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Structural and Functional Changes of the Invariant NKT Clonal Repertoire in Early Rheumatoid Arthritis

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Invariant NKT cells (iNKT) are potent immunoregulatory T cells that recognize CD1d via a semi-invariant TCR (iNKT-TCR). Despite the knowledge of a defective iNKT pool in several autoimmune conditions, including rheumatoid arthritis (RA), a clear understanding of the intrinsic mechanisms, including qualitative and structural changes of the human iNKT repertoire at the earlier stages of autoimmune disease, is lacking. In this study, we compared the structure and function of the iNKT repertoire in early RA patients with age- and gender-matched controls. We analyzed the phenotype and function of the ex vivo iNKT repertoire as well as CD1d Ag presentation, combined with analyses of a large panel of ex vivo sorted iNKT clones. We show that circulating iNKTs were reduced in early RA, and their frequency was inversely correlated to disease activity score 28. Proliferative iNKT responses were defective in early RA, independent of CD1d function. Functional iNKT alterations were associated with a skewed iNKT-TCR repertoire with a selective reduction of high-affinity iNKT clones in early RA. Furthermore, high-affinity iNKTs in early RA exhibited an altered functional Th profile with Th1- or Th2-like phenotype, in treatment-naïve and treated patients, respectively, compared with Th0-like Th profiles exhibited by high-affinity iNKTs in controls. To our knowledge, this is the first study to provide a mechanism for the intrinsic qualitative defects of the circulating iNKT clonal repertoire in early RA, demonstrating defects of iNKTs bearing high-affinity TCRs. These defects may contribute to immune dysregulation, and our findings could be exploited for future therapeutic intervention. *The Journal of Immunology*, 2015, 195: 5582–5591.

Invariant NKT (iNKT) cells are CD1d-restricted T lymphocytes that exert powerful regulatory functions during early innate and ensuing adaptive immune responses. In contrast to conventional T cells, which recognize peptide Ags presented by polymorphic HLA molecules, iNKT cells recognize lipid Ags presented by the nonpolymorphic CD1d protein (1). An essential role for iNKT cells in the induction and maintenance of immunological tolerance has been demonstrated in many animal models of autoimmune inflammation (2). Indeed, immunotherapy targeting

iNKT cells prevents the onset of autoimmunity in animal models of arthritis, type 1 diabetes, and autoimmune encephalitis (2). However, a deeper understanding of human iNKT biology is needed to successfully translate these findings into patients with autoimmune disease (3–7).

It has been established that total iNKT cell numbers are reduced in peripheral blood and tissues in diverse human autoimmune conditions, including rheumatoid arthritis (RA), but to date very little is known about the qualitative defects of this repertoire (8, 9). We have previously shown that the human iNKT repertoire in healthy adults is composed of clones expressing iNKT TCRs (iNKT-TCRs) with widely variable affinities for CD1d (10) that were directly dependent on structural differences within the CDR3 β loop of the iNKT-TCR. Clonally distributed differences in iNKT-TCR affinity to CD1d of up to 40-fold were observed, and these differences in TCR affinity were directly correlated to iNKT function. Indeed, compared with low-affinity clones, iNKT clones bearing high-affinity iNKT-TCRs proliferated more avidly, and secreted increased amounts of cytokines in response to CD1d expressing APC (10). Hence, we hypothesized that such differential activation of low- and high-affinity iNKT clones may skew the human iNKT repertoire and exert an impact on its regulatory functions.

In this cross-sectional study, we examined the iNKT repertoire in early RA, as an example of a classic autoimmune condition, and compared it with age- and gender-matched controls. Our results demonstrate a previously unknown mechanism underlying iNKT dysfunction in early RA that was characterized by a shift in the iNKT-TCR repertoire in early RA patients, and a pronounced loss of higher-affinity iNKT clones. These differences in iNKT-TCR affinity are paralleled by differences in iNKT function, with a reduced iNKT proliferative capacity and a skewed cytokine

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Abbreviations used in this article: CRP, C-reactive protein; DAS, disease activity score; DMARD, disease-modifying antirheumatic drug; ESR, erythrocyte sedimentation rate; iNKT, invariant NKT; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus.

