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Immune Complex-Mediated Cell Activation from Systemic Lupus Erythematosus and Rheumatoid Arthritis Patients Elaborate Different Requirements for IRAK1/4 Kinase Activity across Human Cell Types

Eugene Y. Chiang, Xin Yu, and Jane L. Grogan

IL-1R-associated kinases (IRAKs) are important mediators of MyD88-dependent signaling by the TLR/IL-1R superfamily and facilitate inflammatory responses. IRAK4 and IRAK1 function as active kinases and as scaffolds for protein–protein interactions. We report that although IRAK1/4 kinase activity is essential for human plasmacytoid dendritic cell (pDC) activation, it is dispensable in B, T, dendritic, and monocytic cells, which is in contrast with an essential active kinase role in comparable mouse cell types. An IRAK1/4 kinase inhibitor abrogated TLR7/9-induced IFN- α responses in both mouse and human pDCs, but other human immune cell populations activated via TLR7/9 or IL-1R were refractory to IRAK4 kinase inhibition. Gene ablation experiments using small interfering RNA demonstrated an essential scaffolding role for IRAK1 and IRAK4 in MyD88-dependent signaling. Finally, we demonstrate that autoimmune patient (systemic lupus erythematosus and rheumatoid arthritis) serum activates both pDC and B cells, but IRAK1/4 kinase inhibition affects only the pDC response, underscoring the differential IRAK1/4 functional requirements in human immune cells. These data reveal important species differences and elaborate cell type requirements for IRAK1/4 kinase activity. *The Journal of Immunology*, 2011, 186: 1279–1288.

Interleukin-1R-associated kinase 1 (IRAK1) and IRAK4 are essential for transduction of MyD88-dependent TLR and IL-1R signals that underscore a majority of innate and some adaptive immune responses (1). Recruitment of IRAK4 and IRAK1 to the MyD88-signaling complex elaborates NF- κ B-dependent inflammation (2, 3). MyD88 is also coupled to IFN regulatory factors (IRFs) that mediate differential expression of specific cytokines, depending on the TLR/IL-1R signaling pathway (4, 5). Studies with cells derived from human patients with inherited IRAK4 deficiency have shown that, in the absence of IRAK4, cytokine responses to MyD88-dependent signaling are ablated (6, 7). IRAK4-deficient patients also have defects in B cell central and peripheral tolerance, suggesting a role for IRAK4 in directing B cell responses (8).

How IRAKs facilitate TLR/IL-1R-mediated signaling across other human immune cell types is not well characterized. It is clear that although similarities in IRAK4 activity exist between human and mouse, there are important differences as well. Mice

deficient for IRAK4 are severely impaired in their cellular responses to IL-1, IL-18, and most TLR ligands, sharing an overlapping phenotype with IRAK4-deficient human patients (3). However, although IRAK4-deficient mice display broad susceptibility to viral and bacterial infections, IRAK4-deficient human patients exhibit a narrow infectious phenotype, limited primarily to pyogenic bacterial infections at an early age (6, 7). IRAK1 deficiency in humans has not been described. Intriguingly, however, IRAK1 has recently been identified as a susceptibility allele in human systemic lupus erythematosus (SLE), with a four-single nucleotide polymorphism haplotype showing association with disease (9, 10). SLE is characterized by the presence of immune complexes (ICs) formed by autoantibodies associated with self-DNA and RNA, increased serum IFN- α levels produced principally by plasmacytoid dendritic cells (pDCs), and an IFN- α -inducible gene signature (11, 12).

In current models of SLE pathogenesis, ICs bind to Fc γ Rs expressed on the surface of pDCs, and subsequent internalization of ICs allows DNA to associate with TLR9 and promote IFN- α production by pDCs (13, 14) via a pathway tightly regulated by IRF-7 (15). Increased levels of IFN- α further induce type I IFN-regulated genes, eliciting an inflammatory feedback loop. This IFN- α gene signature is associated with SLE pathogenesis and is also observed in a subpopulation of rheumatoid arthritis (RA) (11, 16). In addition, these ICs can bind to the BCR of autoreactive B cells, resulting in BCR-mediated signaling and endocytosis. Within the endosome, CpG DNA engages TLR9, initiating an MyD88-dependent signaling cascade that leads to B cell activation, proliferation, and differentiation into autoantibody-producing cells. Studies in mouse autoimmune disease models have shown that ICs trigger activation of pDC and autoreactive B cells via TLR7/9 signaling pathways (12, 17). IRAK1, being a key component of the TLR7/9 signaling cascade, participates in elaboration of lupus development as elucidated through the use of IRAK1-deficient mice crossed to SLE-prone mice (9).

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E.Y.C. performed plasmacytoid dendritic cell (DC), B cell, monocyte, conventional DC, and T cell in vitro experiments and contributed to preparation of the manuscript. X.Y. performed experiments on plasmacytoid DC and conventional DC responses. J.L.G. supervised the project and drafted the manuscript.

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Abbreviations used in this article: DC, dendritic cell; IC, immune complex; IRAK, IL-1R-associated kinase; IRF, IFN regulatory factor; KD, kinase-dead; KO, knock-out; pDC, plasmacytoid dendritic cell; RA, rheumatoid arthritis; RF, rheumatoid factor; siRNA, small interfering RNA; SLE, systemic lupus erythematosus; SS, Sjögren's syndrome.

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IC-mediated IFN- α production by pDCs is a key driver of SLE, and IRAK1 and IRAK4 are both essential for regulation of the pDC IFN- α response. IRAK4 kinase activity phosphorylates and activates IRAK1, which, in turn, phosphorylates and activates IRF-7 (18). Given the proximal positions of IRAK1 and IRAK4 in the TLR9 signaling pathway, targeted inhibition of IRAK1/4 kinase activity is attractive for blockade of IFN- α -mediated pathology. However, IRAK1 and IRAK4 have dual roles as active kinases and scaffolding proteins, and the contributions of each to signaling in mouse versus human, as well as in different cell types, is poorly understood. Although IRAK4 kinase activity has been shown to be required for pDC (19) and B cell (20) responses to TLR stimulation, as well as Th17 cell differentiation (21) through the use of IRAK4 kinase-dead (KD) mutant knock-in mice, the observed IRAK4 deficiencies in human patients do not allow discrimination between kinase and scaffold function. Similarly, the use of IRAK1 knockout (KO) mice leaves the kinase-specific role of IRAK1 in various signaling pathways unresolved. In addition, translation of mouse studies to human disease is hindered by the broader expression pattern of TLR9 in mouse versus human cells. Although TLR9 is expressed only by pDCs and B cells in humans, mice also express TLR9 in myeloid dendritic cells (DCs), monocytes, and macrophages.

In this study, we explored the role of IRAK1/4 in TLR/IL-1R signaling in multiple human cell types, and investigate the role of IRAK1/4 in the activation of pDC and B cells by SLE and RA patient serum IC. Our study reveals important species differences in the cellular requirements for IRAK1/4 kinase activity that should be taken into account in the context of IC-mediated autoimmune responses.

Materials and Methods

Subjects

Serum samples were collected from SLE patients under treatment at the Ann Arbor Veterans Affairs hospital with active disease at the time of blood donation. Serum samples for the RA cohort were obtained from patients fulfilling the 1987 American College of Rheumatology criteria for RA (Cureline Human Biospecimens). Clinical features of the SLE and RA patient cohorts are described in Supplemental Table I. Serum from healthy control donors was collected as part of the Genentech blood donor program, with written informed consent and approval from the Western Institutional Review Board.

