Human innate responses and adjuvant activity of TLR ligands in vivo in mice reconstituted with a human immune system

Liang Cheng [a], Zheng Zhang [a], Guangming Li [a], Feng Li [a], Li Wang [a], Liguo Zhang [b], Sandra M. Zurawski [c,d,e], Gerard Zurawski [c,d,e], Yves Levy [d,e], Lishan Su [a,*]

[a] Lineberger Comprehensive Cancer Center, Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, United States
[b] Key Laboratory of Infection and Immunity, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China
[c] Baylor Institute for Immunology Research, Dallas, TX 75204, United States
[d] Vaccine Research Institute, Université Paris-Est, Faculté de Médecine, INSERM U955, Créteil, France
[e] Assistance Publique-Hôpitaux de Paris, Groupe Henri-Mondor Albert-Chenevier, Service d'immunologie clinique, 94010 Créteil, France

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ABSTRACT

TLR ligands (TLR-Ls) represent a class of novel vaccine adjuvants. However, their immunologic effects in humans remain poorly defined in vivo. Using a humanized mouse model with a functional human immune system, we investigated how different TLR-Ls stimulated human innate immune response in vivo and their applications as vaccine adjuvants for enhancing human cellular immune response. We found that splenocytes from humanized mice showed identical responses to various TLR-Ls as human PBMCs in vitro. To our surprise, various TLR-Ls stimulated human cytokines and chemokines differently in vivo compared to that in vitro. For example, CpG-A was most efficient to induce IFN-α production in vitro. In contrast, CpG-B, R848 and Poly I:C stimulated much more IFN-α than CpG-A in vivo. Importantly, the human innate immune response to specific TLR-Ls in humanized mice was different from that reported in C57BL/6 mice, but similar to that reported in nonhuman primates. Furthermore, we found that different TLR-Ls distinctively activated and mobilized human plasmacytoid dendritic cells (pDCs), myeloid DCs (mDCs) and monocytes in different organs. Finally, we showed that, as adjuvants, CpG-B, R848 and Poly I:C can all enhance antigen specific $CD4^+$ T cell response, while only R848 and Poly I:C induced $CD8^+$ cytotoxic T cells response to a CD40-targeting HIV vaccine in humanized mice, correlated with their ability to activate human mDCs but not pDCs. We conclude that humanized mice serve as a highly relevant model to evaluate and rank the human immunologic effects of novel adjuvants in vivo prior to testing in humans.

1. Introduction

The most effective vaccines are live attenuated vaccines such as the yellow fever vaccine YF-17D [1] and smallpox vaccine [2,3], providing long-lasting protective immunity with a single administration. It has become clear that, by activating pathogen-encoded pattern recognition receptors (PRRs) on immune effector cells, these vaccines can efficiently activate the innate immune system to induce efficient antigen-specific humoral and cytotoxic T lymphocytes (CTL) responses [4,5]. Conversely, recombinant antigen-based vaccines are often poorly immunogenic and need co-administered adjuvants to enhance the protective immunity especially the CTL response [5].

Toll like receptor (TLRs) represent an important type of PRRs that can sense the microbial components named pathogen-associated molecular patterns (PAMPs) [6,7]. TLRs are expressed by various cells, especially by the innate immune cells such as monocytes, myeloid dendritic cells (mDCs) and plasmacytoid dendritic cells (pDCs) [6,7]. Stimulation of these innate immune cells with natural or synthetic TLR ligands (TLR-Ls) results in up-regulation of co-stimulatory molecules, enhanced expression of MHC class II molecules, and production of inflammatory cytokines [8,9]. Thus, natural ligands or synthetic agonists for TLRs are being developed as potential new vaccine adjuvants [10,11]. For example, Monophosphoryl Lipid A (MPLA), a derivative lipopolysaccharide which acts through TLR4, has been approved

Abbreviations: LN, lymph node; mDC, myeloid dendritic cell; MPLA, monophosphoryl lipid A; pDC, plasmacytoid dendritic cell; TLR-Ls, Toll-like receptor ligands.

* Corresponding author.
E-mail address: lsu@med.unc.edu (L. Su).

for clinic application as a component of AS04 adjuvant in Cervarix vaccine against cervical cancer and the vaccine against hepatitis B virus [12].

Although the adjuvant effects of TLR-Ls are promising, their immunological effects in vivo in humans are still poorly understood. Mouse models serve as the most widely used tools for mechanistic study and preclinical evaluation of TLR-Ls adjuvants. However, fundamental differences exist between human and mouse since the two species diverged between 65 and 75 million years ago, and knowledge gained from mouse studies does not always apply to humans [13,14]. For example, preclinical toxicology study in mice did not provide any indication that fialuridine would be hepatotoxic to human beings [15], but 5 of 15 clinical trial participants died and the other two required a liver transplant after receiving a nucleoside analogue fialuridine treatment due to acute liver failure. The species-specific expression of a mitochondria nucleoside transporter in human but not in mouse is probably responsible for the human-specific liver toxicity caused by fialuridine [16]. Recently, it is reported that fialuridine induced acute liver damage in human-mouse liver chimeric TK-NOG mice [17].

The immune system of human also has diverse differences from mouse [13]. One obvious difference is that bronchus-associated lymphoid tissue is only developed in mice but not in healthy humans. This has possibly evolved because mice live so much closer to the ground where they experience a higher dose of pathogens [13,18]. It has also been reported that the distribution of several TLRs in innate immune cells is quite different between human and mouse [13]. TLR9 in mouse is widely expressed on pDC, mDC, B cells and also expressed in monocyte/macrophage lineage cells [19,20], whereas in humans, it is preferentially expressed on pDCs and B cells [21,22]. TLR8, which is expressed on mDC and macrophage, can respond to ssRNA stimulation in human but this is not functional in mice [23]. Moreover, TLR10, whose ligands are as yet unknown, is widely expressed in humans but not in mice [24]. The discrepancies in TLRs distribution between mouse and human immune cells may limit the translation of findings into human clinical applications, when based on mouse work.

Another commonly used tool for evaluating the adjuvant effects of the TLR-Ls is the human peripheral blood mononuclear cells (PBMCs) in vitro culture system. However, this cell culture system is not useful to study non-circulating cells that also respond to TLR-Ls stimulation in vivo [25]. The other fundamental limitation of the human PBMCs in vitro culture system is that it cannot authentically reflect the cell-cell interaction environment in vivo. The dynamics and accessibility of the drugs to the cells should also be different in vivo compared to that in vitro. It is also difficult to evaluate vaccine adjuvant activity in inducing human T and B cell responses in vitro.