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response in early RA. To our knowledge, these results show for the first time that the iNKT repertoire undergoes significant *in vivo* changes in patients with early RA, which are related to clonal differences in iNKT-TCR affinity to CD1d.

Materials and Methods

Study participants

Early RA patients with onset of clinical disease manifestations within the last 36 mo before recruitment into the study and age- and gender-matched controls were recruited from the Early Arthritis Clinic in University Hospital Southampton between May 2011 and March 2013 (Table I). The diagnosis of RA in the study participants was based on the 1987 American College of Rheumatology (11) criteria. In all included RA patients, the 28 joint disease activity score (DAS28) was assessed by the same study nurse. DAS28 assessment includes swollen and tender joint score, patient global disease activity score, and erythrocyte sedimentation rate (ESR) or C-reactive protein (CRP). Ethical approval was granted by the National Research Ethics Service Committee South Central (REC Ref 09/H0502/107). Identity of all study participants was anonymized and encrypted immediately after inclusion into the study.

Lipid Ags

Lyophilized KRN7000 and OCH (both from AXXORA) were solubilized in vehicle (150 mM NaCl, 0.5% Tween 20) to 200 μ g/ml using repeated 1-min pulses of sonication and heating to 80°C.

CD1d tetramers and CD1d MACSibeads

CD1d tetramers. First, KRN7000- and OCH-loaded CD1d/ β 2-microglobulin complexes were generated by *in vitro* refolding, as previously described, and biotinylated via an engineered BirA motif on the C terminus of CD1d (12). Biotinylated lipid-loaded CD1d/ β 2-microglobulin complexes were again purified by size exclusion chromatography before conjugation to PE-streptavidin (Sigma-Aldrich) to generate lipid-CD1d tetramers (13).

CD1d MACSibeads. A total of 10 μ g biotinylated KRN7000-CD1d monomers, 5 μ g biotinylated anti-CD2, and 5 μ g biotinylated anti-CD28 mAbs was conjugated to 5×10^7 anti-biotin Ab-coated MACSibeads (Miltenyi Biotec) and stored in PBS containing 2 mM EDTA, 0.3% BSA (Sigma-Aldrich), and 0.4% sodium azide (Melford) at 4°C.

Human cell lines and clones

PBMCs were isolated from fresh human blood by density gradient centrifugation (Ficoll-Hypaque; GE Healthcare).

Human iNKT clones were generated from PBMCs, as previously described (10). Briefly, single live CD3⁺V α 24⁺V β 11⁺ T cells were sorted into 96-well plates using FACSaria and cultured in the presence of 1 μ g/ml PHA (Sigma-Aldrich) and γ -irradiated (35 Gy) autologous feeder PBMCs in complete RPMI 1640 (Lonza) containing 10% FBS (Sigma-Aldrich), 200 IU IL-2 (Proleukin, Chiron), 50 ng/ml IL-7, 50 ng/ml IL-15 (Immunotools), 1 mM sodium pyruvate, 1% nonessential amino acids, 1% L-glutamax, 50 μ M 2-ME, 100 IU penicillin, 100 μ g/ml streptomycin, and 2% human AB serum (all Sigma-Aldrich).

T2 lymphoblasts (T2-) and stably CD1d-expressing T2 lymphoblasts (T2-CD1d) were maintained in complete medium (RPMI 1640 supplemented with 10% FBS, 1% nonessential amino acids, 1% L-glutamax, 1 mM sodium pyruvate, 100 IU penicillin, and 100 μ g/ml streptomycin).

Flow cytometry

The following fluorescent reagents were used for cell staining: PE-conjugated human CD1d tetramers loaded with either KRN7000 or OCH; PE anti-V α 24 and FITC anti-V β 11 (both Beckman Coulter); allophycocyanin anti-CD3 and allophycocyanin anti-CD4 (Immunotools); PE anti-human CD1d (clone CD1d42) and PE-conjugated IgG2bk isotype control (both from BD Biosciences); and propidium iodide (Sigma-Aldrich) was used to exclude dead cells. Samples were acquired on a FACSCalibur flow cytometer, and data processed using CellQuest Pro software (BD Biosciences).