Measurement of autoantibodies in patient sera

Serum samples were analyzed using the QUANTA Plex SLE Profile 8 fluorescent immunoassay (INOVA Diagnostics). Semiquantitative measurements were made for IgG Abs against chromatin, ribosome-P, ribonucleoprotein, Smith (Sm), Sjögren's syndrome (SS)-A 60, SS-A 52, and SS-B. Serum samples were run on a Luminex 100 IS Flow Cytometer System (Luminex Corporation). Results were calculated as recommended by assay instructions and reported as reactivity in Luminex units, and are proportional to the amount of Ab present; values >20 are classified as positive. Abs against dsDNA were measured by using QUANTA Lite dsDNA ELISA (INOVA Diagnostics). Results were calculated as specified by the manufacturer's protocol and reported as IUs (IU/ml). Values >200 IU/ml are classified as positive. Anti-rheumatoid factor (RF)-IgM, anti-RF-IgG1, and anti-RF-IgA Abs and Abs against CCP3 were similarly measured using appropriate QUANTA Lite ELISA kits (INOVA Diagnostics). Anti-RF Ab levels were classified as positive if >6 U; anti-CCP3 levels were classified as positive if >20 U.

Small molecule inhibitors

IRAK1/4 inhibitor *N*-(2-morpholinylethyl)-2-(3-nitrobenzoylamido)-benzimidazole, a cell-permeable compound that potently and selectively inhibits IRAK1 and IRAK4 kinases, was purchased from EMD Biosciences. IRAK1/4 inhibitor showed minimal activity against a panel of 50 other kinases in independent kinase inhibition assays using Invitrogen's SelectScreen Profiling Service. A PI3Kp110 δ small molecule inhibitor

(22) was demonstrated to have high potency and selectivity (K. Reif, unpublished data). All inhibitors were reconstituted in DMSO, aliquoted, and stored at -20°C . Aliquoted inhibitor was thawed once and unused portion was discarded. As positive controls for biological activity of inhibitors, pDCs were assayed in parallel. In addition, human and mouse experiments were performed concurrently. Inhibitor IC₅₀ values and curve fits were determined with Kaleidagraph version 3.6 (Synergy Software) using a four-parameter curve fit formula: $M1 + (M2 - M1)/(1 + (M0/M4)^{M3})$, where M1 = minimum of curve, M2 = maximum of curve, M3 = 1, M4 = estimated IC₅₀.

Cell purification and activation

Blood samples from healthy donors were collected after informed consent was provided and ethical approval granted from the Western Institutional Review Board. Purified human pDC, B cell, conventional DC, monocyte, and T cell populations were obtained using magnetic bead separation kits (Miltenyi Biotec), according to the manufacturer's instructions. Mouse single-cell suspensions were prepared from spleens harvested from 8- to 10-wk-old female C57BL/6 mice. Animals were from The Jackson Laboratory and were maintained in accordance with American Association of Laboratory Animal Care guidelines. All experimental animal studies were conducted under the approval of the Institutional Animal Care and Use Committees of Genentech Lab Animal Research. Specific cell populations were purified using appropriate magnetic bead separation kits (Miltenyi Biotec). Cells were cultured in complete DMEM or complete RPMI media (DMEM or RPMI 1640 media supplemented with 10% FBS, 2 mM glutamine, 55 μM 2-ME, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin).

For pDC activation, 5×10^4 to 2.5×10^5 cells were stimulated with TLR7 agonist Gardiquimod (InvivoGen) at 4 $\mu\text{g}/\text{ml}$, TLR9 agonist CpG-A ODN2216 (InvivoGen) at 500 nM or serum for 40 h, in the absence or presence of inhibitor. For B cell activation, $1-5 \times 10^5$ cells were stimulated with TLR9 agonist CpG-A ODN2216 at 500 nM, TLR9 agonist CpG-B ODN2006 (InvivoGen) at 5 μM , or serum, in the absence or presence of inhibitors. For cytokine analysis and proliferation assays, B cells were cultured for 3 d. Supernatants from wells were harvested, and wells were replenished with 50 $\mu\text{l}/\text{well}$ media containing 1 μCi [^3H]thymidine (Perkin-Elmer). [^3H]Thymidine incorporation was measured 18 h later by liquid scintillation counting. For isotype switching and plasma cell induction, B cells were cultured for 6-7 d. For DC and monocyte activation, $1-2.5 \times 10^5$ cells were stimulated with 50 ng/ml IL-1 β (R&D Systems), 100 ng/ml IL-18 (MBL), or 20 $\mu\text{g}/\text{ml}$ LPS (Sigma) for 40 h, in the absence or presence of inhibitor.

Human and mouse Th1 and Th17 cell polarization was performed as previously described (23). For Th1 cell restimulation, $2-5 \times 10^5$ resting Th1 cells were activated with 10 ng/ml IL-12 or 100 ng/ml IL-18 alone, or in combination, for 24 h. For Th17 cell restimulation, $2-5 \times 10^5$ resting Th17 cells were activated with 10 ng/ml IL-23 or 50 ng/ml IL-1 β alone, or in combination, for 24 h. Supernatants were collected; then [^3H]thymidine was added for an additional 18 h to measure proliferation.

Cytokine and Ig isotype quantitation

Cytokine concentrations were measured in cell culture supernatants with ELISA kits specific for IFN- α (PBL Biomedical Laboratories), IL-6 (eBioscience or R&D), TNF- α (eBioscience or R&D), IFN- γ (BD Biosciences or R&D), and IL-17 (R&D Systems), or with LINCoplex beads (Linco Research) using the Luminex 100 system for analysis according to the manufacturer's recommended protocol (Luminex). Ig isotype concentrations were similarly measured by Luminex.

Flow cytometry

Plasmablast induction was performed using purified B cells stimulated with either 5 μM CpG-B ODN2006 (InvivoGen, San Diego, CA), CpG-B and 200 U/ml IFN- α (Sigma), or patient serum. After 7 d, cells were incubated with Fc block followed by staining with PE anti-CD19 and allophycocyanin anti-CD38 Abs (BD Biosciences). Plasmablasts were gated as CD19 $^+$ CD38 $^+$ cells. Samples were acquired on a FACSCalibur flow cytometer using CellQuest Pro v5.1.1 software (BD Biosciences), and data analysis was performed using FlowJo v6.4.2 software (Tree Star).

For detection of total IRF-7, phospho-IRF-7, and phospho-NF- κB p65, cells were fixed with BD Cytofix buffer and then permeabilized with BD Phosflow Perm Buffer III. Total IRF-7 was detected using PE mouse anti-IRF-7 (clone K40-321; BD Biosciences), phospho-IRF-7 with PE mouse anti-IRF-7 (pS477/pS479; clone K47-671; BD Biosciences), and phospho-NF- κB p65 with PE anti-NF- κB p65 (pS529; clone K10-895.12.50; BD Biosciences).

Small interfering RNA knockdown, RNA isolation, and quantitative real-time RT-PCR

ON-TARGETplus SMARTpool small interfering RNA (siRNA; IRAK1, L-004760-00-0010; IRAK4, L-003302-00-0010; negative control pool, D-001810-10) were from Dharmacon. Cell populations were transfected with siRNA (300 nM final concentration) using appropriate Nucleofector Kit (Amaxa) according to manufacturer's recommended protocol. RNA was isolated from siRNA transfected cells using RNeasy Mini Kit (Qiagen). TaqMan real-time quantitative RT-PCR was performed using the ABI 7500 Real-Time PCR system (Applied Biosystems). TaqMan Gene Expression Assay primer/probe sets for IRAK1 (Hs00155570_m1) and IRAK4 (Hs00211610_m1) were from Applied Biosystems. Data were normalized to ribosomal protein L19 expression values.