Mice reconstituted with a functional human immune system provide a valuable platform to study the development and functions of human immune cells, and more importantly, to investigate human immune response to pathogens, vaccines and other stimulations in vivo [14]. We and others have shown that injection of human CD34$^+$ hematopoietic stem cells into the immunodefecient BALB/c Rag2$^{-/-}\gamma_c^{-/-}$ mice or NOD-scid $\gamma_c^{-/-}$ (NSG) mice as well as NOD-Rag2$^{-/-}$ $\gamma_c^{-/-}$ (NRG) mice can reconstitute all major human myeloid and lymphoid subsets, including monocytes, mDCs, pDCs, T cells and B cells [26–31]. In this study, we used the humanized NRG mice as an in vivo model to explore how the human immune system responds to different PAMPs, specifically, how various TLR-Ls differentially stimulate human innate immune response and regulate adaptive CD4$^+$ helper T cell and importantly cytotoxic T lymphocyte (CTL) response to CD40-targeting HIV candidate therapeutic vaccine in vivo. We demonstrate that human leukocytes developed in humanized mice respond similarly to TLR-Ls stimulation of human PBMCs in vitro. When tested in vivo, however, TLR-Ls induce a significantly different profile of human cytokines and chemokines compared to that induced in vitro. We show that, in humanized mice, various TLR-Ls differentially activate distinct human immune cells in different lymphoid organs. Importantly, humanized mice respond to TLR-Ls stimulation differently from C57BL/6 mice [25] but similarly to that observed in nonhuman primates [32]. Finally, we show that, consistent with their different abilities to activate mDCs, Poly I:C and R848 (but not CpG-B) were able to enhance antigen-specific CTL responses to a CD40-targeting HIV candidate vaccine in humanized mice. Our study indicates that various TLR-Ls differentially activate human innate immune cells to enhance antigen-specific cellular immune responses in humanized mice. The humanized mouse model thus provides a unique platform to evaluate the immunologic effects of novel adjuvants in vivo, prior to human testing.

2. Materials and methods

2.1. Ethics statement

The report followed NIH research ethics guidelines. For the construction of humanized mouse, human fetal liver was obtained from elective or medically indicated termination of pregnancy through a non-profit intermediary working with outpatient clinics (Advanced Bioscience Resources, Alameda, CA). The use of the tissue in the research had no influence on the decision regarding termination of the pregnancy. Informed consent of the maternal donor is obtained in all cases, under regulation governing the clinic. We were provided with no information regarding the identity of the patients, nor is this information traceable. The project was reviewed by the University's Office of Human Research Ethics, which has determined that this submission does not constitute human subjects research as defined under federal regulations [45 CFR 46.102 (d or f) and 21 CFR 56.102(c)(e)(l)] and does not require IRB approval. The University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee (IACUC) has reviewed and approved this research. All animal experiments were conducted following NIH guidelines for housing and care of laboratory animals and in accordance with The University of North Carolina at Chapel Hill with protocols approved by the institution's Institutional Animal Care and Use Committee (IACUC ID: 14-100).

2.2. Construction of humanized mice

We constructed humanized NRG (NOD-Rag2$^{-/-}$ $\gamma_c^{-/-}$) mice by reconstitution with human fetal liver (17 to 22 weeks of gestational age) derived CD34$^+$ hematopoietic progenitor cells (Advanced Bioscience Resources, Alameda, CA) similarly as previously reported [30]. Humanized BLT mice were generated according to a previous report [33]. Briefly, 6 to 8 weeks old NRG mice were sub-lethally irradiated and anesthetized the same day, and $\sim$1-mm^3 fragments of human fetal thymus were implanted under the recipient kidney capsule. CD34$^+$ hematopoietic progenitor cells purified from fetal liver of same donor were injected *retro*-orbital within 3 h. Human immune cell engraftment was detected by flow cytometry 12 weeks after transplantation. All animal studies were approved by the University of North Carolina Institutional Animal Care and Use Committee (IACUC).

2.3. TLR-L treatment in vitro and in vivo

CpG-A (ODN 2216), CpG-B (ODN 2006), CpG-C (ODN 2395), R848, MPLA and Poly I:C used in this study were all purchased from InvivoGen. 1×10^6 total human PBMCs or splenocytes of humanized mice containing 1×10^6 human CD45$^+$ cells were used

for in vitro stimulation. Cells were stimulated with 5 μg/ml of CpG-A, CpG-B, CpG-C, R848, Poly I:C and 2 μg/ml of MPLA for 24 h and supernatants were collected for cytokine/chemokine detections. For in vivo treatment, humanized mice were treated with 50 μg/mouse of CpG-A, CpG-B, CpG-C, R848, Poly I:C or 20 μg/mouse of MPLA through i.p. injection.

2.4. Detection of cytokines/chemokines

Human IFN-α was detected by enzyme-linked immunosorbent assay using the human IFN-α pan ELISA kits purchased from Mabtech. A high sensitivity immunology multiplex assay (Luminex) (Millipore, Billerica, Massachusetts, USA) was used to measure human IL-12, IL-6, IFN-γ, TNF-α, IL-1β, IP-10, MCP-1, IL-8, IL-4 and IL-10 in plasma of humanized mice or cell culture supernatant according to the manufacturer's instructions.

2.5. Flow cytometry

For surface staining, single cell suspensions prepared from peripheral blood, spleen, or bone marrow of humanized mice were stained with surface markers and analyzed on a CyAn ADP (Dako). For intracellular cytokine staining, cells were first stained with surface markers, and then permeabilized with cytofix/cytoperm buffer (BD Bioscience), followed by intracellular staining. FITC-conjugated anti-human CD40, CD56, PE-conjugated anti-human CD303, PE/Cy5-conjugated anti-human CD4, CD86, PE/Cy7-conjugated anti-human CD3, HLA-DR, PB-conjugated anti-human CD4, CD14, IL-2, APC-conjugated anti-human CD123, CD11c, TNF-α and APC/Cy7-conjugated anti-human CD45 were purchased from Biolegend. Pacific orange-conjugated anti-mouse CD45, PE/Texas red-conjugated anti-human CD3, CD19, CD8 and LIVE/DEAD Fixable Aqua (LD7) Dead Cell Stain Kit were purchased from Invitrogen. Data were analyzed using Summit4.3 software (Dako).

2.6. Vaccination and antigen-specific T cell response detection

Recombinant anti-human CD40 antibody fused to 5 HIV peptide regions (αCD40-HIV5pep) was produced as previously reported [34], except that the HIV peptide and flexible linker sequences were reconfigured and the CD40 binding variable regions were changed to a human framework. These changes had no impact on CD40 binding or efficacy in expansion of antigen-specific T cells in vitro. Humanized BLT mice were intramuscularly (half dose) and intraperitoneally (half dose) injected with 10 μg αCD40-HIV5pep alone or with 50 μg of each TLR-L two times at 3 week intervals. Splenocytes from vaccinated humanized mice were collected 10 days after the second vaccination and stimulated ex vivo with 5 specific HIV long peptides [34] plus α-CD28 for 12 h. Brefeldin A was added during the last 4 h of stimulation and IL-2, TNF-α expression by $CD4^+$ and $CD8^+$ T cells were detected by intracellular staining. IFN-γ production was detected by ELISPOT after 24 h of stimulation with 5 specific HIV long peptides plus α-CD28.

2.7. Statistics

Statistical analysis was performed using the two-tailed, unpaired Student's *t* test ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; ns, non-significant) using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA).