Functional studies

In vitro expansion of human iNKTs using autologous CD14⁺ monocytes. PBMC were first depleted of monocytes and B cells using anti-CD14 and anti-CD19 MicroBeads. Then, 1×10^6 CD14⁻CD19⁻ PBMC were cocultured in 48-well plates with 1×10^5 Ag (KRN7000, OCH) or vehicle-pulsed autologous CD14⁺ monocytes and cultured in complete medium at 37°C, 5% (v/v) CO₂ for 14 d.

In vitro expansion of human iNKTs using MACSibeads. A total of 1×10^6 CD14⁻CD19⁻ PBMC was cultured in 48-well plates in complete medium with 5×10^5 MACSibeads. Seven days later, IL-2 (400 IU/ml) was added to cultures. Expansion of iNKT cells was assessed by FACS at day 14 in all cultures.

Cytokine secretion of iNKT clones. T2- and T2-CD1d were used as APC. Fifty-seven randomly selected iNKT clones from patients and controls were plated in triplicates at 1×10^5 and a 1:1 ratio in 96-well round-bottom plates in complete medium. Clones were stimulated with either 1×10^5 KRN7000-pulsed T2-CD1d, 1×10^5 KRN7000-pulsed T2-, or media alone. Supernatants were collected after 24 h, and the concentration of IFN- γ , TNF- α , IL-4, and IL-10 was measured using a Bio-Plex 200 system (Bio-Rad Laboratories).

Stimulation of CD1d expression on monocytes. Freshly purified CD14⁺ monocytes were cultured at 1×10^5 cells/well in a 48-well plate in 1 ml serum-free medium, and either of the following reagents was added: 1 ng/ml TNF- α and IFN- γ (Immunotools); 1 ng/ml PG J2 or 1 ng/ml all-*trans* retinoic acid (Sigma-Aldrich). CD1d cell surface expression on live monocytes was measured 24 h later by FACS.

Statistical analysis and definition of iNKT-TCR affinity

GraphPad Prism and STATA software were used for statistical analysis, and *p* values ≤ 0.05 were considered statistically significant. Fisher's *r*- to *z*-transformation was used to assess the significance of the difference between two correlation coefficients. The pcor.test method in the pcor package in R was used to assess the correlation (Spearman) of DAS28 and circulating iNKT frequency while controlling for age. iNKT clones of low- or high-affinity were identified following staining with the following: 1) KRN7000-CD1d tetramers and 2) OCH-CD1d tetramers. iNKT-TCR affinity values were expressed as the ratio of the mean fluorescence intensity of OCH:KRN7000 staining.

Results

One hundred early RA patients (46 treatment-naïve and 54 treated [24 patients treated with disease-modifying antirheumatic drugs (DMARDs), 25 patients treated with steroids, and 5 treated with both]) and 54 age- and gender-matched control subjects (33 healthy, 19 osteoporosis, 2 osteoarthritis) participated in the study. Table I shows the demographic data for all study participants. Individual experiments were performed on all samples from this cohort. The numbers of early RA patients and age- and gender-matched controls from which data were obtained for each experiment are stated in the figures.

Numerical iNKT deficiency in peripheral blood in early RA correlates with disease activity (DAS28)

Numerical iNKT defects have been previously described in RA and other autoimmune conditions (8, 9). In this work, we used CD1d tetramers to determine the *ex vivo* iNKT frequency in the peripheral blood of a larger cohort of early RA patients and compared it with age- and gender-matched controls (Fig. 1A). In agreement with published reports (8, 14), the frequency of circulating iNKTs was significantly reduced ($p < 0.001$) in early RA patients compared with age- and gender-matched controls (Fig. 1B). No association of iNKT frequency with treatment status, gender, or the duration of treatment was found (Supplemental Fig. 1A–C). In contrast, the frequency of circulating iNKTs was inversely correlated with age in early RA patients (Spearman's $r = -0.3$ [$p < 0.003$]; Fig. 1D) and controls (Spearman's $r = -0.5$ [$p < 0.0001$]; Fig. 1C). Furthermore, we found a significant age-independent, inverse correlation of iNKT frequency with disease activity, as measured by DAS28, in early RA patients (Spearman's $r = -0.2$ and $p < 0.01$; Fig. 1E, 1F). Additionally, in separate analyses, we compared iNKT frequency in early RA patients with their DAS28 scores while controlling for age. The inverse correlation between iNKT frequency and DAS28 was age independent (Spearman's $r = -0.228$ and $p = 0.031$). We also found a significant inverse correlation between iNKT cell frequency and systemic inflammation markers of CRP (Spearman's $r = -0.38$ and $p = 0.01$; Fig. 1G) and ESR (Spearman's $r = -0.3$ and $p = 0.02$; Fig. 1H). This relationship was only observed in the treatment-naïve

