CD4⁺ T-cells in systemic lupus erythematosus (SLE) patients show altered T-cell receptor signaling, which utilizes Fc-receptor γ-chain FcγRIIIa. A role for FcγRIIIa activation from immune complex (IC) ligation and sublytic terminal complement complex (C5b-9) in CD4⁺ T-cell responses is not investigated. In this study, we show that the ICs present in SLE patients by ligating to FcγRIIIa on CD4⁺ T-cells phosphorylate Syk and provide a co-stimulatory signal to CD4⁺ T-cells in the absence of CD28 signal. This led to the development of pathogenic IL-17A⁺ and IFN-γhigh CD4⁺ T-cells in vitro. Cytokines IL-1β, IL-6, TGF-β1, and IL-23 were the only requirement for the development of both populations. SLE patients CD4⁺ T-cells that expressed CD25, CD69, and CD98 bound to ICs showed pSyk and produced IFN-γ and IL-17A. This FcγRIIIa-mediated co-signal differentially up-regulated the expression of IFN pathway genes compared with CD28 co-signal. FcγRIIIa-pSyk up-regulated several toll-like receptor genes as well as the HMGB1 and MyD88 gene transcripts. ICs co-localized with these toll-like receptor pathway proteins. These results suggest a role for the FcγRIIIa-pSyk signal in modulating adaptive immune responses.

Concurrent with the presence of aberrant T-cell responses, elevated serum levels of both immune complexes (ICs) and C5b-9 (non-lytic terminal complement complex) are associated with systemic lupus erythematosus (SLE) (1, 2). These immune-reactants form immune deposits at vascular sites and trigger inflammation (3). Immune deposits are also present in the ectopic germinal centers, the site for plasma B cell development (4). Formation of ICs by autoantibodies activate complement cascade and drive the formation of C5b-9 on cell membrane. We previously showed that non-lytic C5b-9 deposits trigger clustering of membrane rafts (MRs) observed in SLE T-cells. Hence, we examined the role for FcγRIIIa ligation by ICs in CD4⁺ T-cell responses in the presence of sublytic C5b-9 (5, 6).

FcyRIIIa-Syk Co-signal Modulates CD4⁺ T-cell Response and Up-regulates Toll-like Receptor (TLR) Expression* 

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Toll-like receptor (TLR) signaling in innate cells indirectly promotes T-cell differentiation. T-cells express TLRs and promote cytokine secretion. TLR signaling augments the TH1, TH17, and Tregs responses (34, 35). Activation of TLRs by DNA/RNA-ICs leads to autoantibody production. FcγRII (CD32) is a key participant for the delivery of DNA-ICs to many cell types (36). Subcellular localization of TLR9 discriminate between self and non-self DNA (37).

In this report, we demonstrate that the FcγRIIIa-pSyk signal successfully replaced the CD28 requirement for differentiation of CD4+ T-cells. ICs ligation to FcγRIIIa phosphorylates Syk (pSyk), which caused the activation of CD4+ T-cells. In the presence of polarizing cytokines, this activation resulted in the development of CD4+ IFN-γhigh and IL-17A-producing subsets. FcγRIIIa-pSyk co-signal induced colony stimulating factor 2 (Csf2) and IL-2 gene expression, which are associated with pathogenic T_{h17} cells. In SLE patients, CD4+ T-cell showed a subset that expressed activation markers CD25, CD69, and CD98, which also bound to ICs and showed pSyk. Furthermore, these activated cells produced IFN-γ and IL-17A. FcγRIIIa-pSyk-mediated signal differentially regulated the expression of IFN pathway genes. Co-signaling triggered by IC ligation of FcγRIIIa up-regulated expression of TLR signaling genes, suggesting a co-operation among these pathways.

**Experimental Procedures**

**Subjects**—Blood from SLE patients and normal donors was collected with informed consent in the Saint Louis University Rheumatology clinic. The peripheral blood mononuclear cells were isolated using the Histopaque gradient (Sigma). The donors 1–9 were analyzed for IFN-γ and IL-17A (Figs. 1 and 2). IL-21 production was analyzed in donors 1–4. This analysis from these donors is presented in Figs. 1, 2, and 4.
Donors 8, 9, and an additional donor 10 were analyzed for the IFN gene analysis (shown in Fig. 9). Results presented in Figs. 4–6 were obtained from additional donors not represented in Figs. 1 and 2.

**ICs and C5b-9—**ICs were purified from 50 ml of pooled serum or plasma from 5–10 SLE patients that showed high levels of complement opsonized ICs. The purification procedures for ICs and C5b-9 have been previously described.

**FIGURE 2. ICs+C5b-9 induces IL-17A expression.** A, flow cytometry analysis for IL-17A production on day 9 of post polarization. Cells treated with anti-CD3+ICs+C5b-9 generated 7.67% IL-17A+ cells, and anti-CD3+anti-CD28 generated 3.12% IL-17A+ cells, shown in donor 7. B, histogram of CD4+ gated cells showing IL-17A in cells treated with anti-CD3+ICs+C5b-9 (25.6%, IL-17A+) (a) and treated with anti-CD3+anti-CD28 (b) of donor 3. C, percentage of IL-17A-producing cells shown in nine individual donors. D, combined analysis of same 9 donors for IL-17A production as in Fig. 1. The anti-CD3+ICs+C5b-9-treated group showed a statistically significant increase for IL-17A production at a p value of 0.016 compared with anti-CD3 alone. A significant increase was not observed in other groups. E, flow analysis showing double positive IFN-γhighIL-17A+ populations. A small population of IFN-γhighIL-17A+ was observed from co-stimulation by ICs+C5b-9.
(11, 38, 39). The nature of the ICs used has been characterized for their binding to FcγRIII in multiple cell types, compared with AHG and anti-FcγRIIIa antibody (clone 3G8) (40). In addition the ICs were compared for their potential to activate CD4⁺ T-cells with in vitro formed Ova-anti-Ova ICs (11).

**T-cell Culture and Differentiation**—Peripheral blood mononuclear cells were isolated within 12 h of sample collection, and monocytes were removed by overnight plating in a culture dish. The next day the CD4⁺ CD45RA⁻ cells were purified using naïve CD4⁺ T-cell isolation kit II (Miltenyi Biotec, Product no. 130-094-131). Purified cells were maintained in culture with 20 units of IL-2 for 2 days. Thereafter, these cells were stimulated with plate-bound ICs at 10 µg/ml and using purified soluble C5b-9 at 2.5 µg/ml for 1 × 10⁶ cells in the presence of plate-bound anti-CD3 (eBioscience, clone OKT3) at 0.25 µg/ml. Positive control cells were stimulated with plate-bound 1 µg/ml anti-CD28 (clone 28.2) and 0.25 µg/ml anti-CD3. At 24 h post stimulation cells were cultured in the presence of IL-2 (20 IU), IL-1β (50 ng), IL-6 (50 ng), IL-23 (20 ng), and TGF-β1 (10 ng) for each ml of medium (Peprotech, Princeton, NJ). On days 9–11, cells were analyzed by flow cytometry for cytokine production. Cytokine levels were measured in the culture supernatants harvested on day five due to the concern for overgrowth in anti-CD3+anti-CD28 activation.