3. Results

3.1. Human PBMCs and human leukocytes developed in humanized NRG mice respond similarly to TLR-L stimulation in vitro

We reconstituted newborn NRG mice with human fetal liver derived $CD34^+$ hematopoietic stem/progenitor cells and detected the development of human leukocytes 3 months after human cell transplant. All major human $CD45^+$ leukocyte subsets including T cells ($CD3^+$), B cells ($CD19^+$), NK cells ($CD3^-CD56^+$), monocytes/macrophages ($CD3^-CD19^-HLA^-DR^+CD14^+$), mDCs ($CD3^-CD19^-HLA^-DR^+CD14^-CD11c^+$) and pDCs ($CD3^-CD19^-HLA^-DR^+CD303^+$) were developed in humanized NRG mice (Supplemental Fig. 1). In order to test whether the innate immune cells reconstituted in humanized NRG mice were functional, we stimulated splenocytes isolated from humanized mice in vitro with the TLR9-Ligands CpG-A, CpG-B, CpG-C [35,36], the TLR7/8-L R848 [37], the TLR4-L MPLA [38] and the TLR3-L Poly I:C [25]. Human cytokines and chemokines were measured as functional readout for activation of immune cells. In parallel, results were also obtained from human PBMCs stimulated with the same individual TLR-Ls in vitro. As shown in Fig. 1A, various TLR-Ls differentially stimulated the production of cytokines including IFN-α, IL-6, TNF-α and IL-12 from splenocytes of humanized mouse. Importantly, the cytokine induction profile from splenocytes of humanized mice was comparable to that induced from human PBMCs in vitro (Fig. 1B). These results indicate that functional human innate immune cells are developed in humanized NRG mice 3 months after transplantation with human hematopoietic stem/progenitor cells.

3.2. TLR-Ls stimulate different human cytokine production profiles in vivo compared to that in human PBMCs in vitro

To assess the early effects of TLR-Ls on systemic human immune responses in vivo, we treated humanized NRG mice with various TLR-Ls. We monitored the dynamics of each human cytokine and chemokine level at different time points after TLR-Ls treatment in vivo and the peak values were shown (Fig. 2 and Supplemental Fig. 2A). Distinct TLR-Ls stimulated human innate immune response differentially as indicated by inducing different amounts of human IFN-α, IL-12, IL-6, TNF-α, IP-10, MCP-1 and IL-8 in the serum after treatment (Fig. 2 and Supplemental Fig. 2A). Among the TLR-Ls tested, CpG-B, R848 and Poly I:C were more potent than other TLR-Ls to stimulate production of IFN-α and other proinflammatory cytokines and chemokines (Fig. 2 and Supplemental Fig. 2A).

To our surprise, we found that the array of human cytokines and chemokines stimulated by specific TLR-Ls in vivo was significantly different from that induced from human PBMCs or splenocytes of humanized mice in vitro (Figs. 1 and 2). As shown in Fig. 1B, CpG-A was most efficient in stimulating IFN-α production in vitro (16,218 ± 5861 pg/ml). However, it only induced a minimal amount of IFN-α (25 ± 9 pg/ml) in vivo (Fig. 2A). In contrast, R848 induced a low level of IFN-α (162 ± 84 pg/ml) in vitro (Fig. 1B), while it robustly induced IFN-α (10801 ± 4275 pg/ml) in vivo (Fig. 2A). Also in contrast to CpG-A, both CpG-B and Poly I:C induced very low levels of IFN-α from human PBMCs in vitro (Fig. 1B). However, CpG-B and Poly I:C both stimulated much more IFN-α production in humanized mice (1039 ± 276 pg/ml and 522 ± 265 pg/ml respectively) than CpG-A (25 ± 9 pg/ml) (Fig. 2A). TLR4-L MPLA stimulated no significant production of IFN-α both in vitro and in vivo (Figs. 1B and 2A).

The induction profile of human IL-12 production was also different between in vivo and in vitro in response to TLR-Ls. Poly I:

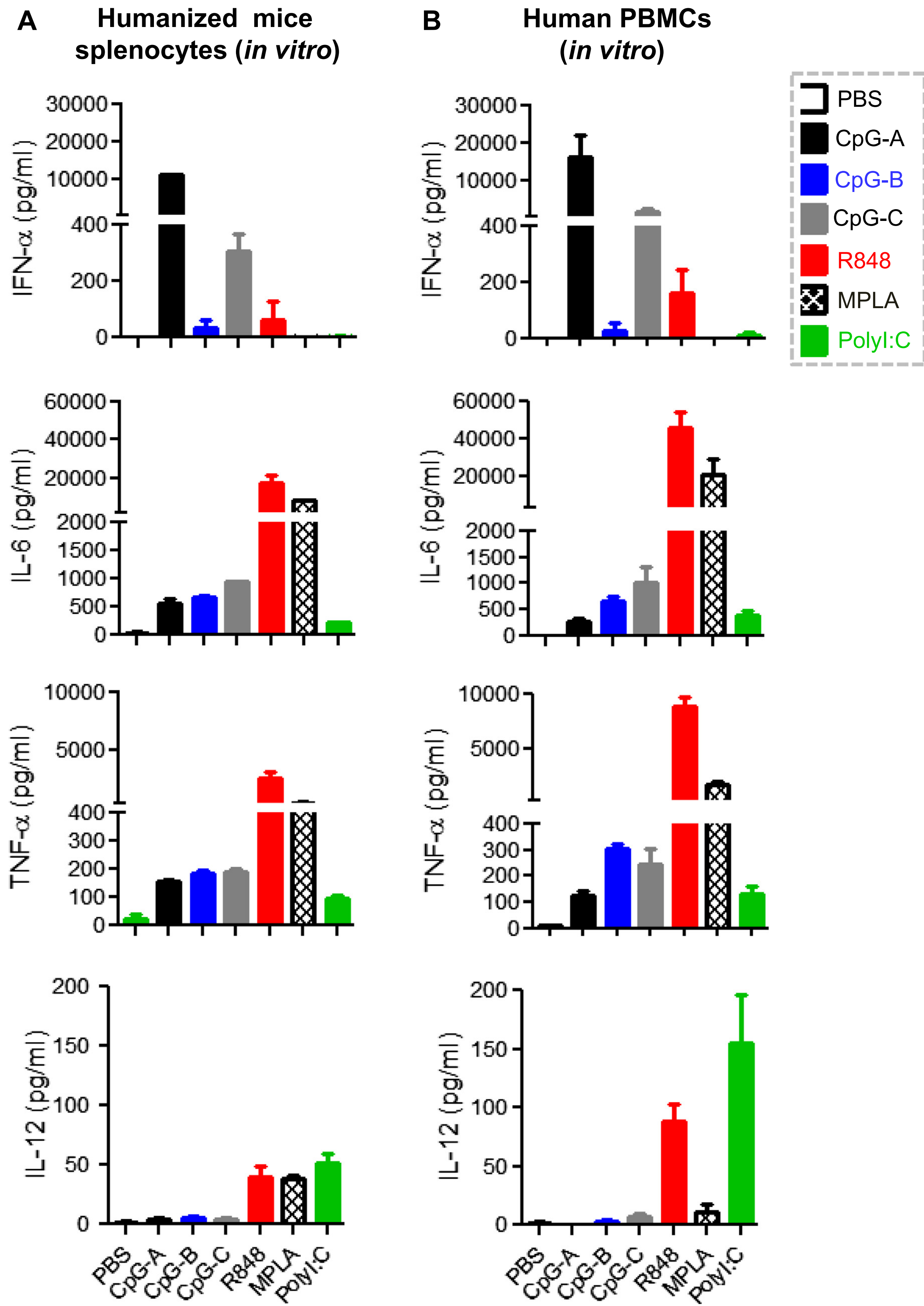

Fig. 1. TLR-Ls induce similar human cytokine production profiles from human PBMCs and splenocytes of humanized mice. Freshly isolated splenocytes from humanized mice or human PBMCs were stimulated with indicated TLR-Ls *in vitro*. Cytokine levels in culture supernatant were detected at 24 h after stimulation. Graph represents mean cytokine levels from splenocytes of humanized mice with 3 different human donor tissues (A) or PBMCs from 5 different donors (B).