Statistics

Statistical analyses were performed using JMP version 6.0.2 software (SAS Institute). We made comparisons for each pair with Student *t* test, with *p* values <0.05 considered significant.

Results

Human pDC, but not B cell, responses to TLR9 signals depend on IRAK1/4 kinase activity

Human and mouse pDCs produce IFN- α in response to stimulation with CpG via triggering of TLR9 and TLR7. IFN- α expression requires IRF-7, whose activation and nuclear translocation is dependent on IRAK1/4 kinase activity (15, 18, 24). In concordance, a specific dual-IRAK1/4 kinase inhibitor potently inhibited both TLR9- and TLR7-mediated IFN- α production from human pDCs stimulated with cognate agonists (Fig. 1A).

As B cells from IRAK4 KD mice have defects in TLR9-triggered responses (20), we tested whether the IRAK1/4 kinase inhibitor would inhibit human B cell responses to CpG. TLR9 agonist CpG-B ODN2006 induced plasma cell differentiation, and the IRAK1/4 inhibitor failed to block the TLR9-mediated generation of plasmablasts (Fig. 1B, 1C). In contrast, inhibition of PI3K catalytic subunit p110 δ , which has a role in TLR-mediated signaling in B cells (25), completely abrogated plasmablast differentiation (Fig. 1B, 1C). Furthermore, human B cell proliferation and cytokine production in response to CpG-B stimulation were not affected by the IRAK1/4 inhibitor, whereas these B cell responses were inhibited by PI3Kp110 δ inhibitor in a dose-dependent manner (Fig. 1D, 1E). Isotype switching after activation with CpG-B was similarly unaffected by IRAK1/4 inhibitor (Supplemental Fig. 1). In contrast, mouse B cell responses to CpG-B were effectively blocked by the IRAK1/4 inhibitor (Fig. 1F, 1G), consistent with observations in IRAK4 KD mice (20). CpG-A stimulation elicited weak proliferative and cytokine responses from both human and mouse B cells (data not shown). We conclude that human B cell responses to TLR9 stimulation do not require IRAK1/4 kinase activity, in contrast with the mouse where TLR9-induced B cell responses are highly dependent on IRAK1/4.

IRAK1/4 kinase activity is not required for TLR9-mediated activation of NF- κ B in human B cells

CpG-A and CpG-B are known to activate different TLR9-mediated signaling pathways in pDCs (26–28). Multimeric CpG-A, which engages TLR9 in the early endosome compartment, induces IRAK1/4 kinase-dependent downstream phosphorylation and activation of IRF-7, resulting in IFN- α production (15, 24). Monomeric CpG-B binds TLR9 in late endosomes/lysosomes, resulting in activation of signaling pathways that include NF- κ B. Early versus late endosome pathway signaling was assessed using Abs specific for phospho-IRF-7 and phospho-NF- κ B p65. Human pDCs were confirmed to constitutively express IRF-7, and phosphorylation of IRF-7 was detectable within 1 h of CpG-A-, but not

CpG-B-mediated activation (Fig. 2A). The IRAK1/4 inhibitor efficiently blocked IRF-7 phosphorylation. In contrast, the IRAK1/4 inhibitor had no effect on CpG-A- and CpG-B-induced NF- κ B p65 phosphorylation (phospho-NF- κ B p65 mean fluorescence intensity = 52 for CpG-B and 29 for CpG-A). Activation of the NF- κ B pathway in pDCs by CpG-A and CpG-B was demonstrated by production of IL-6 and TNF- α (Supplemental Fig. 2).

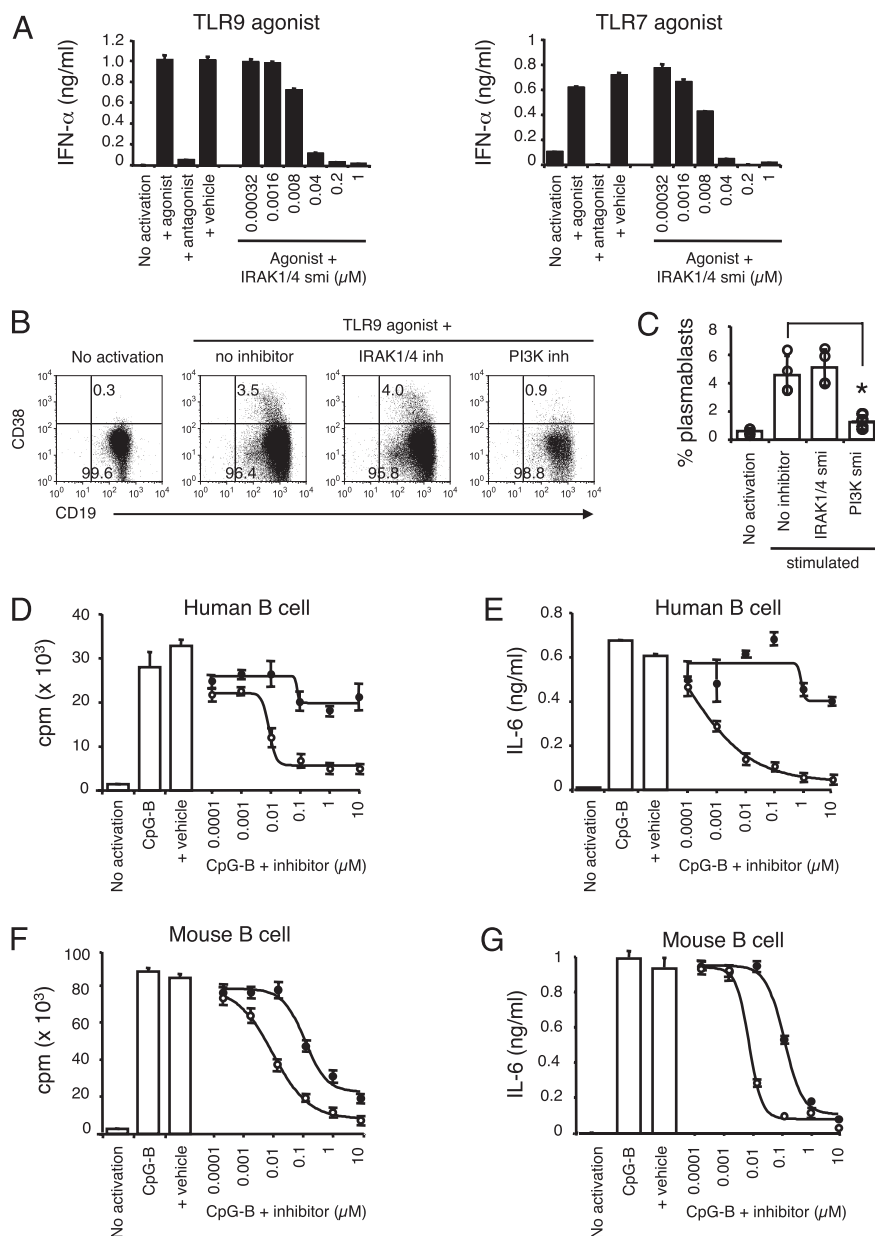
In TLR9-stimulated human B cells, phosphorylation of IRF-7 was not detected despite weak upregulation of IRF-7 expression (Fig. 2B). In addition, although both CpG-A and CpG-B induced NF- κ B p65 phosphorylation (phospho-NF- κ B p65 mean fluorescence intensity equals 142 for CpG-B and 93 for CpG-A), only the PI3Kp110 δ inhibitor reduced NF- κ B p65 phosphorylation induced by CpG-B stimulation, whereas the IRAK1/4 inhibitor showed no activity in the assay; neither had an effect on CpG-A-mediated activation. We conclude that the IRF-7 pathway does not appear to be important in TLR9-mediated signaling in human B cells, and the NF- κ B pathway does not require IRAK1/4 kinase activity.