**Thymidine Uptake**—Naïve CD4⁺ T-cells were activated for 48 h with plate-bound anti-CD3+anti-CD28. Cells were then cultured in the presence of 20 units IL-2 and examined for binding of labeled ICs. Cells on day 7 were activated with plate-bound anti-FcγRIIIa/b (0.5 µg/ml), ICs (10 µg/ml), and anti-CD3+anti-CD28 (0.5 and 1 µg/ml). Thymidine uptake was measured using Click-iT Plus Edu Alexa-488 assay (Product no. C10632, Life Technologies) 96 h post activation. Cells alone and isotype control (0.5 µg/ml) were used as negative controls.

**Flow Staining**—Cell surface staining was done using antibody conjugated directly with fluorochromes at room temperature for 30 min as per the manufacturer’s recommended use. The binding of labeled ICs was performed using 1 µg of protein label/10⁶ cells for 30 min at room temperature. For intracellular cytokine staining, cells were stimulated with 1 µg/ml phorbol 12-myristate 13-acetate (PMA) and 2.5 µg/ml ionomicyn for 4 h. Brefeldin at 5 µg/ml (Golgi Plus BD) was added after 1 h of PMA/ionomicyn stimulation. Cells were collected for staining after 3 h. After cell surface staining the intracellular staining was performed using fixation/permeabilization reagents for IFN-γ, IL-17A, and IL-21 (eBioscience) according to manufacturer-suggested protocol. The following antibodies were used for cell surface or intracellular staining: Per-CP Cy5-anti-CD4, APC-anti-IFN-γ, PE-anti-IL-21, PE-Cy7-anti-CD1, APC-eFluor780-anti-ICOS (eBioscience) PE-Cy7-anti-CD25, BV650-anti-CD69, BB515-anti-CD98, and Alexa Fluor 647-anti-IL-17A (BD Bioscience). PE-pSyk (Tyr-348) was purchased from eBioscience and PE-pSyk (Tyr-525/526) from Cell Signaling Technologies. Cells were stained in two panels: 1) anti-CD4, anti-pSyk (eBioscience), anti-IL-17A, anti-IFN-γ, and ICs; 2) anti-CD4, anti-CD25, anti-CD69, anti-CD98, and ICs. Staining using PE-pSyk (Cell Signaling Technologies) was performed in a separate panel from same samples. Stained cells were analyzed by flow cytometer (BD-LSRII, BD Biosciences). The flow data were analyzed with FlowJo software (Tree Star). CD4⁺-gated T-cells were analyzed for pSyk presence with CD25, CD69, CD98, ICs, IL-17A, and IFN-γ. The graphs were generated using GraphPad Prism 6. p values were calculated using non-parametric t test in Prism software.

**Quantitative Real-time-PCR and PCR Array Analysis**—Total RNA was prepared from cells harvested between days 4–5 post-stimulation using kit from Agilent Technologies (Wilmington, DE). Semiquantitative analysis for gene expression was carried from cDNA generated from total RNA using a high capacity cDNA kit (Applied Biosystems) using the comparative Ct (ΔΔCt) method. For Rorc (Hs01076122), endogenous control GAPDH (Hs02758991) (Applied Biosystems) was used. The RQ, RQ (minimum), and RQ (maximum) were calculated using StepOne software and plotted using GraphPad Prism. For gene expression cDNA was analyzed as per the manufacturer recommendation in the TaqMan Array for human IFN pathway (product no. 4418931) and analyzed in Data-assist. For calculating RQ, the corresponding genes in CD28 co-stimulated sample were used to normalize the expression. GAPDH, GUSB, and HPRT1 were used as endogenous controls for IFN array analysis. Analysis of variance was carried out using Partek Genomic Suite (Life Technologies). cDNA prepared from anti-CD3+anti-CD28 treated and anti-CD3+ICs+C5b-9-treated cells was analyzed for human Toll-Like Receptor Signaling Pathway genes using a RT² Profiler PCR array plate (PAHS-018ZC, SAS Bioscience). Data were analyzed using vendor software, and ACTB, B2M, RPLP0, and GAPDH were used as endogenous controls included in the array.

**Cytokine Measurement**—Culture supernatants were collected from activated cells on day 5 and kept frozen at −70 °C. Cytokine measurements were performed using the multiplexing assay as per the manufacturer’s instruction (EMD Millipore). For statistical analysis a non-parametric t test was performed using GraphPad Prism software.

**Cell Staining**—P116 cells (ATCC, CRL-2676), an acute T cell leukemia ZAP-70 mutant was grown as per the guidelines from ATCC. These cells were activated as described in the previous section. Cells were harvested and washed with PBS and fixed in 4% formaldehyde for 15 min at room temperature. Cells were permeabilized using cold methanol at −20 °C for 10 min. Cells were then kept for 1 h in 1% BSA/PBS and stained using anti-species specific Alexa Fluor fluochrome conjugate (Life Technologies) at appropriate dilutions. Anti-TLR antibodies were purchased from R&D Systems and eBioscences. Anti-MyD88 and anti-HMGB1 was obtained from Cell Signaling Technologies. As a control for labeled ICs we used human IgG-conjugated with Alexa Fluor 488. Isotype controls for mouse monoclonal and purified rabbit IgG fraction were used as negative controls.

We stained human CD4⁺ FcγRIII⁺ cells after treating them with plate-bound anti-CD3 (1 µg/ml) for 1 h with FITC-labeled anti-CD3 and anti-FcγRIIIa/b monoclonal (Clone 245536) (R&D Systems). A secondary anti-mouse-Alexa Fluor 594 was used to stain monoclonal anti-FcγRIIIa/b.
**Results**

ICs and C5b-9 Co-stimulation Generate CD4+ IFNγ-high Population—IFN-γ is an autocrine T_{h1} differentiation factor that requires cytokine IL-12 for differentiation (19, 41). To examine whether ICs+C5b-9 contributes to CD4+ T-cell mediated pathological responses, we first examined the IFN-γ production in the presence of IL-1β, IL-6, IL-23, and TGF-β1 cytokines. Flow analysis showed substantial and reproducible increases in the IFN-γ-producing populations on day nine post polarization (Fig. 1, A and B). We observed a high and moderate IFN-γ producing population (Figs. 1A and 2E). A statistically significant increase in IFN-γ-high population upon anti-CD3+ICs+C5b-9 treatment was observed compared with anti-CD3 treatment in 9 of 12 subjects analyzed (Fig. 1). These donors demonstrated an IFN-γ-high population upon ICs+C5b-9 co-stimulation (Fig. 1C). Donors 2, 7, and 9 also showed IFN-γ production in response to anti-CD3+anti-CD28 treatment. In nine donors that produced IFN-γ, combined analysis showed a statistically significant increase in IFN-γ producing population at a p value of 0.0026 in the anti-CD3+ICs+C5b-9-treated group compared with anti-CD3 group (Fig. 1D). In donor 7, a higher basal level of IFN-γ before activation was observed. This donor also showed elevated Tbx21 transcripts, suggesting an ongoing T_{h1} response at the time of sample collection (not shown).