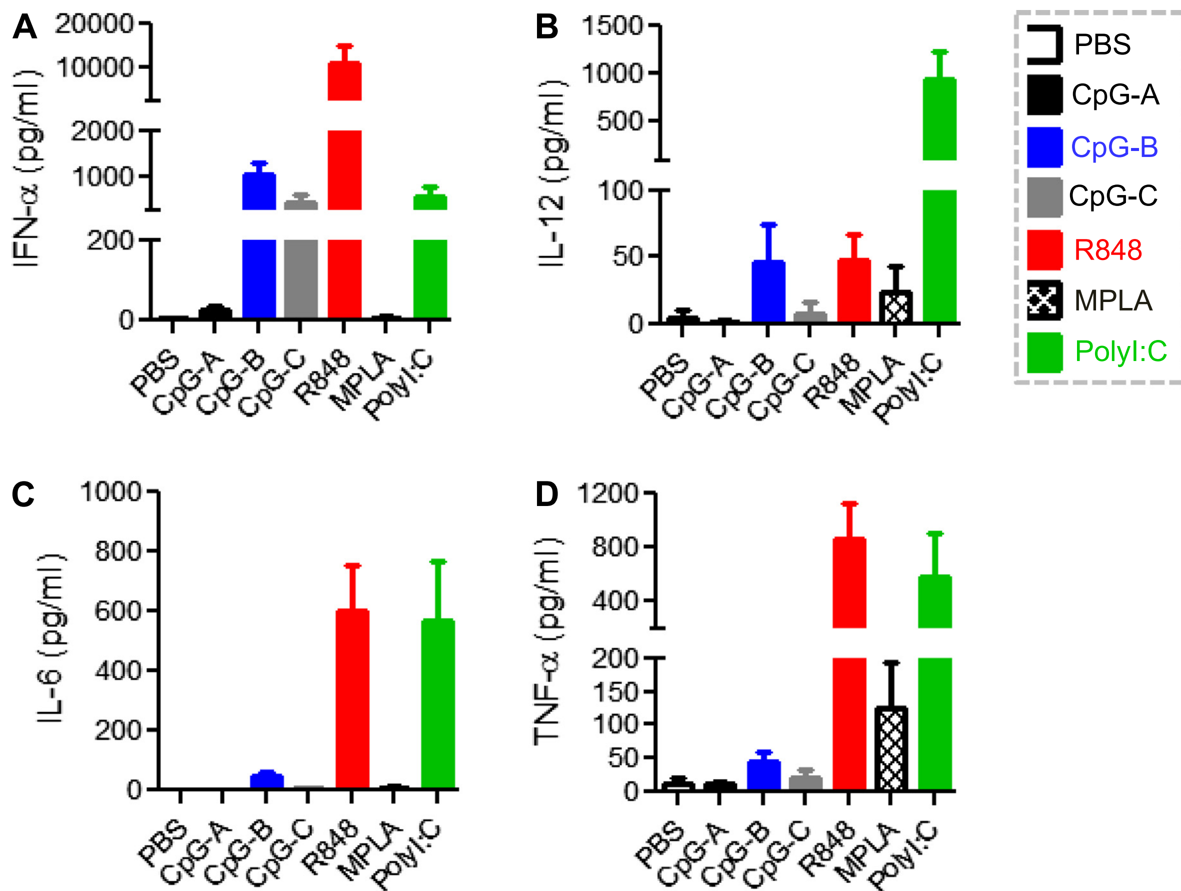

Fig. 2. Various TLR-Ls differently stimulate human cytokine production *in vivo*. Humanized mice were injected intraperitoneally with indicated TLR-Ls or PBS. IFN-α, IL-12 levels in plasma were detected at 9 h after treatment (A and B). IL-6 level was detected at 4 h after treatment (C). TNF-α level was detected at 1 h after treatment (D). Graphs represent the mean of 3–4 mice each group. Three independent experiments were performed with similar results.

C and R848 stimulated similar level of IL-12 while CpG-B did not induce significant level of IL-12 in vitro (Fig. 1B). We found that in vivo Poly I:C induced around 20-fold higher IL-12 production than R848 or CpG-B (Fig.2B). MPLA stimulated a low level of IL-12 in vitro and in vivo (Figs. 1B and 2B).

The proinflammatory cytokines IL-6, TNF-α and chemokines IP-10, MCP-1 and IL-8 induced by distinct TLR-Ls also varied between in vivo and in vitro systems (Figs. 1, 2 and Supplemental Fig. 2). The data above indicate that different TLR-Ls distinctively stimulate cytokine and chemokine production in humanized NRG mice in vivo, but these profiles are different to those observed from human PBMCs, as well as splenocytes from the humanized mouse, in vitro. These data suggest that the immunological effect of TLR-Ls in vitro does not predict their effect in vivo.

3.3. Kinetics of human cytokine production in vivo after TLR-Ls treatment

The results above showed that CpG-B, R848 and Poly I:C are more efficient than other TLR-Ls in stimulating production of human proinflammatory cytokines and chemokines in vivo in humanized mice. We further characterized the induction kinetics of human cytokines and chemokines by these three TLR-Ls in humanized mice. IFN-α was robustly induced by R848 at 1 h after injection, and reached peak level at 4 h (Fig. 3A). After 48 h, the IFN-α level was only slightly reduced (Fig. 3A). In contrast, CpG-B and poly I:C stimulated slower and lower IFN-α expression and the IFN-α level became undetectable after 48 h (Fig. 3A). However, IL-12 induced by Poly I:C was higher and persisted longer in the plasma (Fig. 3B). Both Poly I:C and R848 induced higher level of IL-6 than CpG-B (Fig. 3C). Induction of other cytokines such as TNF-α, IFN-γ and IL-1β; and chemokines such as IP-10, MCP-1, and IL-8 also showed different kinetics after CpG-B, R848 or Poly I:C treatment (Fig. 3 and Supplemental Fig. 3). All the TLR-Ls tested induced only very low levels of anti-inflammatory cytokine IL-10 and they did not induce detectable Th2 cytokine IL-4 (Supplemental Fig. 3).

3.4. Humanized mice respond to TLR-Ls stimulation differently from C57BL/6 mice but similarly to that reported in nonhuman primates

Mouse models have been widely used for mechanistic study and preclinical evaluation of TLR-Ls adjuvants. Since the expression of several TLRs in immune cells is quite different between human and mice [13], we investigated whether TLR-Ls differentially activate human and mouse immune response in vivo. It was reported that CpG-B induced a high level of mouse cytokines IL-6, TNF-α, IL-12 and IFN-γ in C57BL/6 mice [25]. In marked contrast, our result showed that, in comparison with Poly I:C and R848, CpG-B only induced very low levels of human IL-6, TNF-α, IL-12 and IFN-γ in humanized mice (Fig. 3 and Supplemental Fig. 3). Study in nonhuman primates such as Rhesus macaques, which has closer genetic relationship to human, also indicated that CpG-B induced low levels of IL-6, TNF-α, and IFN-γ [32] when administered in vivo. Importantly, CpG-B therapy in mildly asthmatic human patients also did not stimulate TNF-α production [39]. Our in vivo data further explain the fact that CpG-B elicits TNF-α–dependent toxicity in rodents but not in humans [39].

