* and *B*) qPCR assay of *Il12b* (*A*) and *Il12a* (*B*) in PMs transfected with *Car6-b*–specific siRNA (*siCar6-b*) and scramble siRNA (siCtrl) and stimulated with LM or LPS for the indicated time. (*C*) ELISA in the supernatants of cells transfected and stimulated as in *A*. (*D*) qPCR assay of *Il23a* stimulated as in *A*. (*E*) *Il12b* expression in the RAW264.7 $Car6\text{-}b^{-/-}$ cells from two colonies ($Car6\text{-}b^{-/-}$ 1 and $Car6\text{-}b^{-/-}$ 2) and control cells ($Car6\text{-}b^{+/+}$) stimulated by LM. (*F*) *Il12b* expression in *Car6* knockout RAW264.7 cells (KO) and control cells (WT) after transfection with Flag-vector (Flag), Flag–*Car6-b* (*Car6-b*), Flag–*Car6-a* (*Car6-a*), or Flag–*Car6-b*–M28+89 (M28+89) stimulated by LM. (*G*) *Il12b* expression in *Car6-b* or control vector overexpressing RAW264.7 cells after stimulation by LM or LPS for indicated times. Data were shown as mean ± SD ($n = 3$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

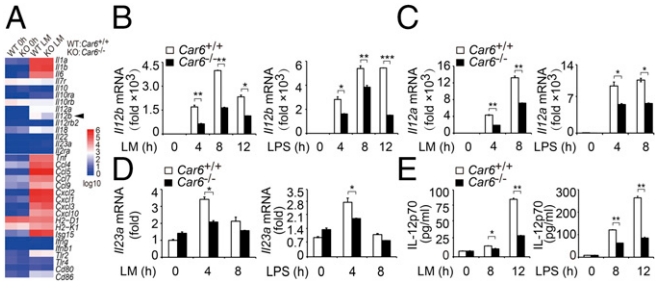

Fig. 3. *Car6* selectively promotes induction of IL-12 in macrophages upon innate stimuli. (*A*) Heatmap of gene expression in $Car6^{+/+}$ and $Car6^{-/-}$ PMs with or without LM stimulation for 8 h. Log10-expression values were calculated from RNA-seq. (*B–D*) qPCR assay of *Il12b* (*B*), *Il12a* (*C*), and *Il23a* (*D*) expression in the $Car6^{+/+}$ and $Car6^{-/-}$ PMs after stimulation by LM or LPS for the indicated time. (*E*) ELISA of IL-12p70 in the supernatants of cells stimulated as in *B*. Data were shown as mean ± SD ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

We next generated $Car6\text{-}b^{-/-}$ RAW264.7 cells by CRISPR/Cas9 technology (Fig. S3*A*). As expected, LPS or LM-induced *Il12b* expression was significantly decreased in $Car6\text{-}b^{-/-}$ RAW264.7 cells compared with wild-type RAW264.7 cells (Fig. 2*E*), whereas overexpression of *Car6-b* into $Car6\text{-}b^{-/-}$ RAW264.7 cells significantly rescued *Il12b* mRNA expression (Fig. 2*F*) and IL-12p70 production (Fig. S2*F*). However, IL-6 and TNF-α expressions were not affected by overexpression of *Car6-b* (Fig. S2 *G* and *H*). We also found that transfection of *Car6-a* did not rescue the expression of *Il12b* mRNA (Fig. 2*F*). In addition, overexpression of *Car6-b* remarkably promoted *Il12b* mRNA expression in RAW264.7 cells upon innate stimuli (Fig. 2*G*).