The flow data were supported by an observed increase in IFN-γ levels in the culture supernatants post day five from the time of polarization. A statistically significant increase in IFN-γ production from anti-CD3+ICs+C5b-9 treatment, 14,398 ± 6,587 pg/ml (p value of < 0.0001), compared with untreated cells 1684 ± 338 pg/ml was observed (Fig. 3D). When compared with the anti-CD3-treated control group, anti-CD3+ICs+C5b-9-treated cells showed a statistically significant increase in IFN-γ at a p value of < 0.0025. The positive control group treated with anti-CD3+anti-CD28 also showed an increase in IFN-γ production compared with untreated cells, 7571 ± 5887 versus 1684 ± 338 pg/ml, respectively, which was significant at a p value of < 0.0001 (Fig. 3D). Untreated cells maintained in IL-2 (20 units/ml) showed minimal amounts of IFN-γ. These results confirm a role for ICs+C5b-9 for IFN-γ production in naive CD4+ T-cells.

ICs and C5b-9 Co-stimulation Generate a T_{h17} like Population—T_{h17} cells contribute to multiple autoimmune pathologies including SLE (28, 42). In humans, IL-17A production is driven by TGF-β (28, 43). Cytokines IL-1, IL-6, and IL-23 expand and stabilize this population (44). Flow analysis showed IL-17A producing cells upon ICs+C5b-9 co-stimulation in 9 of 12 subjects analyzed (Fig. 2, A and B). Donor 1 showed a minimal increase (Fig. 2C). The combined analysis showed a statistically significant increase at a p value of 0.0016 from ICs+C5b-9 co-signal (Fig. 2D). Donors 3, 4, 5, 6, 8, and 9 showed a higher percentage of IL-17A+ cells from in vitro activation by anti-CD3+ICs+C5b-9. IL-17A-producing cells were also observed in donors 5, 7, and 8 in cells that received anti-CD3+anti-CD28 treatment. A higher percentage of IFN-γ- and IL-17A-producing populations were generated from ICs+C5b-9 co-stimulation. Proportionately, only a small fraction of cells were double positive for IFN-γ IL-17A cytokines (Fig. 2E).

Flow data were reconfirmed by the observed increases in the level of the cytokines IL-17A, IL-17F, and IL-22 in the culture supernatants, measured post day five from polarization. This time point was chosen to avoid differences arising from cell division in various activations. The amount of IL-17A produced in response to treatment with anti-CD3+ICs+C5b-9, compared with untreated cells, showed a statistically significant increase from 290 ± 169 to 2220 ± 1930 pg/ml (Mean ± S.E.) at a p value of < 0.0169. The positive control group treated with anti-CD3+anti-CD28 also showed a significant increase, from 290 ± 169 to 1508 ± 955 pg/ml, a p value of 0.0055 (Fig. 3A). IL-17F also showed a statistically significant increase in the anti-CD3+ICs+C5b-9-treated group compared with the untreated control, an increase from 579 ± 79 to 2979 ± 328 pg/ml with a p value of < 0.0001. A comparable increase was observed in the anti-CD3+anti-CD28-treated group, from 579 ± 79 to 2653 ± 2073 pg/ml, with a p value of < 0.0001 (Fig. 3B). The addition of C5b-9 to the anti-CD3+ICs-treated group showed a statistically significant increase from 1937 ± 191 to 2978 ± 1042 pg/ml at a p value of 0.0035, suggesting a role for complement. There was no statistically significant difference in IL-17F production from co-stimulation with either CD28 or ICs+C5b-9. Although an increase in the IL-22 levels was observed in response to co-stimulation with CD28 as well as
ICs+C5b-9, these values were not statistically significant (Fig. 3C). IL-22 production was also observed in flow analysis (not shown).

We also examined IL-21, a cytokine produced by both T_{H17} and T_{fh} cells. IL-21 activates lymphocyte and regulates antigen specific antibody response (45, 46). All four donors examined showed enhanced production of IL-21 from ICs+C5b-9 co-stimulation. Donors 3 and 7 also showed IL-21 production from anti-CD3+anti-CD28 activation. The combined analysis of all four donors showed a statistically significant increase in the percentage of IL-21-producing cells upon ICs+C5b-9 co-stimulation (not shown).

ICs+C5b-9 Co-signal Triggers Expression of Genes Associated with T_{H17} Terminal Differentiation—We further confirmed the identity of T_{H17} cells by examining Rorc expression, a T_{H17} transcriptional regulator. All five donors analyzed showed a 2–8-fold increase in Rorc gene transcripts upon ICs+C5b-9 co-stimulation when normalized with transcript levels present in the anti-CD3-treated cells. Donors 4 and 5 were normalized using a control from another subject, as Rorc gene transcripts were not detectable in either untreated (not shown) or anti-CD3-treated cells used as negative controls (Fig. 4A). Csf2, IL-2, and Tbx21 are markers for the terminally differentiated pathogenic T_{H17} population (30). ICs+C5b-9 co-stimulation increased the expression of gene transcripts for IL-6, Csf2, IL-10, IL-12A, IL-1A, IL-1B, and IL-2 compared with the levels of transcript observed from CD28 co-stimulation (Fig. 4B, n = 3). The increase in the IL-6 transcripts was 4.21-fold and for Csf2 was 3.95-fold. These data suggest that the ICs+C5b-9 co-stimulation contributes to the development of the pathogenic T_{H17} population.

IC Engagement Phosphorylates Syk and Triggers Thymidine Uptake—To further confirm a role for FcγRIIIa-Syk signal in CD4^+ T-cell activation, we examined the T-cell activation markers and pSyk upon in vitro activation of naïve CD4^+ T-cells. Co-stimulation by CD28 or ICs+C5b-9 induced the expression of CD25 and CD69 (Fig. 5A). Co-stimulation by ICs+C5b-9 showed an increase in pSyk population (Fig. 5B). These pSyk+ cells expressed IFN-γ (Fig. 5C). IFN-γ production was observed in the absence of PMA and ionomycin treatment.