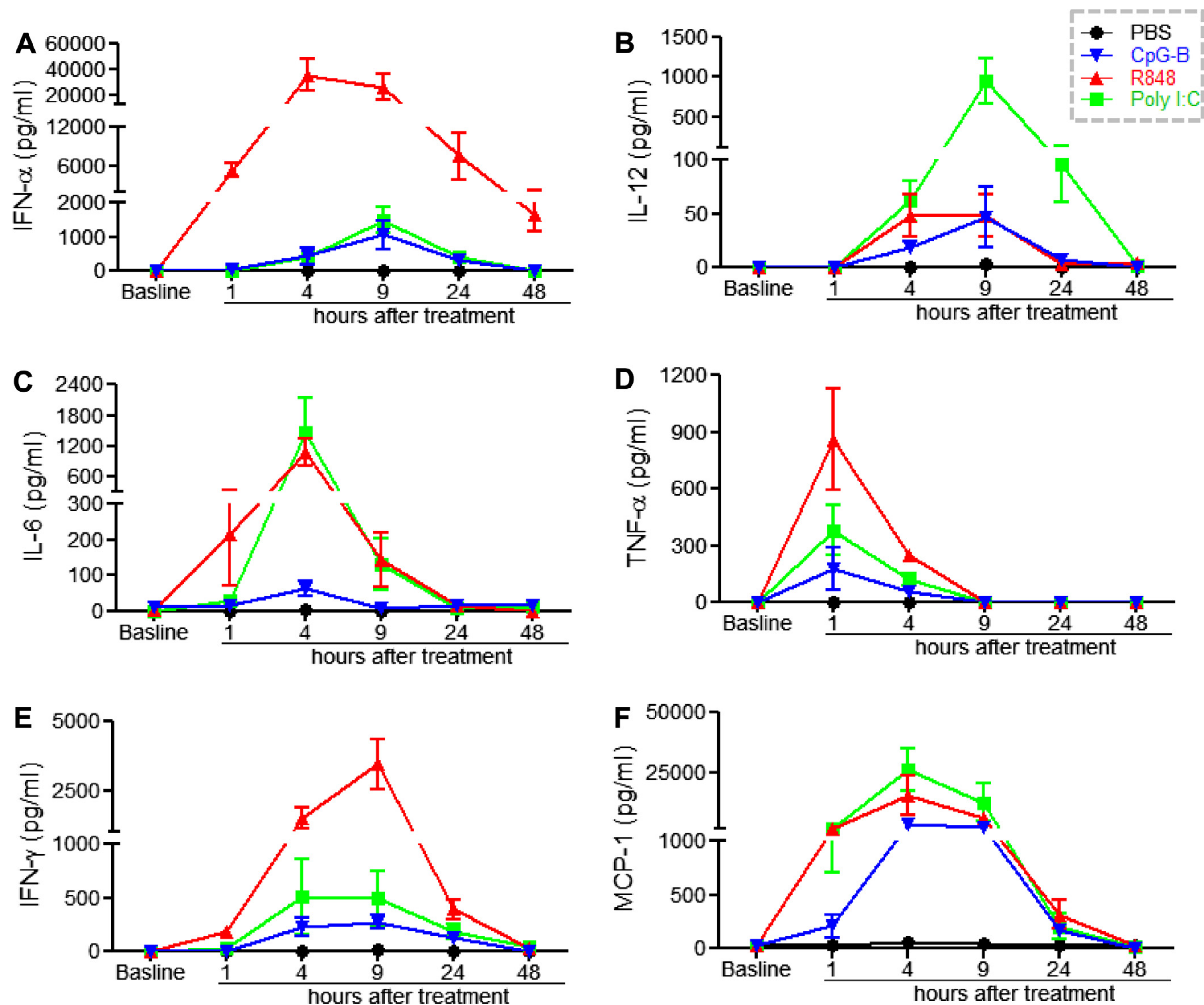

Fig. 3. Kinetics of human cytokine production after TLR-Ls treatment in humanized mice. Humanized mice were injected intraperitoneally with CpG-B, R848, Poly I:C or PBS. Cytokines and chemokines in plasma were measured at the indicated time points after TLR-Ls injection. Graphs represent the mean of 3–4 mice each group. Three independent experiments were performed with similar results.

These results indicate that the immunological effect of CpG-B in humanized mice, but not in mice, is more relevant to the situation in humans.

Poly I:C treatment in C57BL/6 mice leads to higher amounts of mouse IFN-α production than R848 and CpG-B [25]. In contrast, we found here that R848 was the superior human IFN-α inducer in humanized mice (Fig.3A). Similar to the result observed in Rhesus macaques [32], R-848 treatment induced rapid and abundant production of pro-inflammatory cytokines in humanized mice (Fig. 3 and Supplemental Fig. 3). Taken together, these results indicate that mouse and human immune system respond differentially to in vivo administered TLR-Ls stimulation. Thus, knowledge about the immunological effect of TLR-Ls in mouse is not always applicable to humans. The similarity of immune responses to specific TLR-Ls between humanized mice and nonhuman primates support the use of humanized mouse model to study the human immunological effects of TLR-Ls, in vivo.

In summary, different TLR-Ls showed variable abilities to stimulate production of human cytokines and chemokines in vivo in humanized mice. Importantly, the human cytokine and chemokine production profile induced by TLR-Ls in vivo was quite different compared to that induced in vitro from human PBMCs or splenocytes of humanized mouse. The data indicate that the immunological effect of TLR-Ls on human PBMC in vitro cannot simply apply to that in vivo. Results also showed that humanized mice responded to TLR-Ls stimulation differently from C57BL/6 mice, but similarly to that in nonhuman primate (Table 1). Thus, humanized mice provide a unique platform to study the human immunological effect of TLR-Ls and novel adjuvants in vivo.

3.5. Different TLR-Ls distinctively activate and mobilize human monocytes/ macrophages, mDCs and pDCs in peripheral blood, spleen and lymph nodes

We further characterized the effects of TLR-Ls in vivo by measuring the expression level of surface activation markers (CD40, CD86, HLA-DR) on various human innate immune cells in different organs. We found that CpG-B preferentially activated $CD303^+$ pDCs in the peripheral blood, spleen and mesenteric lymph nodes (mLNs) while it had minimal effect on $CD14^+$ monocytes/

Table 1
Cytokines induced by TLR-Ls in humanized mice in vivo resemble that in Rhesus macaque but different from that in C57BL/6 mice.