To distinguish the roles of the kinase domain as compared with the scaffolding function of IRAK1 and IRAK4 in human B cells, we used siRNA knockdown of gene expression. Targeted siRNA knockdown of IRAK1 reduced mRNA levels by at least 65% in B cells under various stimulation conditions (Fig. 2C), whereas IRAK4 was reduced by ~55% (Fig. 2D), and the knockdown was specific for each kinase. Reduction of either IRAK1 or IRAK4 expression significantly impaired (*p* < 0.001) proliferation (Fig. 2E) and cytokine (Fig. 2F) responses to CpG-B stimulation, suggesting that the nonkinase roles (e.g., scaffolding function) of IRAK1 and IRAK4 are necessary for downstream signaling pathways, such as those involving NF- κ B, in human B cells.

Activation of B cells by autoimmune patient serum does not require IRAK1/4 kinase activity

SLE patient serum contains stimulatory ICs that elicit production of IFN- α by pDCs (13, 14); however, the roles of IRAK1 and IRAK4 in IC-mediated B cell responses are unknown. Given the differential roles of IRAK1/4 observed in human pDCs compared with B cells stimulated with TLR9 agonists, we tested whether IRAK1/4 kinase activity inhibition would similarly impair IC-mediated activation of human cells. As previously reported (13, 14), SLE patient sera, despite their heterogeneous IC content profiles, induced IFN- α production from normal donor pDCs (Fig. 3A, Table I). Treatment of pDCs with IRAK1/4 kinase inhibitor strongly inhibited the IFN- α response, as expected (Fig. 3A). The abrogation of pDC IFN- α production by the IRAK1/4 inhibitor was comparable with the effects of chloroquine (data not shown), an inhibitor of endosomal acidification and TLR activation (14). When normal donor B cells were incubated with SLE serum, plasmablast differentiation was induced, as previously observed after CpG-B stimulation (Fig. 3B, Table I). Treatment with the PI3Kp110 δ inhibitor prevented plasmablast differentiation, but blockade of the kinase activity of IRAK1/4 with the dual inhibitor had no effect. Normal donor sera, which had no measurable IC content, did not activate pDCs or B cells. Although most SLE patient serum samples activated pDC (10/12) and B cells (12/12), we did not find correlation with any specific autoantibody or clinical parameters. Furthermore, RA patient sera containing anti-nuclear autoantibodies in addition to RFs and anticyclic citrullinated peptide were found to have immunostimulatory effects on pDCs and B cells that were also differentially affected by IRAK1/4 kinase inhibitor (Supplemental Fig. 3, Supplemental Table II). Similar to the SLE data, RA serum-mediated effects did not correlate with autoantibody content or other factors.

FIGURE 1. Effects of IRAK1/4 inhibitor on pDC and B cell responses to CpG stimulation. **A**, pDC IFN- α response to activation with TLR9 or TLR7 agonist. pDCs were stimulated with TLR9 agonist CpG-A ODN2216 (*left panel*) or TLR7 agonist gardiquimod (*right panel*) in the absence or presence of TLR inhibitor (ODNTTAGGG for TLR9, chloroquine for TLR7) or indicated concentration of IRAK1/4 inhibitor. IRAK1/4 inhibitor IC_{50} = 12.8 nM for TLR9 inhibition; IC_{50} = 10.0 nM for TLR7 inhibition. Data are shown as mean \pm SD of triplicate measurements from one experiment and are representative of data from ≥ 3 donors. **B** and **C**, Plasmablast induction in B cells after 7-d stimulation with TLR9 agonist CpG-B ODN2006 in absence or presence of IRAK1/4 or PI3K p110 δ inhibitor (1 μ M). **B**, Plasmablasts are gated as CD19 $^{+}$ CD38 $^{+}$ cells; numbers denote percentage of cells in respective quadrant. **C**, Effect of inhibitors on plasmablast induction. Bar denotes mean \pm SD of four donors; circles represent individual donors. **D–G**, B cell responses to TLR9 agonist CpG-B ODN2006 stimulation in absence or presence of IRAK1/4 (filled circles) or PI3K p110 δ inhibitor (open circles). **D**, Proliferation response of human B cells. **E**, IL-6 response of human B cells. All data are shown as mean \pm SD of duplicate measurements. Each experiment was performed with three different donors. **F** and **G**, Effect of IRAK1/4 inhibitor on mouse B cell proliferation (**F**) and cytokine production (**G**) in response to CpG-B stimulation. Data are shown as mean \pm SD of duplicate measurements and are representative of three separate experiments. * p < 0.0001.



IL-1R/IL-18R signaling in human cells requires IRAK1/4, but kinase activity is dispensable

Given the evidence that IRAK1/4 kinases have different roles in human pDCs as compared with B cells, and that differences were also observed between human and mouse B cells, we next sought to determine the roles of IRAK1/4 in other human and mouse cell MyD88-dependent responses. In addition to TLR signaling, the IRAK1/4 pathway also mediates signaling downstream of IL-1R/IL-18R, potentiating diverse immune responses (29). Human conventional DCs and monocytes stimulated with IL-1 β or IL-18 proliferated in response to proinflammatory cytokines such as TNF- α , and these responses were not affected by the presence of IRAK1/4 inhibitor (Fig. 4A, 4B). The effects of the IRAK1/4 inhibitor at high concentrations (>2.5 μ M) are well above the nanomolar potency observed in pDCs; therefore, we cannot rule out possible off-target, nonspecific effects at these high concentrations. In contrast with human cells, mouse CD11c $^{+}$ DC and CD11b $^{+}$ monocyte/macrophage responses to IL-1 β or IL-18 were suppressed by IRAK1/4 kinase inhibitor in a dose-dependent manner (Fig. 4C, 4D).

Because the IRAK1/4 kinase inhibitor did not impair human monocyte or DC responsiveness to IL-1R/IL-18R signaling, siRNA knockdown of either IRAK1 or IRAK4 was performed to determine whether nonkinase functions were important. siRNA knockdown in monocytes reduced IRAK1 expression by $\sim 75\%$ and IRAK4 expression by $\sim 65\%$ (Supplemental Fig. 4A). The siRNA-mediated reductions of either IRAK1 or IRAK4 impaired the ability of monocytes to produce TNF- α in response to IL-1 β (Supplemental Fig. 4B), indicating that although their kinase activities are dispensable, both proteins are necessary for signaling through MyD88-dependent pathways.