We further examined whether FcγRIIIa^+ CD4^+ T-cells upon receptor ligation trigger thymidine uptake. Both anti-FcγRIIIa/b antibody (12.4%) and ICs (10.7%) ligation triggered thymidine uptake (Fig. 6). Cells activated with anti-CD3+anti-CD28 showed 18.6% cells in proliferation. Isotype antibody control (1.09%) and untreated cells (0.82%) did not show thymidine uptake. These results further confirm the presence of FcγRIIIa on CD4^+ T-cells.

Confocal microscopic examination of z-series sections for CD3 complex and FcγRIIIa staining showed co-localization of these proteins (Fig. 6F). These results are in accordance with our previous observation and published report (11, 47).

CD4^+ pSyk^+ T-cells in SLE Are an Activated Phenotype That Produces IFN-γ and IL-17A—To establish a role for Syk signaling in vivo, we next examined the presence of pSyk using two antibodies in the CD4^+ T-cells within the peripheral blood mononuclear cells of SLE patients. Antibodies used recognized the Tyr-348 residue in Vav1 binding of human Syk and a second antibody that recognized the Tyr-525/526 residue in the kinase domain (Fig. 7A). Both of these antibodies confirmed the presence of pSyk in activated peripheral CD4^+ T-cells that expressed CD25 (a and b), CD69 (c and d), and CD98 (e and f), all T-cell activation markers. We then examined the IC binding to CD4^+ T-cells that expressed T-cell activation markers and pSyk (Fig. 7A, panels g, h, i, and j). Activated CD4^+ pSyk^+ T-cells bound ICs, suggesting the presence of FcγRIIIa. These results suggest that in CD4^+ SLE T-cells, Syk signaling contributes to cell activation (11, 48). Activated CD4^+ T-cells express FcγRIIIa (40). The presence of pSyk in activated CD4^+ T-cells in the patient population is also supported by our previous studies where we showed Syk phosphorylation during T-cell activation (38). A role for Syk in the development of T_{H17} and T_{fh} responses via dendritic cell activation has been also been suggested (49).
We next examined whether the activated pSyk CD4+ T-cells in SLE patients produced IFN-γ and IL-17A. Flow analysis showed that the pSyk cells both at Tyr-348 or Tyr-525/526 produced IFN-γ and IL-17A (Fig. 7B). Data from two patients show 7.77 and 9.31% pSyk IFN-γ cells in donor 1 (panels a and b) and 4.48 and 4.82% in donor 2 (panels e and f), respectively. Donor 1 showed 7.10% and 7.82% of pSyk IL-17A cells (panels c and d) and 3.32% and 3.38% population in donor 2 (panels g and h). A minor population of IL-17Ahigh was also observed in several subjects. These cells were analyzed without activation by PMA, ionomycin, and brefeldin A treatment. Analysis of 29 patients showed that pSyk cells were activated CD4+ T-cells, which produced IFN-γ and IL-17A cytokines. Individual and combined analysis of these 29 subjects as a group demonstrated that the percentage of pSyk FcγRIIIa (CD16a) Co-localizes with TLRs in CD4+ T-cells

We also examined IFN-γ production in P116 cells, a ZAP-70 mutant of Jurkat cells that can only signal via Syk (17). Again, co-stimulation from ICs+C5b-9 in P116 cells produced IFN-γ cells (not shown).

ICOS+ but Not PD1high Cells Show pSyk in SLE-CD4+ T-cells—ICOS and PD1 are key membrane regulators of CD4+ T-cell response. Thus we next examined whether pSyk FcγRIIIa+ cells express these proteins. In all 15 donors analyzed, ICOS+ CD4+ T-cells also showed pSyk (Fig. 8A). Cells that expressed ICOS bound to ICs (Fig. 8B). However, those
cells that expressed high levels of PD1 (PD1\textsuperscript{high}) lacked pSyk. pSyk\textsuperscript{-} cells expressed low levels of PD1 (Fig. 8, panels C and D). A higher percentage of PD1\textsuperscript{high} cells with high mean fluorescence intensity values was observed compared with PD1\textsuperscript{low} pSyk\textsuperscript{-} cells (Fig. 8H). In only two patients the mean fluorescence intensity for PD1 was equal or slightly higher in cells with pSyk. A role for PD1 in down-regulation of Syk phosphorylation via SHP2 has been shown (50). PD1\textsuperscript{high} cells did not bind to ICs, although in some patients both moderate PD1 levels and an IC binding population was observed (Fig. 8 panels E and F). A paired t-test showed a statistically significant correlation among pSyk and ICOS expression at a p value of <0.0001, with a strong correlation (r = 0.77). However, a correlation between pSyk and PD1 expression was not observed (r = 0.22) (Fig. 8, panel G). It is likely that PD1 dephosphorylated Syk via SHP2. These data suggest a possible role for Fc\textgamma R\textgamma III\alpha-Syk signaling in modulating responses of CD4\textsuperscript{+} T-cell membrane regulators (Fig. 8).

ICs+C5b-9 Provides a Distinct Co-stimulatory Signal for IFN-pathway Gene Expression—IFN inducible gene “signature” serves as a marker of lupus nephritis (25). We analyzed expression of IFN signaling pathway genes from ICs+C5b-9 co-stimulation and compared it with the CD28 co-signal. Three donors, 8, 9, and 10, showed strong expression of IFN pathway genes, which were differentially expressed from two co-stimulations (Fig. 9). Interestingly, donor 8 showed expression of type I IFN genes, IFNA1, IFNA2, IFNA4, IFNA5, IFNA6, IFNA7, IFNB1, and IFNW, which were further up-regulated after ICs+C5b-9 co-stimulation (Fig. 9, blue bars donor 8). The IFNA1, -2, -4, -5, -6, -7, -8, -14, and -17 were up-regulated over 6-fold with only a 1.53- and 1.26-fold increase in IFNAR1 and IFNAR2, respectively. These receptors are utilized by type I IFNs. Donor 9 showed increased expression of type II IFN responses (Fig. 9, red bars). MAP2, MAP3, and PIK3 kinases showed a >5-fold increase in donors 9 and 10 (Fig. 9). In donor 8, insulin receptor substrate 2 (IRS2) showed a 20-fold increase. IRS2 along with IRS1 acts as an adaptor substrate for the type I IFN signaling. IRS2 is negatively regulated by the cyclic AMP response element-binding protein 3-like 4 (CREB3L4), which was shut down by ICs+C5b-9 co-stimulation (Fig. 9). Donor 9 with a long history of SLE and nephritis showed an 87-fold increase of IFN pathway genes from ICs+C5b-9 co-stimulation and compared it with the CD28 co-signal. Three donors, 8, 9, and 10, showed strong expression of IFN pathway genes, which were differentially expressed from two co-stimulations (Fig. 9). Interestingly, donor 8 showed expression of type I IFN genes, IFNA1, IFNA2, IFNA4, IFNA5, IFNA6, IFNA7, IFNB1, and IFNW, which were further up-regulated after ICs+C5b-9 co-stimulation (Fig. 9, blue bars donor 8). The IFNA1, -2, -4, -5, -6, -7, -8, -14, and -17 were up-regulated over 6-fold with only a 1.53- and 1.26-fold increase in IFNAR1 and IFNAR2, respectively. These receptors are utilized by type I IFNs. Donor 9 showed increased expression of type II IFN responses (Fig. 9, red bars). MAP2, MAP3, and PIK3 kinases showed a >5-fold increase in donors 9 and 10 (Fig. 9). In donor 8, insulin receptor substrate 2 (IRS2) showed a 20-fold increase. IRS2 along with IRS1 acts as an adaptor substrate for the type I IFN signaling. IRS2 is negatively regulated by the cyclic AMP response element-binding protein 3-like 4 (CREB3L4), which was shut down by ICs+C5b-9 co-stimulation (Fig. 9). Donor 9 with a long history of SLE and nephritis showed an 87-fold increase.
FcγRIIa (CD16a) Co-localizes with TLRs in CD4⁺ T-cells