Cytokines induced	Stimulation conditions											
	Humanized mouse *in vivo*				Rhesus macaque *in vivo* (32)				C57BL/6 mouse *in vivo* (25)			
	CpG-B	R848	Poly I:C	MPLA	CpG-B	R848	Poly I:C	MPLA	CpG-B	R848	Poly I:C	LPS
IFN-α	++	++++	++	–	++	++++	NA	–	+/–	++	++++	–
IL-12p70	+	+	+++	+/–	NA	NA	NA	NA	–	–	–	–
IL-6	+	+++	+++	+/–	+	+++	NA	+/–	+++	+++	+++	++
IFN-γ	+	+++	++	+/–	+	+++	NA	–	++++	++	++	+

Summary of cytokines (IFN-α, IL-12p70, IL-6 and IFN-γ) induced by different TLR-Ls in humanized mice in vivo, in Rhesus macaque (32) and C57BL/6 mice (25) in vivo. ++++, +++, ++, + and – indicate the relative levels of cytokine. Levels of cytokines induced in humanized mice, rhesus macaques, and C57BL/6 mice shown here represent their peak levels after treatment in vivo. NA, assays not performed in the experiments.

macrophages and CD11c$^+$ mDCs in peripheral blood and spleen (Fig. 4A–C). However, monocytes/macrophages from the mLNs expressed a higher level of this activation marker in the CpG-B treated group than control mice (Fig. 4A). Poly I:C was most potent in activating mDCs, but not pDCs, in the peripheral blood, spleen, and mLNs (Fig. 4A–C). It significantly up-regulated the expression of CD40, CD86 and HLA-DR on monocytes/macrophages from treated mLNs but not peripheral blood or spleen (Fig. 4A). Since monocytes do not express TLR-9 and TLR-3, their activation by CpG-B and Poly I:C in the mLN may be indirect. R848 triggered the activation of a broader spectrum of immune cells, including pDCs, mDCs and also monocytes/macrophages from peripheral blood, spleen and mLNs (Fig. 4A–C).

In addition, we found that frequency of monocytes and mDCs decreased in the peripheral blood and spleen 24 h after R848 and Poly I:C treatment. In contrast, R848 and Poly I:C treatment increased the frequency of monocytes and dendritic cells in the mesenteric LNs (Fig.4D). These data suggest that after R848 and Poly I:C treatment, human monocytes and cDCs migrated to the LNs.

In summary, the results above indicated that TLR-Ls systemically and differentially activated human immune cells, in vivo, in different organs; with CpG-B preferentially activating pDCs, Poly I:C being more potent in activating mDCs, while R848 activated both mDCs and pDCs. Thus, by using the humanized mouse model we can investigate the human immunological effects of TLR-Ls on different immune target cells in different lymphoid organs and evaluate or develop novel human adjuvants based on such multiple parameters.

3.6. Poly I:C, R848 and CpG-B have different efficacy for enhancing antigen-specific T cell response

Recombinant antigen-based vaccines are often poorly immunogenic and need adjuvants to induce efficient protective immunity, especially the CTL response [5]. Since TLR-Ls CpG-B, R848, and Poly I:C are better than other TLR-Ls tested here to activated human innate immune system in vivo, we further tested their ability to enhance antigen-specific T cell response to a CD40-targeting HIV vaccine, which is based on a recombinant anti-human CD40 antibody fused via the heavy (H) chain C-terminus to a string of the 5 HIV peptides (αCD40-HIV5pep) [34]. Humanized BLT (CD34$^+$ HSC, fetal liver, and thymus) mice were immunized with αCD40-HIV5pep with or without individual TLR-L as adjuvant. Antigen-specific T cell responses were evaluated one week after the boost vaccination. As shown in Fig. 5A and B, after ex vivo stimulation with antigen-specific peptides, CD4$^+$ cells from vaccinated mice with all three TLR-Ls as adjuvant, produced significantly higher levels of intracellular IL-2 and TNF-α than non-vaccinated control mice or vaccinated mice without adjuvant. In contrast, antigen-specific CD8$^+$ T cell responses were only detected in vaccinated mice with Poly I:C and R848, but not CpG-B, as adjuvant (Fig. 5C and D). PolyI:C as adjuvant also enhanced the ability of antigen-specific T cells to produce IFN-γ after stimulation with peptides ex vivo (Fig. 5E).

3.7. The different ability of Poly I:C, R848 and CpG-B to enhance antigen-specific CTL response correlates with their ability to activate mDC

To induce antigen-specific CTL responses, exogenous protein vaccines need to be processed and cross-presented with MHC class I molecules by specific dendritic cells [10]. It is reported that, in vitro, both mDCs and pDCs from human lymphoid organs can cross-present soluble antigens [40,41]. Our data indicated that, in vivo, Poly I:C and R848 but not CpG-B as adjuvant enhanced antigen-specific CTL response to a protein based vaccine (Fig. 5). Of these three adjuvants, Poly I:C only activated human mDCs, R848 activated both human mDCs and pDCs, while CpG-B preferentially activated human pDCs in different lymphoid organs in vivo (Fig. 4). These results suggest that, in vivo, activation of human mDCs but not pDCs is important for inducing antigen-specific CTL response to protein based vaccination.

CpG-B has been reported to activate and promote the cross-presentation ability of mouse pre-CD8α$^+$ mDC [42] and mouse bone marrow-derived mDCs [43]. It also induces a sustained high level of IL-12 expression in mouse [44]. Thus, as adjuvant, CpG-B can significantly enhance mouse CTL responses to protein or peptide vaccine in mice [45,46] which is different from our data in humanized mice here. Again, our results suggest that the adjuvant activity of TLR-Ls in mice is distinct from that in humans.

Taken together, our data indicate that, consistent with their different abilities for activating human mDCs, Poly I:C and R848 but not CpG-B have the ability to induce human CTL response to antigen-based vaccine. Thus, humanized mice provide a unique and highly relevant in vivo model to test human innate response, which can define intrinsic adjuvant activity and resulting enhancement of vaccine antigenicity prior to human testing.

4. Discussion

TLR-Ls, which can activate the innate immune system through specific receptors, are widely recognized as potential vaccine adjuvants [10]. Therefore, understanding the immunological effect of TLR-Ls, in vivo in humans, is essential for applying them in the clinic. Mouse models have served as the most widely used tools for mechanistic study and preclinical evaluation of TLR-Ls as adjuvants. However, since human and mouse diverged between 65 and 75 million years ago, fundamental differences exist between the two species, in particular in their innate immune systems [13]. In the present study, we took advantage of the well-developed humanized mouse model to study how distinct TLR-Ls stimulated the human immune system and as adjuvants in vivo. We report the