To further investigate the role of *Car6-b*, we tried to generate $Car6\text{-}b^{-/-}$ mice. Given the structure shown in Fig. 1*A*, we could not knock out *Car6-b* specifically without influencing *Car6-a*. On the other hand, *Car6-a* is not expressed in immune cells, and *Car6-b* is the only type expressed. Therefore, we generated $Car6^{-/-}$ mice in the C57BL/6J background using the CRISPR-Cas9 method (Fig. S3*B*) to represent knocking out *Car6-b* in immune cells. $Car6^{-/-}$ mice and *Car6*-sufficient littermate ($Car6^{+/+}$) mice were fertile and normal in size. They were viable and normal, consistent with previous reports (16). FACS analysis showed no difference in immune cells including $CD19^{+}$ B cells, $CD4^{+}$ T cells, $CD8^{+}$ T cells, NK cells, and macrophages between $Car6^{+/+}$ and $Car6^{-/-}$ mice (Fig. S4); thus, *Car6* deficiency does not affect the development of the immune system.

We collected PMs from $Car6^{+/+}$ and $Car6^{-/-}$ mice to investigate the function of *Car6-b* in innate immune responses because there is no expression of *Car6-a* in macrophages. We performed RNA-sequencing (RNA-seq) of $Car6^{+/+}$ and $Car6^{-/-}$ PMs with or without LM stimulation. The cluster heatmap of different samples was shown in Fig. S5A. There were 1,810 genes differentially expressed between $Car6^{+/+}$ and $Car6^{-/-}$ PMs without LM stimulation. There were 1,011 genes differentially expressed between $Car6^{+/+}$ and $Car6^{-/-}$ PMs stimulated with LM for 8 h (Fig. S5B). $Car6^{-/-}$ PMs had 887 genes up-regulated and 124 genes down-regulated compared with $Car6^{+/+}$ PMs after stimulation with LM. We then analyzed the immune-related genes and found that *Il12b* was down-regulated in $Car6^{-/-}$ PMs after stimulation by LM (Fig. 3A). However, there were no significantly different expressions of other cytokines, including *Il6*, *Tnf*, *Il1*, and *Il10*, between $Car6^{+/+}$ and $Car6^{-/-}$ PMs, which was further confirmed by qPCR. Notably, LM- or LPS- induced *Il12b*, *Il12a*, and *Il23a* mRNA expression and IL-12p70 production were significantly decreased in $Car6^{-/-}$ PMs compared with $Car6^{+/+}$ PMs (Fig. 3 *B–E*). In addition, *Car6* deficiency did not affect innate signal-induced production of IL-6 and TNF-α (Fig. S5 *C–F*), and *Car6-b* deficiency did not affect the expression of *Nos2*, *Ifnb1*, *Il1a*, and *Cd80* in $Car6^{-/-}$ PMs compared with that in $Car6^{+/+}$ PMs after innate stimuli (Fig. S5 G–J). The innate signal-induced expression of *Il12b* mRNA was also significantly reduced in $Car6^{-/-}$ bone marrow-derived macrophages (BMMs) (Fig. S6*A*) but not in bone marrow-derived DCs (BMDCs) (Fig. S6*B*). Taken together, we demonstrate that *Car6-b* selectively promotes IL-12 expression in macrophages upon innate stimuli.

***Car6-b* Protects Mice Against Infection with LM.** To further elucidate the significance of *Car6* in innate responses in vivo, we challenged $Car6^{+/+}$ and $Car6^{-/-}$ mice with the lethal load of LM by i.p. injection. The survival rate of $Car6^{-/-}$ mice was much lower than that of $Car6^{+/+}$ mice after LM infection (Fig. 4*A*), consistent with the increased bacterial load in various organs and more obvious infiltration of mononuclear inflammatory cells in the lungs of $Car6^{-/-}$ mice (Fig. 4 *B* and *C*). Moreover, a much lower level of serum IL-12p70 and IFN-γ but similar levels of IL-6 and TNF-α production were detected in $Car6^{-/-}$ mice (Fig. 4*D*). Therefore, *Car6-b* significantly protected mice from LM infection by selectively promoting IL-12 production in vivo.