A

CD25

CD69

CD98

pSyk

B

IFN-γ

IL-17A

pSyk
increase in IFN-γ expression and downstream signaling genes (Fig. 9, donor 9 red bars). Other notable increases were observed in expression of JAK1, JAK2, IRF-9, TYK2, STAT1, and STAT2. Donor 10 (Fig. 9, green bars) also showed an increase in the expression of IFN-γ. Pronounced expression of PI3K in response to ICs/C5b-9 co-stimulation was observed. These results suggest a distinct up-regulation of IFN genes in each donor from ICs/C5b-9 stimulation.

ICs/C5b-9 Up-regulate TLR Signaling Pathway Genes—
TLRs play a role in adaptive immune responses (35). To examine whether TLR signaling synergizes the FcγRIIIa-Syk-mediated signal in modulating T-cell responses, we analyzed the expression of TLR signaling genes. We analyzed naïve CD4+ T-cells from five paired samples under identical culture conditions. Cells were co-stimulated with ICs/C5b-9, and the gene expression levels were compared with cells co-stimulated using ani-CD28 from the same subject. Combined analysis of five samples showed increased expression of TLR-interacting proteins and adaptors such as Bruton agammaglobulinemia tyrosine kinase (Btk) (2.92), HMGB1 (3.62), Harvey ras sarcoma virus oncogene homolog (HRAS) (4.91), and Myd88 (2.34). Myd88-dependent signaling TLRs, TLR2 (5.50), TLR4 (2.23), TLR5 (5.17), TLR7 (2.55), and TLR10 (5.16), showed significant increases, whereas TLR9 (1.03) did not show any increase. TIRAP (5.21), which is essential for TLR2 and TLR4 signaling, was up-regulated. Expression of TRAF6 (4.65), a TNF receptor-associated family factor, which signals via the Toll/IL-1 family, was also increased. Proteins that influence the adaptive responses, TRAF6 (4.65), IL10 (2.99), IL12B (2.31), IL1A (3.05), and IL1B (2.68), showed increased expression (Fig. 10). TLR3 (9.89), a MyD88-independent signal, showed the highest increase in the gene expression (Fig. 10). Two donors, 8 and 12, showed the maximum up-regulation of TLRs (Fig. 10). In these two donors we compared the up-regulation of TLR signaling genes from untreated cells with CD28 and ICs/C5b-9 co-stimulation. IL-12A was significantly up-regulated.

**FIGURE 7—continued**

- **C** shows the staining of CD4+ T-cells expressing FcγRIIIa (CD16a) co-localizes with TLRs in CD4+ T-cells.
- **D** shows the staining of pSyk and IC-bound populations.
FIGURE 8. pSyk$^+$ CD4$^+$ T-cells express ICOS and not PD1. pSyk$^+$ CD4$^+$ T-cells express ICOS (A) and bind to ICs (B). PD1$^{\text{high}}$ cells do not show pSyk and IC binding. Cells with low PD1 expression show pSyk (showing 2 of 15 analyzed, C and D). PD1$^{\text{high}}$ cells do not bind to ICs (E and F). The plot shows PD1$^+$ and pSyk$^+$ CD4$^+$ T-cells and pSyk$^+$ ICOS$^+$ CD4$^+$ T-cells (G, n = 15). Data in C and E and in D and F are paired donors. pSyk$^-$ cells show low levels of PD1 expression. Shown is mean fluorescence intensity (MFI) plotted from paired samples from pSyk$^-$ and pSyk$^+$ population (H).
regulated by both co-signals. Cells activated with the ICs+C5b-9 co-stimulation showed a significant increase in many genes, notably HMGB1 (20.66), IL-1B (11.25), IL-10 (10.71), TLR3 (88.22), TLR7 (13.16), TLR8 (80.49), TLR9 (17.59), TLR10 (19.17), and TRAF6 (20.74) (Fig. 10 and Table 1). TLR3 showed the most increase and is shown to aggravate lupus nephritis (51). To examine the presence of TLR proteins and their association with FcγRIIIa in CD4+ T-cells, we stained P116 cells after co-stimulation with ICs+C5b-9. ICs co-localized with MyD88 (supplemental Movie 1), HMGB1 (supplemental Movie 2), TLR3 (supplemental Movie 3), TLR5 (supplemental Movie 4), and TLR9 (supplemental Movies 5 and 6), MyD88, and HMGB1 along with IC localized on the cell membrane (Fig. 11). TLR3 and TLR9 colocalized with ICs on membrane (Fig. 11A, panels e and f and panels k and l; supplemental Movies 3 and 6). Both of these proteins were also present in the endolysosome. This was confirmed using LysoTracker deep red (Molecular Probes). These proteins appear in microclusters. IC binding showed a pattern of receptor capping. Even though we did not see up-regulation of TLR9 transcripts upon ICs+C5b-9 co-stimulation at the protein level in naïve CD4+ T-cells, we observed the TLR9 protein in P116 cells and activated human CD4+ T-cells. We observed two staining patterns for TLR9. The first pattern was with membrane staining and intracellular staining for ICs (Fig. 11A, panels i and j). In the second staining pattern ICs showed membrane staining and TLR9 in endolysosomes forming a ball-like structure (Fig. 11A, panels k and l, supplemental Movie 5). TLR9 co-stained with ICs, suggesting their co-localization. Untreated P116 cells showed mostly membrane staining for ICs and TLR9. A similar staining pattern was also observed for TLR3. In Western blot analysis, both HMGB1 and MyD88 were observed in immunoprecipitates prepared using anti-CD16 (clone 3G8) antibody (data not shown). These data suggest a role for FcγRIII-Syk signaling in the up-regulation of TLR signaling pathways in CD4+ T-cells.