Table I. Demographics of the study population

	Early RA			
	Controls	All RA	Treatment-Naive	Treated
Number of subjects, <i>n</i> (%)	54 ^a	100	46 (46)	54 (54)
Age median, years (range)	53 (21–91)	56 (20–82)	59 (20–82)	54 (20–82)
Female/male (%)	69/31	61/39	74/26	50/50
RF positive, <i>n</i> (%)	ND	57 (57%)	24 (42%)	30 (58%)
ACCP positive, <i>n</i> (%)	ND	54 (54%)	23 (44%)	33 (56%)
ACCP concentration μ/ml, median (range)	ND	54 (0.1–411)	57 (0.7–340)	46.2 (0.1–411)
DAS28 median (range)	NA	4.6 (0.7–7.9)	4.6 (2–9)	4.1 (0.7–7.1)
CRP median (range)	NA	9 (1–218)	8 (1–92)	9.5 (1–218)
ESR median (range)	NA	24 (1–81)	24.5 (2–74)	22.5 (1–81)
DMARDs, <i>n</i> (%)	NA	24 (24%)	0 (0%)	24 (44%)
Steroids, <i>n</i> (%) ^b	NA	25 (25%)	0 (0%)	25 (46%)
DMARDs and steroids, <i>n</i> (%)	NA	5 (5%)	0 (0%)	5 (10%)

DMARDs: methotrexate, hydroxychloroquine, sulfasalazine. Steroids: depo-medrone, prednisolone.

^a33 healthy, 19 osteoporosis, 2 osteoarthritis.

^b5–15 mg/day.

ACCP, anticyclic citrullinated peptide Ab; NA, not available; RF, rheumatoid factor.

early RA patient cohort. The inverse correlation between iNKT cell frequency and CRP or ESR in patients undergoing treatment with immunosuppressive drugs was not significant (data not shown). Taken together, these results were consistent with the hypothesis that iNKT cells exert regulatory functions in early RA, and that loss of these iNKT functions may exacerbate the disease.

Impaired Ag-specific proliferative response of iNKT cells in early RA

Previous studies have shown that, unlike those from healthy donors, iNKT cells from RA patients have a defective *in vitro* response to the potent iNKT agonist KRN7000 (8, 9). However, various factors may have underlain these differences, for example, different iNKT:APC ratios used in the *in vitro* assays, differences in CD1d expression on RA and control APCs, or intrinsic iNKT repertoire differences between RA patients and controls.

To address this, we first stimulated monocyte- and B cell-depleted PBMC (CD14⁺CD19⁺ PBMC) at a fixed ratio with Ag- or vehicle-pulsed autologous CD1d⁺ monocytes, and subsequently determined iNKT expansion in these cultures after 2 wk (Fig. 2A, 2B). The results of these experiments showed that iNKT cells in early RA patients were significantly impaired in their *in vitro* expansion in response to Ags compared with age- and gender-matched controls, independent of the iNKT:APC ratio (Fig. 2C, 2D). Interestingly, these differences between early RA patients and controls were markedly more pronounced after stimulation with the partial iNKT agonist OCH ($p = 0.006$; Fig. 2D) compared with the full iNKT agonist KRN7000 ($p = 0.01$; Fig. 2C). Conversely, the patients' treatment status did not seem to exert a significant impact (Supplemental Fig. 2A). These results confirmed earlier reports and indicated the presence of qualitative functional defects within the circulating iNKT pool in early RA.