***Car6-b* Promotes IL-12 Production Independent of Its CA Activity and Not via NF-κB/MAPK Pathways.** We wondered whether the underlying mechanism for *Car6-b* selectively promotes IL-12 expression in innate response. CA-VI A is secreted into saliva to maintain pH homeostasis by its CA enzyme activity (16), and CA-VI B also contains this enzyme domain. We wondered whether the pH of cytoplasm and the nucleus of PM might be involved in *Car6-b*–mediated promotion of IL-12 expression. However, there was no obvious difference in pH in either the cytoplasm or the nucleus of $Car6^{+/+}$ and $Car6^{-/-}$ PMs with or without infection (Fig. S7). We also constructed catalytically inactive mutant *Car6-b*–M28+89 (H28A, Y89A) and found that *Car6-b*–M28+89 displayed a similar function as wild-type *Car6-b* in rescuing the *Il12b* mRNA expression (Fig. 2*F*) and IL-12p70 production (Fig. S2*F*) in $Car6\text{-}b^{-/-}$ RAW264.7 cells. However, the catalytically active *Car6-a* did not rescue the IL-12 expression. These results showed that *Car6-b* promotes IL-12 expression independent of its CA catalytic activity.

NF-κB and MAPK signaling pathways are known to induce the expression of IL-12 (2). However, no obvious difference was found in the activation of the inhibitor of NF-κB (IκB), kinase (IKK), TF (c-Rel), MAPKs (ERK, JNK, and p38) (Fig. S8 *A–D*), or the nucleus translocation of c-Rel, p65, and C/EBPβ (Fig. S8 *E* and *F*) between $Car6^{-/-}$ and $Car6^{+/+}$ PMs upon LM or LPS

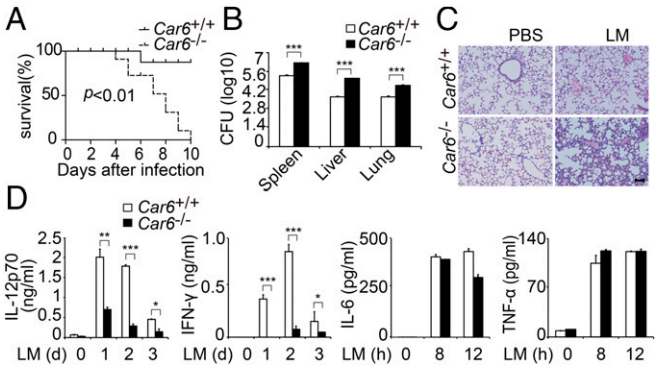

Fig. 4. *Car6* protects mice against infection with LM. (*A*) Survival rate of $Car6^{+/+}$ and $Car6^{-/-}$ mice post-i.p. injection of LM ($n = 10$). (*B*) The cfus in the spleen/liver/lung were determined after 3 d of infection. (*C*) Pathology of $Car6^{+/+}$ and $Car6^{-/-}$ mice after LM infection. Hematoxylin and eosin staining of lung sections from mice after 2 d of infection. (Scale bar, 100 μm.) (*D*) ELISA of indicated cytokine production in serum from $Car6^{+/+}$ and $Car6^{-/-}$ mice stimulated as in *A*. Data were shown as mean ± SD ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

stimuli. Therefore, *Car6-b* does not affect NF-κB and MAPK signaling pathways.

***Car6-b* Promotes c-Rel Recruitment to the *Il12* Promoter upon Innate Stimuli.** Based on the critical function of c-Rel in transcription of genes encoding IL-12 and other cytokines, we performed luciferase *Il12b* reporter assay to explore the detailed mechanism of *Car6-b*–promoted IL-12 production. Ectopic expression of *Car6-b* had no effect on transcriptional activity of the *Il12b* reporter that was induced by c-Rel, and *Car6-b* itself had no function on *Il12b* reporter (Fig. 5*A*), nor did CA-VI B interact with c-Rel in HEK293T cells as detected by co-immunoprecipitation (co-IP) assay (Fig. 5*B*). Thus, *Car6-b* might not directly affect c-Rel transcriptional activity.