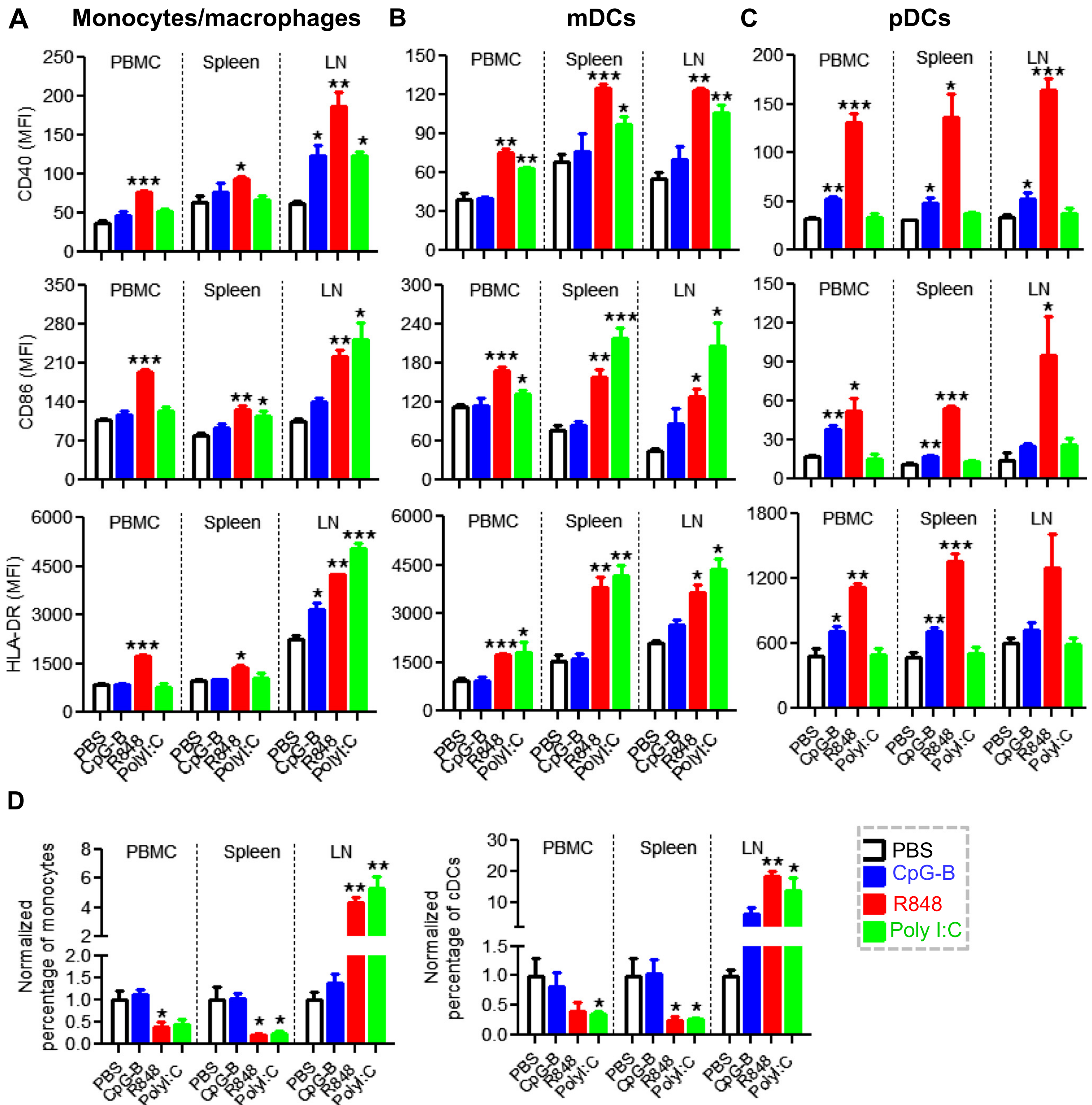

Fig. 4. Distinct TLR-Ls differentially activate human monocytes, mDC, and pDCs in different lymphoid organs. Humanized mice were injected intraperitoneally with CpG-B, R848, Poly I:C or PBS. Leukocytes from peripheral blood, spleen, and LNs were isolated for flow cytometry analysis 24 h after treatment. The MFI for CD40, CD86 and HLA-DR on CD14$^+$ monocytes (A), CD11 c$^+$ mDCs (B) and CD303$^+$ pDCs (C) in blood, spleen, and LNs was shown. (D) Relative frequencies (compared with PBS group) of monocytes (left panel) and cDCs (right panel) in blood, spleen, and LNs 24 h after TLR-Ls treatment were summarized. Graphs represent the mean of 3–4 mice each group. Two independent experiments were performed with similar results.

following novel findings: (1) we proved that leukocytes from spleens of humanized mice respond similarly to 6 different TLR agonists as human PBMCs in vitro. (2) We found that TLR-Ls stimulated distinct human cytokine production profiles in humanized mice in vivo, compared with that in vitro. (3) Humanized mice responded to TLR-Ls stimulation differently from C57BL/6 mice, but similarly to that reported in nonhuman primates. (4) We demonstrated that, consistent with their different abilities to activate human mDCs, Poly I:C and R848 but not CpG-B were able to enhance antigen-specific CTL responses to a protein based vaccine in humanized mice.

We report here that TLR-Ls activated the human immune response differently from that in mice. It is reported that CpG-B treatment in mice induced high levels of pro-inflammatory cytokines IL-6, TNF-α, IL-12 and IFN-γ, comparable to that induced by R848 or Poly I:C [25]. However, CpG-B induced much lower levels of human IL-6, TNF-α, IL-12 and IFN-γ in humanized mice in comparison to R848 and Poly I:C (Fig. 3). The distinct expression patterns of TLR-9 in mouse and human probably contribute to the different immunological effect induced by CpG-B [13]. Our results explained why CpG-B elicits TNF-α-dependent toxicity in mice but not in humans [39]. Thus, knowledge about the immunological