The impaired iNKT proliferative response in early RA is independent of CD1d Ag presentation

The reduced iNKT proliferative response in early RA patients shown above may simply reflect defective CD1d Ag presentation in RA. To address this, we first examined CD1d cell surface expression on monocytes and B cells from early RA patients and age- and gender-matched controls by flow cytometry (Fig. 3). No significant differences in cell surface CD1d expression on freshly isolated monocytes or B cells from early RA patients and controls were observed (Fig. 3A). Furthermore, CD1d expression on monocytes from early RA patients and controls was highly similar after

overnight stimulation of the monocytes with either TNF- α and IFN- γ , PG J2 (PGJ2), or all-*trans* retinoic acid (15, 16), that is, inflammatory mediators previously reported for their ability to upregulate CD1d cell surface expression (Fig. 3B). Finally, we devised a cell-free system for the Ag-specific stimulation of iNKTs and used it to compare the proliferative responses of iNKTs from early RA patients and controls. For this, we conjugated recombinant KRN7000-CD1d protein-lipid complex, anti-CD2 Abs, and anti-CD28 Abs to MACSibeads and used these (CD1dMACSibeads) to stimulate CD14⁺CD19⁺ PBMCs from early RA patients and controls. The results of these experiments showed that CD1dMACSibeads were significantly less efficient in expanding iNKTs from early RA patients compared with controls ($p = 0.01$; Fig. 3C, Supplemental Fig. 2B). Together, these results indicated that the observed numerical and qualitative defects of the circulating iNKT repertoire in early RA are due to iNKT cell-intrinsic factors rather than a defective CD1d expression or Ag presentation.

The iNKT repertoire in early RA is characterized by a reduced iNKT-TCR affinity to CD1d

To examine the possible involvement of the iNKT-TCR in the above described alterations of the iNKT repertoire in early RA, we generated a large panel of iNKT clones from early RA patients and controls. In total, 296 iNKT clones from 27 patients and 21 controls were used for these analyses. First, we examined iNKT-TCR expression levels on early RA and control clones by flow cytometry, staining each clone with anti-TCR V α 24 Abs or KRN7000-CD1d tetramers in two separate experiments. Similar to our previous results, anti-V α 24 TCR staining strongly correlated with the binding of these iNKT clones to KRN7000-CD1d tetramers (Spearman's $r = 0.7$; Supplemental Fig. 3A) (10). Importantly, TCR expression levels on early RA and control iNKT clones were highly similar (Fig. 4A).

Next, we assessed the iNKT-TCR-binding strength to CD1d for each of these clones by measuring each clone's binding to OCH-CD1d tetramers in relation to KRN7000-CD1d tetramers (OCH:KRN7000 ratio). We have previously demonstrated that results obtained with this assay correlate linearly with iNKT-TCR/CD1d affinity measurements in surface plasmon resonance (BiaCore) (10).

As shown in Fig. 4B, the iNKT clonal repertoire in early RA exhibited a highly significant reduction of the OCH:KRN7000 ratio compared with age- and gender-matched control iNKT clones ($p < 0.001$), consistent with a relevant difference in iNKT-TCR-binding affinity to CD1d between the two groups. Of note,