Our results thus suggest that FcγRIIIa-pSyk is a distinct co-signal in CD4+ T-cells that drives the differentiation of naïve cells into IFN-γhigh and IL-17A+ populations. FcγRIIIa-pSyk signal up-regulated the genes associated with terminal differentiation of pathogenic T_h17 cells. FcγRIIIa-pSyk is a distinct and potent signal for up-regulation of the IFN signaling pathway. The FcγRIIIa-pSyk population is present in SLE patients. The ligation of FcγRIIIa by ICs up-regulated the
TLR signaling pathway genes. These data suggest a possible synergistic role of TLR and FcγRIIIa signaling in human CD4+ T-cells.

Discussion

In this report we show that the ICs+C5b-9 acts as a co-stimulator of naïve CD4+ T-cells. ICs+C5b-9 generates a co-stimulatory signal that is mediated via FcγRIIIa-Syk phosphorylation. This ICs+C5b-9-mediated signal efficiently replaced the CD28 requirement for the development of CD4+IFN-γhigh and a Tn17 like population. The FcγRIIIa-pSyk is a distinct co-signal from CD28, as it differentially expressed IFN genes and up-regulated TLR signaling pathways genes. Naïve CD4+ T-cell activation, survival, subset differentiation, and effector function are regulated by the co-signaling proteins present on the CD4+ T-cell membrane (52). A co-stimulatory signal from CD28 (signal 2 of two signal hypothesis) is a key requirement for naïve CD4+ T-cell activation without which cells become anergic. In an autoimmune background, CD4+ T-cells bypass the need of CD28 co-signal to become fully activated (10). However, the mechanism underlying this activation is unknown. Our results suggest that in an autoimmune response, FcγRIIIa-Syk signal is important for the activation of naïve CD4+ T-cells. In CD4+ T-cells that express FcγRIIIa, ICs ligation triggers FcγR chain phosphorylation, which then co-localizes and signal via Syk (38, 53). Although C5b-9 is essential to trigger MR clustering, ICs engage FcγRIIIa and trigger Syk activation (11). Both ICs and C5b-9 are required for phosphorylation of TCR signaling proteins and triggering of T-cell activation-associated changes (11). Co-localization of ICs with in situ assembled C5b-9 and CD3 complex suggests a cooperative response among these complexes (11). On CD4+ T-cell membrane ICs binding occurred at the site of FcγRIIIa staining, confirming the presence of these receptors (40). FcγRIIIa colocalize with the CD3 complex on the cell membrane (Fig. 6F). Previous studies have also shown colocalization of FcR with TCR on activated T-cells (47). In SLE-CD4+ T-cells, the Fcγ chain associates with the CD3 ζ-chain of TCR and signals via Syk (53). Rewiring of TCR-CD3 complex, where the CD3-ζ chain is replaced by the Fcγ chain, which signal via Syk in SLE T-cells is shown (16). Up-regulation of both the Fcγ chain and Syk is observed in TnE cells, unlike naïve cells (16). The T-cell activation via CD28 signaling upon TCR engagement in the absence of ZAP-70 signaling can utilize Syk. The engagement of FcγRIIIa can only

FIGURE 10. ICs+C5b-9 up-regulates TLR signaling genes. A, ICs+C5b-9 co-stimulation of naïve CD4+ T-cells induced expression of TLR genes severalfold. Both MyD88-dependent and independent genes as well as adaptors show enhanced gene expression (n = 5). B, heat map comparing CD28 versus ICs+C5b-9 co-stimulated gene expression in five donors (a) comparing gene expression in donors 8 and 12 in cells versus ICs+C5b-9 (b), and cells versus CD28 (c).
generate the Syk-mediated signal. In the presence of IL-2 and IL-12, ICs + C5b-9 co-signals with suboptimal CD3 ligation generated a Th1-like population (40). Intravenous gamma globulin therapy, which works by blocking low affinity FcRs, reciprocally regulates human pathogenic Th1, Th17, and Treg cells (54). Based on our results and the existing literature, we propose that the low affinity FcγRIIIa-mediated phosphorylation of Syk is an important signal for the development of pro-inflammatory CD4+ T cells (17, 48). Additionally, TLR signal up-regulation from FcγRIIIa ligation by ICs may result in the development of proinflammatory cells that may be refractory to suppression by Tregs. A role for IL-1, IL-6, and LPS signal that utilizes MyD88 an adaptor for Toll/IL-1 receptor is implicated in overcoming the suppression by Tregs (55–57). Cooperation among FcγR-TLR signaling in M1 and M2 macrophages activates Syk kinase, which then produces proinflammatory cytokines (58). An association of TLR4 with FcγRIII upon ICs stimulation of mouse macrophages is observed (59). A similar cooperation between FcγRIIIa and TLR signals in CD4+ T-cells could modulate adaptive immune responses in humans. The expression of co-stimulatory proteins on naïve CD4+ T-cells is limited, and CD28 is the only primary protein known to prime these cells (60). ICs + C5b-9 successfully primed human peripheral naïve CD4+ T-cells in the absence of CD28 co-signal. The co-signal generated by ICs + C5b-9 was efficient and potent enough to support the development of IFN-γ-high and IL17A+ producing cells, which only required IL-1β, IL-6, IL-23, and TGF-β1, without IL-4 and IFN-γ suppression (Figs. 1 and 2). For differentiation of mouse naïve CD4+ T-cells into Th17 cells, IFN-γ and IL-4 suppression is required. We did not observe this requirement.

Altered CD4+ T-cell responses are a common feature of autoimmune pathology, which is often accompanied by elevated IC levels and complement activation byproducts such as C5b-9 (61, 62). The elevated serum and urine levels of C5b-9 are associated with disease activity. In our model the C5b-9 contributes to cell activation by lateral clustering of MRs, which brings the receptors and signaling proteins to close proximity (11). MRs are uniformly distributed on T-cells from healthy individual and are aggregated and clustered in SLE T-cells (63). MRs aggregation is observed in the mouse model of SLE (64). Atorvastatin reversed MR signaling abnormalities in SLE T-cells (65). FcγR engagement by ICs in many cell types drive IFN production (66). The CD4+ FcγRIIIa+IFN-γhigh cells generated via FcγRIIIa-pSyk signaling represent a new subset of T-cells. Co-expression of TLR proteins in these cells could render them refractory to Treg suppression (55).

ICs are often present in the immune deposits along with C5b-9 proteins. Recent studies have also shown that ICs are held without phagolysis by subcapsular sinus macrophages, B cells, and follicular dendritic cells on the cell membrane (67, 68). The retention and passive exchange of intact ICs occur among several cell types within the germinal centers. Follicular dendritic cells recycle the ICs, which make them accessible to antigen-specific B cells (68). In many disease tissues the formation of ectopic germinal centers are often observed. At these sites and in systemic circulation, ICs can drive differentiation of naïve CD4+ T-cells and produce IFN-γ, IL-17A, and IL-21, which can augment antigen-specific antibody responses (45).