To clarify whether *Car6-b* affects chromatin conformation, we detected the DNA accessibility of the *Il12b* promoter by DNase I sensitivity analysis (17). Interestingly, DNA accessibility of the *Il12b* promoter was significantly decreased in *Car6*$^{-/-}$ PMs upon innate stimuli (Fig. 5*C*). We also found that the chromatin accessibility of promoter sites of *Il6* and *Tnf* was not influenced by *Car6* deficiency in PMs upon innate stimuli (Fig. 5 *D* and *E*). C-Rel particularly binds to the region containing an NF-κB binding site of the *Il12b* promoter (8). Therefore, we performed chromatin immunoprecipitation (ChIP) assay to examine c-Rel binding to the region between position –150 and –50 of the *Il12b* promoter, which contains an NF-κB binding site. We found that the recruitment of c-Rel to the promoter of *Il12b* was substantially decreased in *Car6*$^{-/-}$ PMs stimulated with LM or LPS (Fig. 5*F*). However, recruitment of c-Rel to the promoters of *Il6* and *Tnf* was comparable between *Car6*$^{+/+}$ and *Car6*$^{-/-}$ PMs upon innate stimuli (Fig. 5 *G* and *H*). Collectively, we demonstrate that *Car6-b* selectively promotes chromatin accessibility to facilitate c-Rel binding to the *Il12b* promoter.

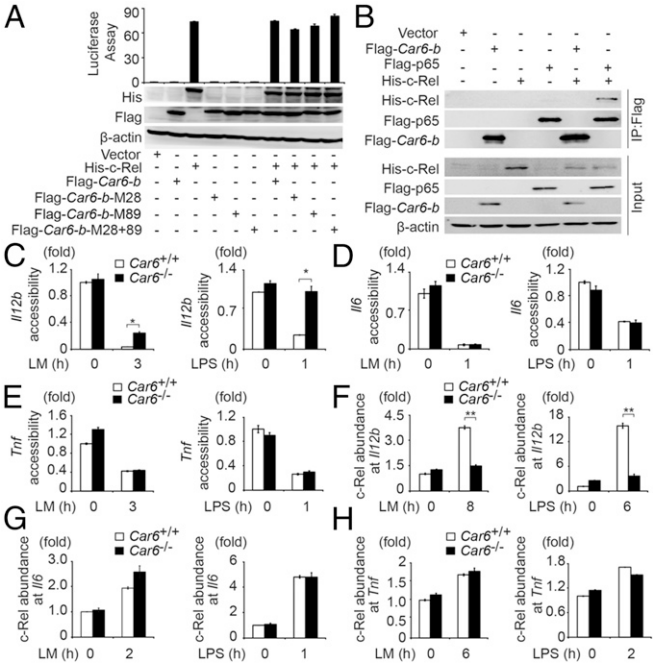

Fig. 5. *Car6* promotes c-Rel recruitment to the *Il12* promoter upon innate stimuli. (*A*) Dual luciferase reporter gene expression assay of the *Il12b* promoter and immunoblot assay in HEK293T cells transfected with plasmids as indicated. (*B*) HEK293T cells were transfected with indicated plasmids. Protein interactions were detected by co-IP experiments. (*C*–*E*) DNA accessibility assay at promoter sites of *Il12b* (*C*), *Il6* (*D*), and *Tnf* (*E*) in PMs after stimulation by LM or LPS for the indicated time. (*F*–*H*) ChIP assay of c-Rel recruitment to the *Il12b* (*F*), *Il6* (*G*), or *Tnf* (*H*) promoter sites in *Car6*$^{+/+}$ and *Car6*$^{-/-}$ PMs after stimulation for the indicated time with LM or LPS. Data were shown as mean ± SD ($n = 3$). *$P < 0.05$, **$P < 0.01$.

CA-VI B Associates with PRMT5 to Promote c-Rel Recruitment to *Il12* Promoters by Histone Modification. Because CA-VI B is located in the nucleus of macrophages and promotes c-Rel recruitment to the *Il12b* promoter, we speculated that *Car6-b* might be involved in histone modification to regulate chromatin accessibility. We performed mass spectrometry screening of the candidate proteins interacting with CA-VI B in HEK293T cells (Fig. S9 *A*–*C*). One potential candidate, PRMT5, attracted our attention. PRMT5, a type II arginine methyltransferase that symmetrically dimethylates arginine residues on histone H2AR3, H3R8, and H4R3, has been reported to regulate gene transcription through histone modification (18). Importantly, methylosome protein 50 (MEP50/WDR77) is required for PRMT5 function (19).