Th1 and Th17 cells are also responsive to synergistic activation with IL-18 and IL-12 or IL-1 β and IL-23, respectively (30, 31). As with myeloid cells, IRAK1/4 kinase inhibitor had minimal impact on IL-1R/IL-18R-induced activation of human Th1 or Th17 when administered at submicromolar concentrations (as defined by proliferation and IFN- γ or IL-17 production; Fig. 5A, 5B), but did have an effect on mouse Th1 or Th17 cells (Fig. 5C, 5D). To investigate the nonkinase roles of IRAK1/4 in human Th cells, resting polarized Th1 and Th17 cells were transfected with IRAK1

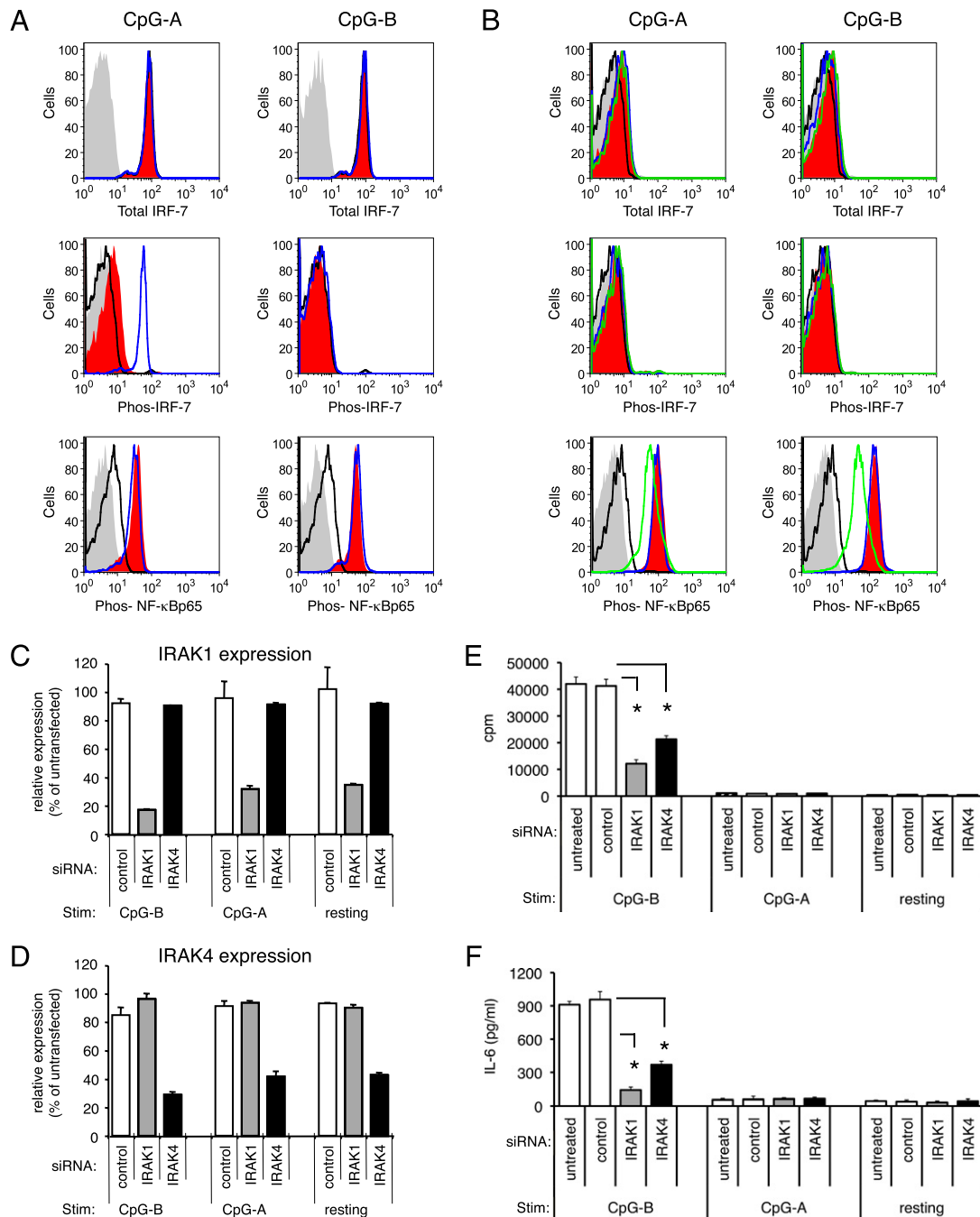


FIGURE 2. Human B cell responses to TLR9 agonist require IRAK1/4 kinase activity-independent activation of NF- κ B. TLR9 stimulation of B cells activates NF- κ B, but not IRF-7, signaling pathway. Flow cytometry of pDC (A) or B cells (B) activated with TLR9 agonist CpG-A ODN2216 or CpG-B ODN2006. Staining for total IRF-7 and phospho-IRF-7 was performed after 1-h culture in absence or presence of inhibitors. Staining for phospho-NF- κ Bp65 was performed after 30-min culture. Blue line histograms represent stimulation in absence of inhibitors; red shaded histograms represent stimulation in presence of IRAK1/4 inhibitor; green line histograms represent stimulation in presence of PI3K p110 δ inhibitor; black line histograms represent basal expression level in resting cells; gray shaded histograms represent isotype control staining. Data are representative of experiments using three different donors. Effects of siRNA knockdown of IRAK1 or IRAK4 expression on B cell responses to TLR9 stimulation. IRAK1 (C) and IRAK4 (D) expression was measured in resting B cells or B cells stimulated with CpG-A or CpG-B 4 d after transfection with IRAK1 (gray bars) or IRAK4 (black bars) siRNA; white bars represent transfection with negative control pool siRNA. Relative expression of targeted genes is shown as a percentage of expression levels of untransfected cells (100%). E, Effects of IRAK1, IRAK4 siRNA on B cell proliferation responses. F, Effects of IRAK1, IRAK4 siRNA on B cell IL-6 production. C–F, Data are mean \pm SD of triplicate measurements and are representative of three different donors. * $p < 0.001$.

or IRAK4 siRNA. siRNA targeting of IRAK1 reduced IRAK1 mRNA levels by $\sim 65\%$ in Th1 and Th17 cells, whereas IRAK4-targeted knockdown reduced IRAK4 expression by $>90\%$ (Fig. 6A, 6D). Knockdown efficiency was stable, with comparably reduced levels observed 3 d after stimulation. Th1 cells restimulated with IL-18 plus IL-12 were significantly impaired ($p < 0.001$) in

their proliferation response when IRAK1 or IRAK4 expression was reduced (Fig. 6B), as was Th17 cell proliferation after IL-1 β plus IL-23 stimulation (Fig. 6E); cytokine production was even more profoundly affected (Fig. 6C, 6F). Thus, reduced expression of IRAK1 and IRAK4, but not inhibition of kinase activity, inhibits the ability of Th1 and Th17 cells to respond to IL-1R/IL-

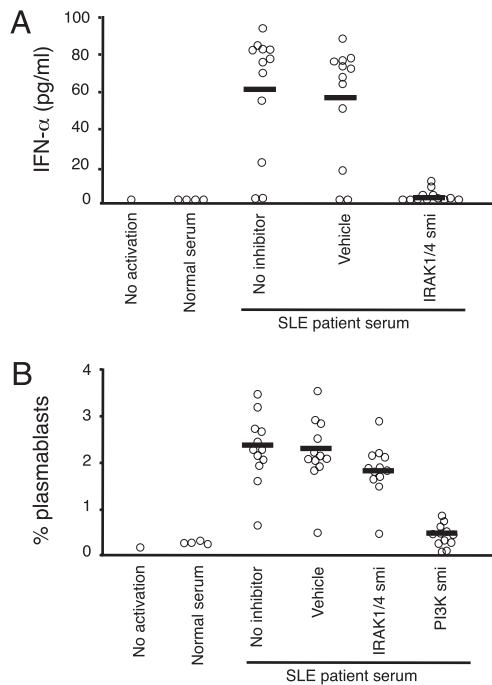


FIGURE 3. Effects of IRAK1/4 inhibitor on pDC and B cell responses to SLE serum-mediated stimulation. *A*, pDC IFN- α response to stimulation with SLE patient sera. pDCs were cultured with serum in absence or presence of 10 μ M IRAK1/4 inhibitor. Each circle represents IFN- α production induced by an individual patient ($n = 12$) or healthy control donor ($n = 4$) performed in parallel. Individual data points are technical means of duplicate measurements. Data are representative of at least two experiments. *B*, B cells were cultured with SLE patient serum in the absence or presence of 1 μ M IRAK1/4 inhibitor or PI3K p110 δ inhibitor. Percentage of cells having the CD19⁺CD38⁺ plasmablast phenotype was determined. Each circle represents an individual patient ($n = 12$) or healthy control donor ($n = 4$) performed in parallel. Data are representative of at least two experiments. Bars represent the mean.