During T<sub>j</sub>1 response, IFN-γ is produced in two waves, and the secondary IFN-γ production is driven by an autocrine IFN-γ signal (41). Two populations of IFN-γ-producing cells in SLE CD4+ T-cells IFN-γmoderate and IFN-γ-high were observed both in vivo and upon in vitro activation (Figs. 1, 2, and 7). The IFN-γmoderate population likely represents the bystander secondary T-cell response (Fig. 2E). In an in vitro experiment, a secondary short term challenge by ICs produced IFN-γmoderate cells, which was accompanied by a proportionate loss of IC binding (not shown). We examined cytokines in the SLE T-cells without PMA and ionomycin activation to avoid the influence of these potent T-cell activators. Our in vitro data on cytokine production and ICs binding was supported by the in vivo presence of such cells in SLE patients. Those SLE CD4+ T-cells that expressed the T-cell activation markers, CD25, CD69, and CD98, also showed pSyk, bound to labeled ICs, and produced cytokines, suggesting a role for Syk signaling (Fig. 7A). A role for anti-CD3 antibodies in rewiring of the CD3 complex with Syk has been suggested in SLE T-cells (48). However, the presence of anti-CD3 antibodies in SLE pathology has not been documented. In our experiments, anti-CD3-treated cells alone did not generate IFN-γ or IL-17A-producing populations. Therefore, we propose that this Syk rewiring of the TCR complex occurred from IC ligation of FcγRIIIa. Labeled ICs co-localize with CD3 complex in activated CD4+ T-cells, suggesting the presence of FcγRIIIa (11). ICOS-expressing cells showed pSyk, implying the co-presence of these proteins in activated T-cells. pSyk+ cells showed low levels of PD1 expression. Cells expressing high levels of PD1 did not show pSyk. PD1 is an inhibitory co-stimulating signal that acts by recruiting phosphatase SHP2. ZAP-70-deficient patients show abnormal peripheral CD4+ T-cells and express high levels of Syk, which drives T-cell activation (17). Kinase activity of Syk is 100-fold higher than that of

### TABLE 1

Expression of TLR pathway genes from two co-stimulations

| Gene symbol | Cells vs. CD28 | Cells vs. ICs + C5b-9 | Cells vs. ICs + C5b-9 | CD28 vs. ICs + C5b-9 |
|-------------|---------------|-----------------------|-----------------------|-----------------------|
| Btk         | 1.15          | 6.12                  | 5.34                  |                       |
| CD80        | 1.32          | 1.75                  | 1.32                  |                       |
| CD86        | 2.35          | 2.79                  | 1.18                  |                       |
| HSPD1       | 4.46          | 3.78                  | 0.85                  |                       |
| HRA5        | 0.74          | 2.85                  | 3.86                  |                       |
| HMGB1       | 2.81          | 20.66                 | 7.35                  |                       |
| IL-1A       | 1.56          | 5.92                  | 3.79                  |                       |
| IL-1B       | 2.6           | 11.25                 | 4.32                  |                       |
| IL-10       | 2.17          | 10.71                 | 4.95                  |                       |
| IL-12A      | 18.8          | 56.22                 | 2.99                  |                       |
| MAP3K7      | 0.31          | 0.93                  | 3.01                  |                       |
| MyD88       | 0.88          | 4.06                  | 4.6                   |                       |
| TICAM1      | 0.58          | 2.34                  | 4.02                  |                       |
| TLR3        | 0.24          | 4.13                  | 17.49                 |                       |
| TLR4        | 3.18          | 88.22                 | 27.77                 |                       |
| TLR5        | 1.28          | 5.34                  | 4.16                  |                       |
| TLR7        | 0.85          | 4.76                  | 5.62                  |                       |
| TLR7        | 3.45          | 13.16                 | 3.82                  |                       |
| TLR8        | 3.73          | 80.49                 | 21.56                 |                       |
| TLR9        | 4.45          | 17.59                 | 3.95                  |                       |
| TLR10       | 0.86          | 19.17                 | 22.24                 |                       |
| TRAF6       | 4.63          | 20.74                 | 4.48                  |                       |
ZAP-70, and Syk demonstrates a differential intrinsic activity compared with ZAP-70 (69). Syk is essential for innate responses and is a key signaling protein in B-cells (70). Further support for the role of Syk in CD4\(^+\) T-cell differentiation also comes from production of IFN-\(\gamma\) by P116 cells upon co-stimulation with ICs+C5b-9. These cells express Fc\(\gamma\)RIIIa upon activation (40). We speculate that the Fc\(\gamma\)RIIIa-pSyk-mediated IFN-\(\gamma\) production observed upon IC ligation is driven by the occupancy of the \(-53\) CpG site in the IFN-\(\gamma\) promoter by ATF2 (71). A 5.3-fold increase in ATF2 \((n = 5)\) was observed upon IC+C5b-9 co-stimulation normalized to the level of transcripts observed from CD28 co-stimulation (Fig. 9A). ATF2 also activates IL-23p19 promoter and has three binding sites in the IL-17 promoter.

A strong association of IL-17A and other T\(_{H}17\) cytokines in SLE pathogenesis in mouse model has been reported (20, 72). IL-23 cytokine, which is elevated in SLE patient sera, contributes to the terminal differentiation of pathogenic T\(_{H}17\) cells.
FcRs on dendritic cells is essential for production of IL-1 in inflammatory diseases. Simultaneous engagement of TLRs and members of these two receptor families has started to emerge in responses has been documented. Interplay between the membrane up-regulated expression of IFN pathway genes, suggesting a bifurcation of signaling events.

IFNs are critical in RA and SLE pathogenesis (25, 73, 74). DNA- and RNA-containing ICs trigger IFN-α production from plasmacytoid dendritic cells by engaging FcγRIIa with TLR7 and TLR9 (34, 75). We observed the expression of all 13 type I IFN genes, which were up-regulated by ICs + C5b-9 co-stimulation in one donor (Fig. 9, blue bars). This donor also showed a 20-fold increase in IRS2. Both IRS1 and IRS2 act as an adaptor for type I IFN-mediated signaling events. IRS2 is negatively regulated by cAMP response element-binding protein 3-like 4 (CREB3L4). A genetic interaction of the CREB, a co-activator with IL-12/STAT4 protein during T₃₁ differentiation, prolongs IFN-γ synthesis (76). We are not aware of any previous report of type I IFN expression in human CD4⁺ T-cells. IFN pathway gene array analysis showed a unique gene signature up-regulation in all three subjects analyzed (Fig. 9). The IFN gene expression profile suggests the ICs + C5b-9 co-signal differentially up-regulated expression of IFN pathway genes, suggesting a bifurcation of signaling events.