Co-IP experiments confirmed that Flag-tagged *Car6-b* interacted with both Myc-tagged PRMT5 and V5-tagged MEP50 (Fig. S9*D*). Moreover, we performed co-IP experiments to confirm the interaction between endogenous *Car6-b* and PRMT5 using CA-VI antibody in the lysates of *Car6*$^{+/+}$ and *Car6*$^{-/-}$ PMs with or without LM stimulation (Fig. 6*A*). By immunofluorescence, we identified that CA-VI B and PRMT5 were colocalized in the nucleus of PMs after stimulation with LM or LPS (Fig. S9*E*). By proximal ligation assay (PLA), we further confirmed the interaction of CA-VI B and PRMT5 in the nucleus of *Car6*$^{+/+}$ PMs but not in *Car6*$^{-/-}$ PMs or PRMT5-slienced *Car6*$^{+/+}$ PMs (Fig. 6*B*). To further investigate the interaction site of CA-VI B and PRMT5, we constructed C-terminal deletion *Car6-b* (ΔC)-, N-terminal deletion *Car6-b* (ΔN)-, and N-terminal deletion PRMT5 (ΔN)-expressing plasmids. Our co-IP experiment showed that the C-terminal domain of *Car6-b* was indispensable for its interaction with the N-terminal domain of PRMT5 (Fig. S9 *F* and *G*). Furthermore, *Car2* and *Car13* were expressed in macrophages and located in the cytoplasm (14). So we constructed *Car2*- and *Car13*-expressing plasmids to detect the interaction with PRMT5. We found only *Car6-b* interacted with PRMT5 but not *Car2* and *Car13* (Fig. S9*H*). These data showed the specificity interaction between *Car6-b* and PRMT5, and overexpression of *Car6-b* ΔC did not promote IL-12 expression in RAW264.7 *Car6-b* KO cells upon LM stimulation (Fig. S9*I*). Furthermore, overexpression of *Car6-b* ΔC did not promote IL-12 expression in RAW264.7 and did not show dominant negative effects (Fig. S9*J*). These results indicate that the C-terminal domain is essential for *Car6-b* to interact with the N-terminal domain of PRMT5 and promotes IL-12 expression. Thus, *Car6-b* binds the same domain of PRMT5 as MEP50 (19). To further clarify whether *Car6-b* affected the activity of PRMT5, we used PRMT5 antibody for co-IP in RAW264.7 cells and found that PRMT5 interaction with *Car6-b* increased after innate stimuli. However, the interaction between PRMT5 and MEP50 was reduced simultaneously (Fig. 6*C*). As known, MEP50 is necessary for PRMT5 activity (19). Thus, *Car6-b* and MEP50 competitively interact with PRMT5, which leads to a decrease in the PRMT5 activity.

Furthermore, we performed ChIP assay to detect the recruitment of PRMT5 in promoters of *Il12b*, *Il6*, and *Tnf*. We found that the recruitment of PRMT5 to the *Il12b* promoter site was increased in *Car6*$^{-/-}$ PMs (Fig. 6*D*). However, the recruitment of PRMT5 to the promoters of *Il6* and *Tnf* was not affected in *Car6*$^{-/-}$ PMs (Fig. 6 *E* and *F*). Furthermore, we used PRMT5 antibody for ChIP-seq of *Car6*$^{+/+}$ and *Car6*$^{-/-}$ PMs with or without LM stimulation. We found PRMT5 consensus-binding element AAAAAAAAAAAA in *Il12b* promoter sites but not in *Il6* and *Tnf* promoters (Fig. S9*K*). Additionally, we silenced *Prmt5* in *Car6*$^{+/+}$ and *Car6*$^{-/-}$ PMs and found that silenced *Prmt5* in *Car6*$^{-/-}$ PMs selectively promoted IL-12 production and did not affect IL-6 and TNF-α production upon innate stimuli (Fig. S10). These results suggested *Car6-b* selectively regulated IL-12 expression by interaction with PRMT5, which is specifically recruited to the *Il12b* promoter. We further elucidate