18R-mediated signaling, suggesting that the kinase-independent roles of IRAK1 and IRAK4 are important.

Discussion

We report that IRAK1/4 kinase activity in human cell populations is restricted to the IRF-7-dependent pathway specifically activated

in pDCs, but both IRAK1 and IRAK4 play important nonkinase functions (e.g., adaptor, scaffolding roles) in non-pDC cell responses to MyD88-dependent signaling. The differential functional roles of IRAK1/4 in human cells are in direct contrast with the mouse system, where TLR9/IL-1R/IL-18R signaling pathways in all cell types examined are dependent on IRAK1/4 kinase activity. These studies contribute to a greater understanding of IRAK roles in various MyD88-dependent signaling pathways, including activation of TNFR-associated factor 6 and downstream activation of NF- κ B and MAPK (2, 3), as well as activation of IRF-7-mediated pDC IFN- α responses (4, 5, 15, 32).

Dissection of IRAK1/4 kinase versus scaffold function, using a synthetic kinase inhibitor or gene ablation by siRNA, revealed an important functional dichotomy between human and mouse requirements for IRAK1/4 kinase activity. IRAK4 KO mice are severely impaired in their cellular responses to IL-1, IL-18, and most TLR ligands, sharing a similar phenotype as MyD88-deficient mice (3). IRAK1 KO mice, in contrast, have only partial defects in responses to TLR/IL-1R/IL-18R-mediated signaling, with significant impairments observed only under certain situations such as TLR9 activation of pDCs (32–36). These gene-deficient mice, however, do not allow discrimination of kinase versus nonkinase functions. IRAK4 KD knock-in mice have revealed an essential role for IRAK4 kinase activity in pDC IFN- α and macrophage responses to IL-1 β and TLR7 (19, 20, 37), whereas its role in T cell responses is unclear because of conflicting data (20, 38–40). Because IRAK1 KD knock-in mice have not been reported, the kinase-specific role of IRAK1 in various signaling pathways is unresolved. However, overexpression of kinase inactive IRAK1 mutant in fibroblasts or T cells does not impair IL-1 β -stimulated activation of the NF- κ B pathway, indicating that IRAK1 kinase activity may be dispensable for IL-1R signaling in certain cell types (41, 42). In this study, we demonstrated that polarized mouse Th1 and Th17 cells are dependent on IRAK1/4 kinase activity for IL-1R/IL-18R-mediated signaling, but the dual IRAK1/4 kinase inhibitor does not discern the individual requirements of IRAK1 or IRAK4, or both.

In contrast with mouse cells, human non-pDC cell types do not require IRAK1/4 active kinase function. However, kinase-independent functions are required because siRNA-mediated silencing of IRAK1 or IRAK4 impairs non-pDC cellular responses. Our data support the limited data reported using human IRAK-deficient cells. Kinase activities of IRAK1 and IRAK4 were in-

Table I. SLE patient serum stimulation of pDC IFN- α response and B cell plasma cell induction

Patient ID	IFN- α (Mean \pm SD, pg/ml)	Plasma Cell Induction (% Plasma Cells)	Chromatin (LU)	Ribosome-P (LU)	RNP (LU)	Sm (LU)	SS-A 60 (LU)	SS-A 52 (LU)	SS-B (LU)	dsDNA (IU/ml)
SLE 1	20.0 \pm 3.5	0.63	41	9	164	91	230	156	116	<1.o.d.
SLE 2	54.5 \pm 1.2	3.16	146	10	201	178	118	7	62	11
SLE 3	0.91 \pm 0.01	3.44	314	184	6	30	14	17	6	378
SLE 4	82.3 \pm 2.5	2.25	11	8	5	5	200	184	63	103
SLE 5	81.7 \pm 0.2	2.7	585	233	97	66	12	7	7	379
SLE 6	75.2 \pm 1.1	1.91	95	110	81	29	6	7	6	270
SLE 7	93.3 \pm 0.5	2.42	159	29	5	14	24	25	6	303
SLE 8	82.0 \pm 0.8	2.13	16	9	6	27	5	6	6	66
SLE 9	69.4 \pm 0.9	2.04	503	13	5	23	315	75	12	172
SLE 10	77.1 \pm 0.9	2.25	68	10	6	6	6	7	10	30
SLE 11	84.2 \pm 0.7	2.64	278	9	18	17	258	323	15	240
SLE 12	0.5 \pm 0.2	1.58	316	8	8	21	204	181	294	273
Normal 1	<1.o.d.	0.37	<1.o.d.	<1.o.d.	<1.o.d.	<1.o.d.	<1.o.d.	<1.o.d.	<1.o.d.	<1.o.d.
Normal 2	<1.o.d.	0.37	<1.o.d.	<1.o.d.	<1.o.d.	<1.o.d.	<1.o.d.	<1.o.d.	<1.o.d.	<1.o.d.
Normal 3	<1.o.d.	0.54	<1.o.d.	<1.o.d.	<1.o.d.	<1.o.d.	<1.o.d.	<1.o.d.	<1.o.d.	<1.o.d.
Normal 4	<1.o.d.	0.26	<1.o.d.	<1.o.d.	<1.o.d.	<1.o.d.	<1.o.d.	<1.o.d.	<1.o.d.	<1.o.d.

1.o.d., limit of detection; LU, Luminex units; RNP, ribonucleoprotein; Sm, Smith; SS, Sjögren's syndrome.