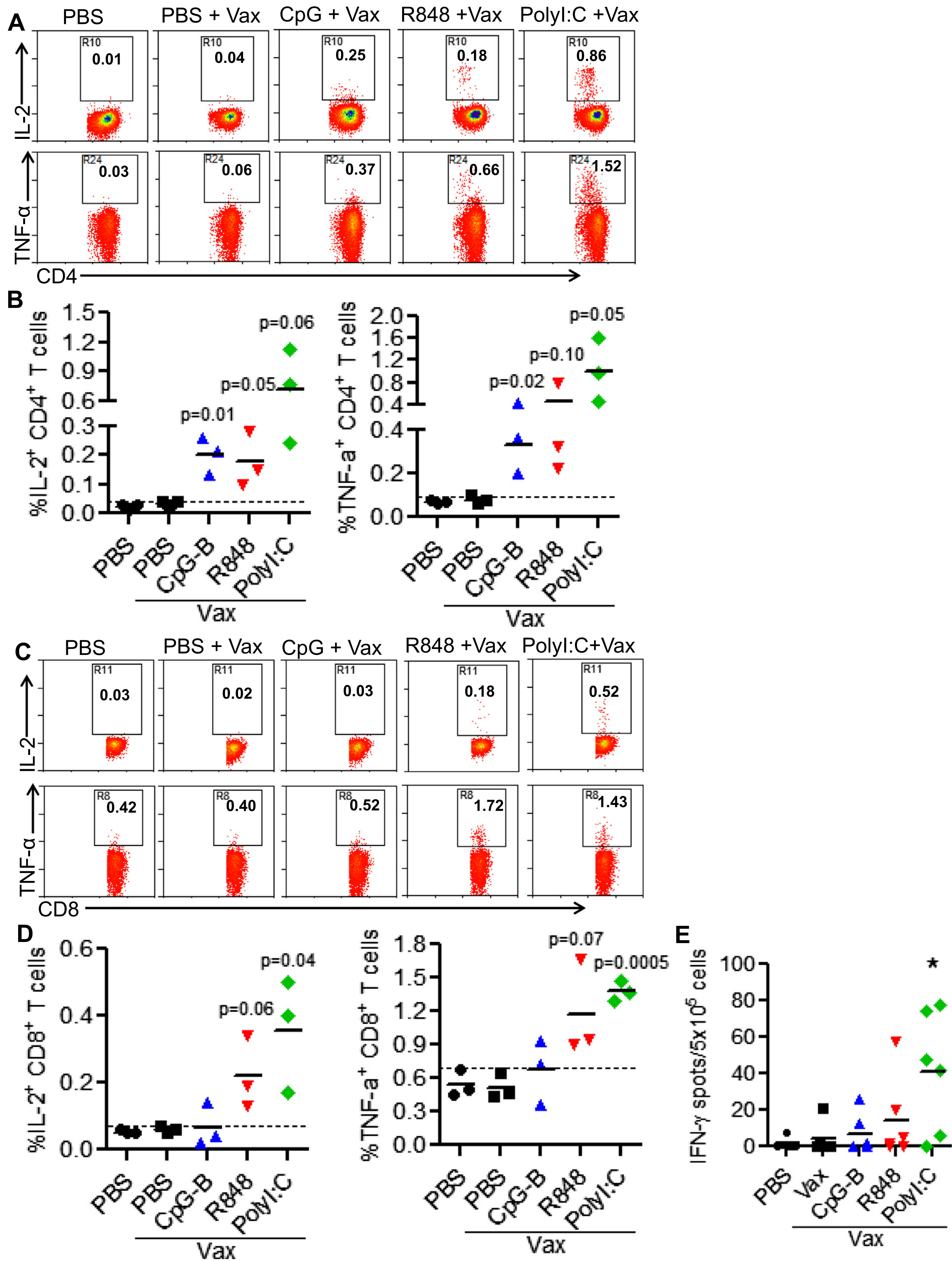

Fig. 5. CpG-B, R848 and Poly I:C differentially enhance antigen-specific $CD4^+$ and $CD8^+$ T cell responses to vaccination. Humanized BLT mice (with both human HSC and thymus co-transplant) were vaccinated with αCD40-HIV5pep alone or with indicated TLR-Ls twice at 3 weeks interval. Mice were euthanized 10 days after boost. Splenocytes from vaccinated humanized mice were stimulated ex vivo with the 5 specific HIV long peptides plus α -CD28 mAb. IL-2 and TNF-α expression by $CD4^+$ and $CD8^+$ T cells were detected by intracellular staining. IFN-γ production was detected by ELISPOT assay. (A and B) Representative plots and summarized data show percentages of $IL\text{-}2^+$ and TNF-α^+ $CD4^+$ T cells. (C-D) Representative plots and summarized data show percentages of $IL\text{-}2^+$ and TNF-α^+ $CD8^+$ T cells. Each dot represents one individual mouse with 3 mice each group. Two independent experiments with similar results were performed. (E) IFN-γ production after 5 specific HIV long peptides plus α -CD28 stimulation. Shown are combined data from two independent experiments with 6 mice from each group.

effect of TLR-Ls in humanized mice will be critical to evaluate human innate immune activation and adjuvant activity relevant to humans.

We also compared our data with reported work in nonhuman primates [32]. We found that TLR-Ls stimulated similar innate immune response in humanized mice as in Rhesus macaque, which has closer genetic relationship to human. Herein, we conclude that the humanized mouse serves as a highly relevant small animal model to test human immunological activity of TLR-Ls, in vivo.

In this study, we also demonstrated that TLR-Ls stimulated the human innate immune response differently in vivo compared to that in vitro. Three major classes of stimulatory CpG oligodeoxynucleotides (CpG ODN) have been identified based on their structural characteristics and activity on human pDCs and B cells [35,47]. Type I IFNs play important roles in stimulating dendritic cell maturation and migration, and enhancing humoral immunity as well as modulating effector and memory T cell response [48]. Therefore, CpG-A, which can induce robust type I IFN response in vitro, may have potential benefits as vaccine adjuvant. However, therapeutic potential of CpG-A remained largely unexplored. Here we showed that CpG-A, which can stimulate high levels of human IFN-α production in vitro, did not induce significant levels of IFN-α in vivo in humanized mice (Figs. 1 and 2). CpG-A can form uncontrollable aggregation that complicates its manufacture [49,50]. Thus, one possible reason that CpG-A behaves poorly in vivo activity could be its highly aggregated structure, which may restrict its access to spleen, lymph node or bone marrow [51,52]. In contrast, CpG-B induced high levels of IFN-α in humanized mice in vivo (Fig. 2). In addition to stimulating IFN-α production, CpG-B also induced other cytokines and chemokines such as IL-6, IL-12, IFN-γ, and IP-10 in vivo in humanized mice. These results further support the development of CpG-B as vaccine adjuvant in clinical trials.

In comparison to CpG-A, the TLR7/8-L R848 was poor in inducing IFN-α, in vitro, both from human PBMCs and spleen cells from humanized mice. However, it stimulated fast, robust, and durable IFN-α production, in vivo, in humanized mice (Fig. 3A). In addition to CpG-B and R848, we report here that Poly I:C is also a potent human innate immune stimulator, in vivo, in humanized mice. Poly I:C treatment induced significant levels of IFN-α, in vivo, but not in vitro (Figs. 1–3). Most importantly, it stimulated much higher and more durable levels of IL-12 than CpG-B and R848, in vivo (Fig. 3B). Study has indicated that IL-12 plays an essential role for the development of antigen-specific $CD8^+$ T cell immune responses to vaccination in humans with cancer [53].

It will be of interest to explore the mechanisms by which TLR-Ls stimulate different cytokine production profiles, in vivo, compared to that in vitro. Lots of variables such as; physiological conditions, cell-cell interactions and dynamics, and accessibility of the drugs to the cells, might contribute to the differences observed here. Thus, the immunological effect of TLR-Ls, in vitro, cannot simply predict their activity in vivo. Using the humanized mouse model, we will further investigate how distinct TLR-Ls differentially stimulate the human innate immune response in vivo.

From a vaccine development state point, induction of robust, potent and durable CTL responses using a non-viral vector is challenging. We hypothesize that targeting antigens through a DC receptor combined with DC stimulators may be required to optimize T cell responses. We show here that Poly I:C and R848, which can significantly stimulate human mDCs, induced antigen-specific CTL response to a DC-targeting HIV vaccine. In contrast, CpG-B, which activated human pDCs but not mDCs, enhanced antigen-specific human $CD4^+$ helper T cell but not CTL response in humanized mice (Fig. 5). The results indicate that human pDCs activated by CpG-B did not cross-present antigen to $CD8^+$ T cells efficiently, although several reports showed that in vitro pDCs can cross-present soluble antigen [40,41]. Taken together, our results suggest that activation of human mDCs but not pDCs in vivo is important for inducing antigen-specific CTL response to protein-based vaccines. Our data is consistent with the result from human clinical trials, which show that CpG-B preferentially enhances antibody responses and $CD4^+$ T cell responses to the NY-ESO-1 protein [54]. Although $CD8^+$ T cell responses are also detected in some vaccinated people at later time points during vaccination, it is believed that the cross-presentation of antigen is predominantly mediated by vaccine-induced antibody [54].

The expression profile of several TLRs in mouse immune cells is different from that in human immune cells. It may lead to different adjuvant activity of TLR-Ls in mice and humans. For example, TLR-9 is widely expressed in nearly all myeloid cells in mice but it preferentially expressed in pDCs and B cells in humans. That explains why CpG-B has been used as a potent adjuvant to enhance CTL response in mice [45,46], which is different from our observation here in humanized mice. Humanized mice thus will provide a critical model to test human adjuvant activity of TLR-Ls and other novel adjuvants, in vivo, prior to clinical trials.

In conclusion, we demonstrate that different TLR-Ls distinctively activate human innate immune responses and enhance antigen-specific $CD4^+$ or $CD8^+$ T cell responses in humanized mice. The results will greatly help the development of novel human adjuvants in human clinical settings.

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Conflict of interest statement

G.Z., S.Z., and Y.L., are named inventors on CD40-targeting vaccine patents and patent filings held jointly by INSERM and the Baylor Research Institute.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vaccine.2017.09.052.

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