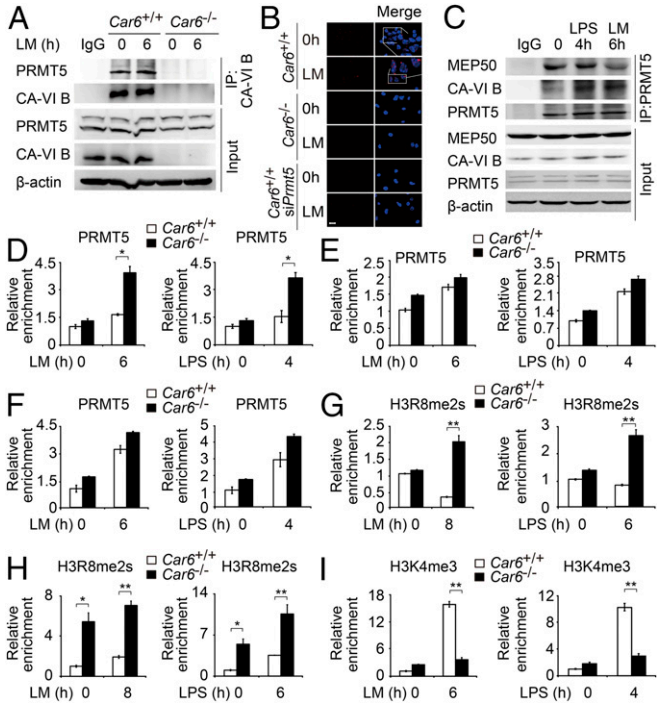

Fig. 6. CA-VI B associates with PRMT5 to promote *Il12* promoter accessibility by histone modification. (*A* and *B*) Interaction between CA-VI B and PRMT5 was detected by co-IP experiments in $Car6^{+/+}$ and $Car6^{-/-}$ PMs with or without the indicated stimuli. (*B*) Interaction between CA-VI B and PRMT5 was detected by PLA in $Car6^{+/+}$ PMs, $Car6^{-/-}$ PMs, and PRMT5-silenced $Car6^{+/+}$ PMs (si*Prmt5*) with or without LM stimulation for 2 h. The interactions were visualized as red spots. (Scale bar, 10 μm.) (*C*) Co-IP experiments in RAW264.7 cells with or without the indicated stimuli. (*D–F*) ChIP assay of PRMT5 recruitment at promoter sites of *Il12b* (*D*), *Il6* (*E*), or *Tnf* (*F*) in $Car6^{+/+}$ and $Car6^{-/-}$ PMs after stimulation for the indicated time with LM or LPS. (*G* and *H*) ChIP assay of H3R8me2 modification at *Il12b* (*G*) or *Il12a* (*H*) promoter sites in $Car6^{+/+}$ and $Car6^{-/-}$ PMs after stimulation for the indicated time with LM or LPS. (*I*) ChIP assay of H3K4me3 modification at the *Il12b* promoter site in $Car6^{+/+}$ and $Car6^{-/-}$ PMs after stimulation for the indicated time with LM or LPS. Data were shown as mean ± SD ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$.

whether histone arginine methylation on promoters of *Il12b* and *Il12a* was regulated by CA-VI B and PRMT5. Indeed, H3R8me2s of *Il12b* and *Il12a* promoters were significantly increased in $Car6^{-/-}$ PMs upon innate stimuli (Fig. 6 *G* and *H*). In addition, we also found that trimethylation of histone H3 lysine 4 (H3K4me3) at the *Il12b* promoter was significantly decreased in $Car6^{-/-}$ PMs upon innate stimuli (Fig. 6*I*). Taken together, CA-VI B inhibits H3R8me2s modification at promoters of *Il12* genes by interaction with PRMT5, which increases the chromatin accessibility upon innate stimuli, consequently enhancing c-Rel binding to the promoter of *Il12b* and promoting IL-12 expression.