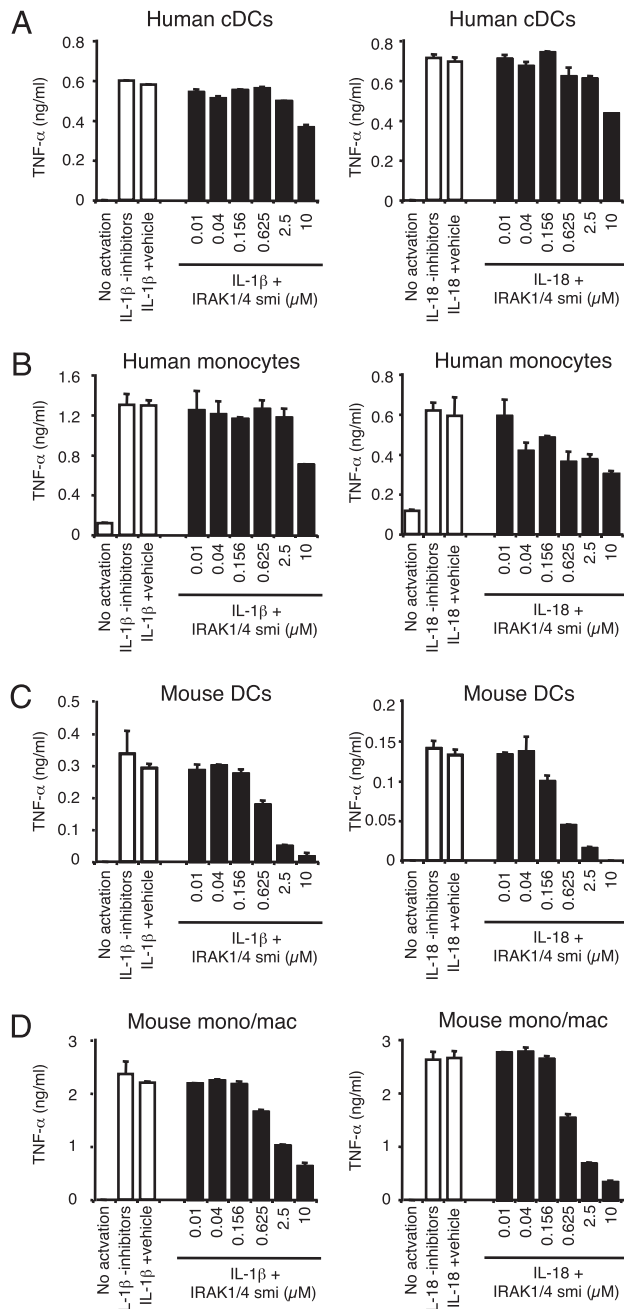


FIGURE 4. Effects of IRAK1/4 inhibitor on IL-1R or IL-18R signaling in DCs and monocytes. TNF- α production by human conventional DCs (cDCs) (A) or monocytes (B) stimulated with IL-1 β or IL-18 in the presence of indicated concentration of IRAK1/4 inhibitor was measured after 40-h culture. Data are shown as mean \pm SD of duplicate measurements. Each experiment was performed with three different donors. Mouse CD11c⁺ DCs (C) and CD11b⁺ monocytes/macrophages (D) were similarly treated with IL-1 β or IL-18. Error bars denote SD of duplicate measurements. Data are representative of two different donors.

dividually dispensable for IL-1 β or TLR8 signaling, suggesting that although their scaffolding adaptor functions were essential, their kinase roles were redundant (43, 44). Furthermore, siRNA silencing of IRAK1 or IRAK4 in HUVEC cells followed by complementation with kinase-inactive mutants demonstrated that the scaffolding function of IRAK4, but not its kinase activity, was required for IL-1 β -induced cytokine production (45). Ablation of IRAK1 expression had no effect on IL-1 β -mediated responses but did impact responses to TNF- α stimulation.

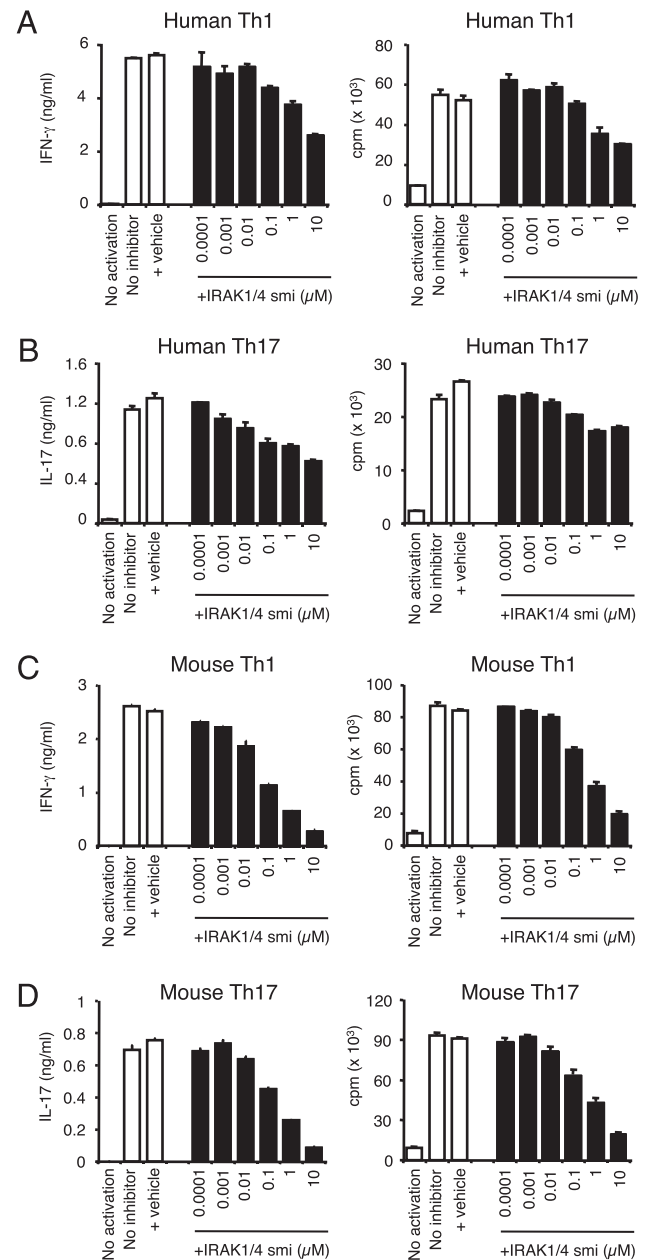


FIGURE 5. Effects of IRAK1/4 inhibitor on IL-1R or IL-18R signaling in Th1 and Th17 cells. A, IRAK1/4 inhibitor effects on human Th1 responses to IL-18 stimulation. IFN- γ production (left panel) and proliferation response (right panel) by resting Th1 cells restimulated with IL-12 plus IL-18 for 24 h. B, IRAK1/4 inhibitor effects on Th17 responses to IL-1 β stimulation. IL-17 production (left panel) and proliferation response (right panel) by Th17 cells restimulated with IL-23 plus IL-1 β . A and B, Data are shown as mean \pm SD of duplicate measurements. Each experiment was performed with polarized Th cells derived from three different donors. C and D, IRAK1/4 inhibitor effects on mouse polarized Th1 (C) or Th17 (D) cells restimulated with IL-12 plus IL-18 or IL-23 plus IL-1 β , respectively. Data are representative of two experiments, with error bars denoting SD of duplicate measurements.

Disparate use of IRAK1/4 kinase activity by different human cell types in response to TLR9 agonists may be caused by compartmentalization of the CpG type used for stimulation. CpG-A and CpG-B are known to activate different TLR9-mediated signaling pathways in pDCs (26–28). In pDCs, multimeric complexes of CpG-A, which induce IFN- α production, are localized to early endosomes together with IRF-7 (24, 28). In contrast, monomeric