An individual role for TLRs and FcRs in inflammatory responses has been documented. Interplay between the members of these two receptor families has started to emerge in inflammatory diseases. Simultaneous engagement of TLRs and FcRs on dendritic cells is essential for production of IL-1β and IL-23 (77). A cross-talk between TLRs and FcRs initiated by ICs and pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) in autoimmune diseases is now proposed (77). Human CD4⁺ T-cells and Jurkat cells express RNA that encodes most of TLRs (78). TLRs play a role in T-cell activation and differentiation during autoimmunity (35, 78). TLRs also modulate CD4⁺ T-cell response (79, 80). In human naïve CD4⁺ T-cells, ICs + C5b-9 co-stimulation up-regulated TLR2, -3, -5, -8, -10, HMGB1, and MyD88 gene transcripts (Fig. 10). This suggests that the activation of CD4⁺ T-cells by ICs + C5b-9 sensitize them for danger signals. The TLR5 ligand, flagellin in the presence of suboptimal antigen presentation in APC triggers cell proliferation, as well produces IFN-γ and IL-8 (81). Chromatin-IgG complexes activate B-cells by dual engagement of B-cell receptor and TLR (82). Such synergism between TCR and FcRs with TLRs in CD4⁺ T-cells has not been shown but could occur in human FcγRIIa⁺CD4⁺ T-cells. FcγRIIa-TLR engagement will have a wide-ranging implication in autoimmunity. DNA-ICs ligate FcγRIIa in plasmacytoid dendritic cells and up-regulate TLR9, which then drive the IFN response in SLE. Our results suggest that in CD4⁺ T-cells FcγRIIa could co-operate with TLRs to drive proliferation and IFN production (11, 40). TLR adaptor molecule-1 (TICAM1), which interacts with TLR3 and other MyD88-dependent TLRs, were up-regulated. In response to double-stranded RNA viruses, TLR3 produces IFN-β via IRF3. Poly I:C, a TLR3 ligand, activates human CD4⁺ T-cells (83). In the same study the TLR5 expression was also observed on CD4⁺ T-cells, but the activation with flagellin was not sufficient to activate these cells. Even though TLR responses in the CD4⁺ cells has been documented, the receptor by which the nucleic acids are delivered to endolysosomes is unknown. Our results suggest a possible role of FcγRIIIa in delivering DNA-ICs to endolysosomes in activated CD4⁺ T-cells where they could interact with TLR9 (Fig. 11). DNA- or RNA-containing ICs via HMGB1 can efficiently deliver self nucleic acid to TLR containing endolysosomes (84). HMGB1 is a DNA chaperon that is capable of organizing dynamic active chromatin structures. It is diffusely distributed in cytoplasm and is released from inflamed cells actively or from apoptotic and necrotic cells. Elevated serum levels of HMGB1 are observed in SLE patients during flares. HMGB1 has a pro-inflammatory effect, which is mediated via TLR2, -4, and -9 (78). ICs + C5b-9 co-signal up-regulated HMGB1 gene transcripts in naïve CD4⁺ T-cells and the HMGB1 protein co-localized with ICs in human CD4⁺ T-cells and P116 cells. Ablation of MyD88 in CD4⁺ T-cells impairs both T₃₁ and T₅₁₇ responses (56). We observed the overexpression of MyD88 transcripts and MyD88 protein co-localized with ICs. Both HMGB1 and MyD88 proteins were observed in co-immunoprecipitates obtained using anti-FcγRIIIa/b antibodies from P116 cells. A role for MyD88 in proliferation and IFN-γ production in mice infected with *Ehrlichia muris* has been observed (85). TLR9 agonist in CD4⁺ T-cells enhanced proliferation, survival, and IL-2 secretion (79). Subcellular localization of TLR9 using HEK293T cells has been shown to be critical in discriminating self versus non-self DNA (37). TLR9 reside in endoplasmic reticulum, and upon stimulation from CpG DNA it is recruited to lysosomes (86). Upon ICs + C5b-9 co-stimulation in P116 cells TLR9 protein localized in endolysosomes with ICs. This pattern was confirmed in human CD4⁺ T-cells. We also observed membrane staining for TLR9 with cytoplasmic IC binding. This suggests a possible role for FcγRIIIa in recruiting TLR9 to endo lysosomes. The significance of these events in the development of autoimmune response remains to be determined. Our results suggest a critical role for FcγRIIIa-pSyk signal in CD4⁺ T-cell-mediated adaptive immunity.

In summary, our results establish a co-stimulatory role for ICs + C5b-9 in the development of the CD4⁺ IFN-γ high cell subset and a T₅₁₁十七 like population. ICs + C5b-9 provides a distinct co-stimulatory signal for the up-regulation of the IFN and TLR signaling pathway genes. The data provide a link for ICs in driving TLR-dependent T-cell activation in autoimmunity (35). T-cell signaling responses by TLRs result in tolerance breakdown and bystander activation of auto reactive T₃₁ and T₅₁₁十七条 cells. An abnormal activating co-stimulatory signal from ICs + C5b-9 during immune contraction can override the inhibition by CTLA-4 and PD1, resulting in peripheral tolerance breakdown. A further understanding of ICs + C5b-9 signaling in CD4⁺ T-cells will lead to a better understanding of the role of CD4⁺ T-cells in diseases like SLE. These findings will not only be relevant to autoimmune disorders but also in cardiovascular diseases, cancers, and viral infections. Both PD1 and CTLA-4 proteins are therapeutic targets. A role for activating FcRs is also suggested in the therapies targeting CTLA-4. It is important to further explore the role of FcγRIIIa signaling in CD4⁺ T-cells.

3 A. K. Chauhan, unpublished observation.
**Introduction**

Regulatory T cells (Tregs) play a critical role in maintaining immune tolerance and preventing autoimmunity. They are characterized by the expression of the transcription factor FoxP3 and specific cell surface markers such as CD4 and CD25. Tregs are essential in the control of autoimmune diseases, but their plasticity and the mechanisms governing their differentiation and function are still under investigation.

**Results and Discussion**

Recent studies have provided new insights into the regulation of Treg plasticity. For instance, the interplay between FoxP3 and other transcription factors, such as RORγt, has been shown to govern the fate of Tregs. Additionally, the role of cytokines and environmental factors in shaping Treg lineage has been highlighted. These findings suggest that Tregs are not static cells but can adapt to different immunological environments.

**Conclusions**

Understanding the mechanisms of Treg plasticity is crucial for developing therapeutic strategies to modulate autoimmune responses. Future studies should focus on identifying the key factors that govern Treg differentiation and function, with the ultimate goal of developing novel therapeutic interventions for autoimmune diseases.

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