Discussion

IL-12 is mainly produced by activated macrophages, monocytes, and DCs (2). Here we identify a previously unknown role of *Car6-b* in promoting host defense against bacterial infection by selectively enhancing IL-12 expression via interaction with PRMT5 to inhibit arginine methylation at *Il12* promoter regions. This is a report about the function of CA members in innate immune response by selectively inducing IL-12 production independent of its CA activity.

Car6 has two types. In our study, we demonstrate that only *Car6-b* is expressed in the immune system and preferentially expressed in macrophages. A previous report showed that the mRNA expression of *Car6* was up-regulated in *Helicobacter pylori*-infected RAW264.7 cells (20). *Car6-b* located in the nucleus has been discovered in mouse embryo fibroblasts in response to stress by unknown mechanisms, whereas its biological functions in the immune system remain unknown. Our study uncovered the function of *Car6-b* in innate immune response.

Car6-b deficiency particularly impairs IL-12 production in macrophages upon innate stimuli but not in DCs. This cell-type selectivity is consistent with the higher *Car6-b* expression in macrophages than that in DCs. DCs and macrophages show different developmental and functional properties in the regulation of innate and adaptive immunity (21). The mechanisms for IL-12 production in DCs and macrophages are different upon different TLR signaling (22). The selective role of *Car6-b* may thereby provide a new mechanism for the differential capacity of DC and macrophages in IL-12 production upon different innate stimuli.

The NF-κB complex contains RelA (p65), RelB, c-Rel, p100, and p105. C-Rel is expressed in hematopoietic cells (23), which might contribute to cell type-specific expression or function of many innate signaling regulators. C-Rel is necessary for *Il12b* transcription in macrophages, whereas *Il12a* is c-Rel–dependent in DCs (24, 25). Both *Il6* and *Tnf* expression are mainly regulated by activation of p65 and p38 in NF-κB and MAPK signaling pathways, although c-Rel also has some functions in this process (23, 26). Both p50/p65 and p50/c-Rel bind to the promoter of *Il12b*, whereas c-Rel has a crucial role in induction of IL-12 expression in macrophages compared with p65 (7, 25). Consistently, deletion of *Car6-b* neither inhibits the production of TNF-α and IL-6 in macrophages upon LM or LPS stimulation nor affects c-Rel binding to the promoter region of *Tnf* and *Il6*. Indeed, the selectivity of regulating gene expression by epigenetic and transcriptional mechanisms has long been a challenging issue in the field. A complex cooperation is shown to be involved in the dynamic and spatial regulation of gene expression. For example, induction of de novo 5-hydroxymethylation upstream of the *Il6* locus by methylcytosine dioxygenase TET2 recruits HDAC2 to specifically inhibit *Il6* transcription via histone deacetylation and therefore is critical for termination of the high transcription of *Il6* during the late phase of inflammatory response (27). In addition, there is a report showing that *linc RNA-Cox2* can regulate *Il12b* expression (28). Our work showed that CA-VI B competitively binds PRMT5 with MEP50 to reduce the methylation activity of PRMT5 specifically recruited to the promoter of *Il12b*, leading it to selectively regulate *Il12* production in innate immune response.

Lysine methylation modification on histone has been extensively investigated, whereas the role of arginine methylation on histone in regulation of immune response has not been clearly clarified. In the present study, we identified that CA-VI B inhibited H3R8me2s at the *Il12* promotor region by interaction with PRMT5, thus adding insights into this histone modification type in regulation of innate immunity and inflammation. Increased activity of the PRMT5–MEP50 complex is found in many kinds of cancer cells and highly correlated with poor prognosis of human cancers, and it is the potential target for treatment of cancers (19). PRMT5 participates in inflammatory response by methylation modification of R35 or R30 of p65 to regulate interleukin-1α (IL-1α), TNF receptor-associated factor 1 (TRAF1), or CXCL10 expression (29). H3R8me2s and H4R3me2s repress gene expression, although the mechanisms mediated with PRMT5 remain to be elucidated (30). Our study reports PRMT5 in regulation of IL-12 expression by arginine modification of histone. Moreover, a crosstalk between arginine and lysine methylation exists to regulate gene expression (31). We found decreased H3K4me3 modification at the *Il12b* promoter in $Car6\text{-}b^{-/-}$ macrophages upon innate stimuli. Further investigations are required to uncover whether and how H3K4me3s and H3R8me2s, and possibly other types of histone modifications, may interact to regulate IL-12 expression.