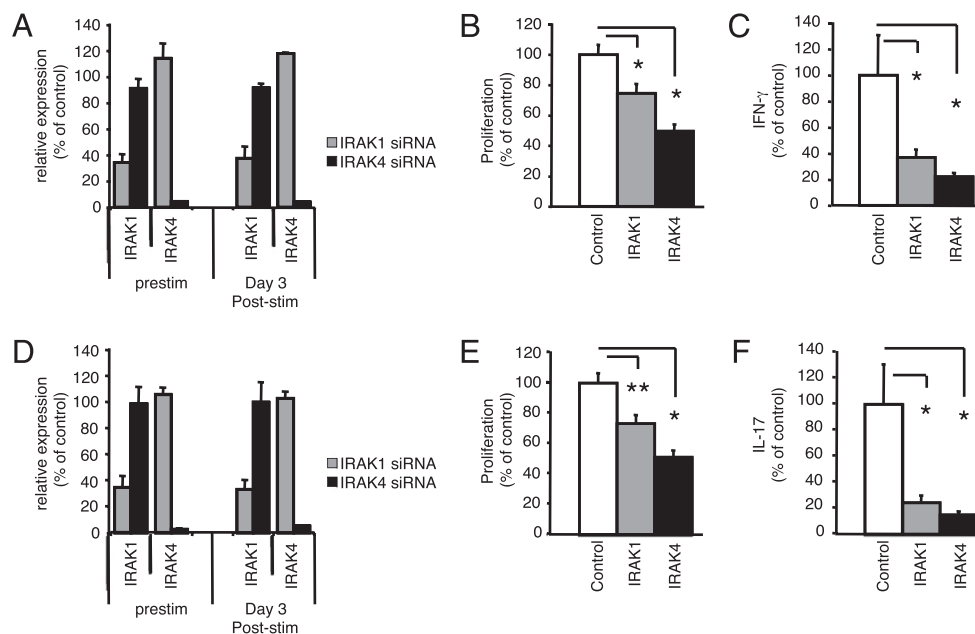


FIGURE 6. Effects of siRNA knockdown of IRAK1 or IRAK4 expression on IL-1R or IL-18R signaling in human Th1 and Th17 cells. **A**, Reduction of IRAK1 and IRAK4 mRNA expression by siRNA in Th1 cells. Resting polarized Th1 cells were transfected with IRAK1 or IRAK4 siRNA and then restimulated with IL-18 plus IL-12. IRAK1 and IRAK4 expression was measured before restimulation or 3 d after restimulation. Relative expression of targeted genes is shown as a percentage of expression levels of negative control pool siRNA nucleofected cells (100%). **B**, Effects of IRAK1, IRAK4 siRNA on Th1 proliferation responses. Proliferation (cpm) was normalized to negative control pool siRNA response (100%; mean cpm = 22,183). **C**, Effects of IRAK1, IRAK4 siRNA on Th1 cytokine responses. IFN- γ production was normalized to negative control pool siRNA response (100%; mean IFN- γ = 4.04 ng/ml). **D**, IRAK1 and IRAK4 siRNA knockdown in Th17 cells. Relative expression of targeted genes is shown as a percentage of expression levels of negative control pool siRNA nucleofected cells (100%). **E**, Effects of IRAK1, IRAK4 siRNA on Th17 proliferation responses. Proliferation was normalized to negative control pool siRNA response (100%; mean cpm = 18,573). **F**, Effects of IRAK1, IRAK4 siRNA on Th17 cytokine responses. IL-17 production was normalized to negative control pool siRNA response (100%; mean IL-17 = 200 pg/ml). For all panels, data are mean \pm SD of triplicate measurements and are representative of experiments using two different donors. * p < 0.001; ** p = 0.002.

CpG-B, which induce maturation and production of proinflammatory cytokines, are directed to late endosomes (28). Our data show that CpG-A-induced IFN- α production is IRAK1/4 kinase dependent, whereas CpG-B-mediated responses, as measured by NF- κ B activation, were unaffected. Thus, IRAK1/4 kinase activity may be essential for early endosomal rather than late endosomal signaling events upstream of IRF-7. Whether similar compartmentalization occurs in human B cells has not been thoroughly explored, but studies in mouse B cells suggest that spatiotemporal regulation of TLR9 responses may occur as well. For mouse B cells, CpG-B is the dominant trigger for B cell responses such as proliferation and IL-6 production. However, CpG-A can stimulate B cell production of NF- κ B pathway-dependent cytokines such as TNF- α (26, 46), consistent with our observation that NF- κ B p65 is phosphorylated after CpG-A stimulation (Fig. 2B). The differential effects of CpG-A and CpG-B on B cell responses may be because of intrinsic properties that affect internalization and endosomal localization. For instance, when CpG-A is presented as an IC, BCR cross-linking mediates delivery of the complex into autophagosomes where TLR9 is colocalized, resulting in B cell proliferation similar to that induced with CpG-B (46, 47).

Restriction of IRAK1/4 kinase function to pDCs in humans suggests that IRAK1/4 kinase inhibitors may have potential therapeutic benefit. SLE patient serum containing ICs directly elicited IFN- α production from pDCs, confirming previously published reports (13, 14), and this was inhibited with a dual IRAK1/4 kinase inhibitor. We also found that some RA patient sera had detectable, albeit low, levels of DNA-containing ICs and were thus capable of activating pDCs. It has been reported by others that RA patient sera did not have stimulatory effects on pDCs; however, in

this small cohort, sera were negative for anti-DNA Abs (13). In contrast, infiltration of pDCs and associated greater levels of IFN- α in synovial fluid of RA patients have been documented (48, 49). In our RA patient cohort, sera that contained circulating autoantibodies had sufficient ICs to drive TLR9-dependent inflammatory responses. Interestingly, no correlation was found between pDC/B cell responses to SLE or RA patient sera and any particular autoantibody species, although sample sizes used in these studies were not large. It will be of further interest to study the TLR7/9-mediated responses of pDC and B cells harvested directly from patients, especially in light of the recent publication highlighting the role of TLR signaling in steroid-resistant lupus (50).

Self-DNA capable of activating TLR9 signaling may also occur in the absence of IC formation, as seen in psoriasis where DNA uptake by pDCs is facilitated by the antimicrobial peptide LL37 (51). Although we cannot formally exclude that such an LL37-dependent mechanism is occurring with our RA (or SLE) samples, the LL37-mediated uptake of DNA is into early endosomes, resulting in activation of TLR9. Our findings with RA patient sera allow for the possibility of LL37-mediated conversion of otherwise inert self-DNA into potent triggers of pDC IFN- α responses. As has been speculated for pDC activation in SLE (51), it would be interesting to investigate whether LL37 is involved in RA, because LL37 expression has been reported in synovial membranes (52). Furthermore, in RA and other autoimmune diseases, proinflammatory cytokines such as TNF- α , IL-1 α , and IL-1 β are often increased and used as surrogate markers of disease activity (53), and therapeutic use of disease-modifying antirheumatic drugs and steroidal or nonsteroidal anti-inflammatory drugs can modulate these levels (50, 53). Overall, these inflammatory cyto-

kines may affect peripheral lymphocytic cell activation involving both IRAK-dependent and -independent pathways.

In summary, we show that human immune cells have differential requirements for IRAK1/4-mediated signaling, and that different immune cell types can potentiate signals through a scaffold function rather than through kinase activity. pDCs require kinase activity, whereas B, T, and myeloid cells activated through MyD88 pathways do not necessarily require kinase activity, although presence of protein is absolutely required. Taken together with data showing a broader expression pattern of TLR9 on human and murine cell immune cells, our work highlights caution when translating mouse genetics into human biology. The restricted role of IRAK1/4 kinase activity to TLR9-mediated activation of the IRF-7–dependent IFN- α pathway has interesting therapeutic implications in autoimmune diseases driven by an IC/IFN- α feedback loop. Targeted inhibition of IRAK1/4 kinase activities may allow specific abrogation of IFN- α production by pDCs, but the intact scaffolding role served by IRAK1/4 would allow unimpeded cellular responses to other IRAK1/4-dependent signals.

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Disclosures

All authors are current salaried employees of Genentech, Inc. and have stock interests in the parent company, Roche.

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