According to published gene-profiling data, *Car6* expression is up-regulated in inflammatory macrophages in an acute TB infection mouse model (1.3-fold higher than that of control cells;

GEO accession no. GSE23014). In addition, *Car6* is down-regulated in chronic TB infection (1.3-fold lower than that of acute infection period patients, GEO accession no. GSE54992). Moreover, IL-12 is induced in macrophages in response to *Toxoplasma gondii* infection (32). *Car6* expression is also up-regulated in *T. gondii*-infected RAW 264.7 cell lines (1.4-fold higher than that of their control cells; GEO accession no. GSE55298) and in zymosan-induced active macrophages (1.2-fold higher than that of control cells; GEO accession no. GSE28621). These data indicate that *Car6* can be up-regulated in acute infection, helping the host to defend against the invading pathogens, whereas down-regulation of *Car6* expression may be one of the reasons for the host with chronic infection not to effectively eliminate the invading pathogens. These data, together with our results, suggest a potentially important connection between *Car6* and human infection diseases and a possible clinical application of activating *Car6* in treatment of chronic infectious disease with intracellular bacterial infection.

Materials and Methods

Mice. C57BL/6J mice were purchased from Beijing Vital River Animal Center. $Car6^{-/-}$ mice were generated by using the CRISPR/Cas9 system. $Car6^{-/-}$ mice generation information is described in *SI Materials and Methods*.

All animal protocols were approved by the Animal Care and Use Committees of the Institute of Laboratory Animal Science of Chinese Academy of Medical Sciences (ILAS-GC-2015-002).

In Vivo Challenge with *Listeria* Monocytogenes. LMs were obtained and cultured as described previously (33). Mice were infected with 5×10^4 virulent LMs intraperitoneally. Bacteria in the spleen, liver, and lung were quantified as described previously (33).

ChIP. PMs were processed according to the protocol described in the ChIP Assay kit (Beyotime P2078). Antibody–chromatin complexes were pulled down using magnetic protein G beads (Invitrogen), washed, and then eluted. After cross-link reversal and protein K treatment, immunoprecipitated DNA was extracted with phenol-chloroform and ethanol-precipitated. ChIP primers used for *Il12a* were described previously (8), and for *Il6*, *Tnf*s were similar to ref. 27. Other mouse primers were as follows: *Il12b* pro (0-0.2K) forward, 5′-GGGGAGGGAGGAACTTCTTA-3′; *Il12b* pro (0-0.2K) reverse, 5′-TGATGGAAACCCAAAGTAGAAACTGAC-3′.

Chromatin Accessibility (DNase I Sensitivity) Assay. Chromatin accessibility assay and analysis were performed as described previously (17). In brief, chromatin accessibility of the *Il12b* promoter region in $Car6^{+/+}$ and $Car6^{-/-}$ macrophages stimulated with LPS for 1 h or LM for 3 h were assayed by qPCR.

Transfection. Cells were seeded and kept overnight, and then RNAi MAX was used for gene-specific siRNA transfection for 36–48 h. Jetprime (Poly plus) and lipofectamine 3000 transfection reagent (Invitrogen) were used for transfection in 293T cells and RAW264.7 cells, respectively.

Statistical Analysis. Statistical significance between two-group comparisons was analyzed by two-tailed Student *t* test. The statistical significance of survival curves was compared with the generalized Wilcoxon test. The value $P < 0.05$ was considered statistically significant.

Additional methods are described in *SI Materials and Methods*.

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