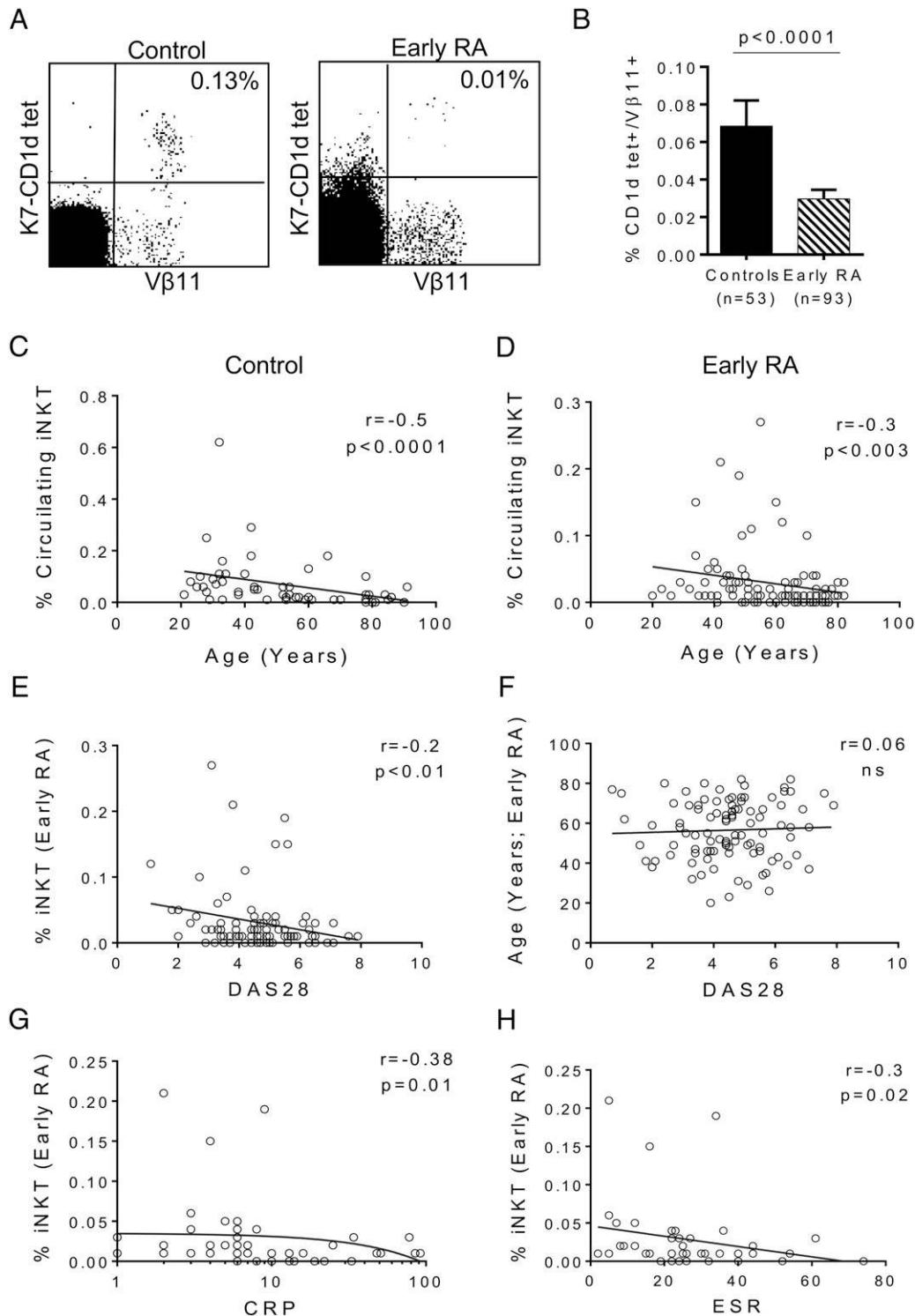


FIGURE 1. iNKT cell frequency in early RA patients and controls. **(A)** Ex vivo flow cytometry staining of live CD3⁺ iNKT cells (KRN7000-CD1d tet⁺ Vβ11⁺) from the peripheral blood of a representative control individual and an early RA patient. **(B)** Cumulative staining data of iNKT frequency in early RA and controls demonstrate lower iNKT frequency in early RA patients. **(C and D)** Spearman’s correlation of ex vivo iNKT frequency with age in controls (C) and early RA (D). In both groups, iNKT frequency declines with age. **(E and F)** Spearman’s correlation of DAS28 with ex vivo iNKT frequency (E) and age (F) in early RA, demonstrating an inverse correlation between DAS28 score and iNKT frequency in early RA patients, but no correlation with age. **(G)** Spearman’s correlation of ex vivo iNKT frequency with CRP in treatment-naïve early RA patients. **(H)** Spearman’s correlation of ex vivo iNKT frequency with ESR in treatment-naïve early RA patients. iNKT frequency inversely correlates with both ESR and CRP in treatment-naïve early RA patients.

these differences were independent of treatment status because iNKT clones from treatment-naïve and treated patients exhibited highly similar iNKT-TCR affinities (Fig. 4C). In separate analyses, the clones with the highest iNKT-TCR affinity (OCH:KRN7000 ratio) in each early RA patient and control were compared. The

results from this analysis confirmed a significant difference between the two groups ($p < 0.04$), suggesting a selective loss of high-affinity iNKTs in early RA (Fig. 4D). Furthermore, a similar analysis of clones with the lowest iNKT-TCR affinity in each early RA patient and control corroborated the above results, thereby

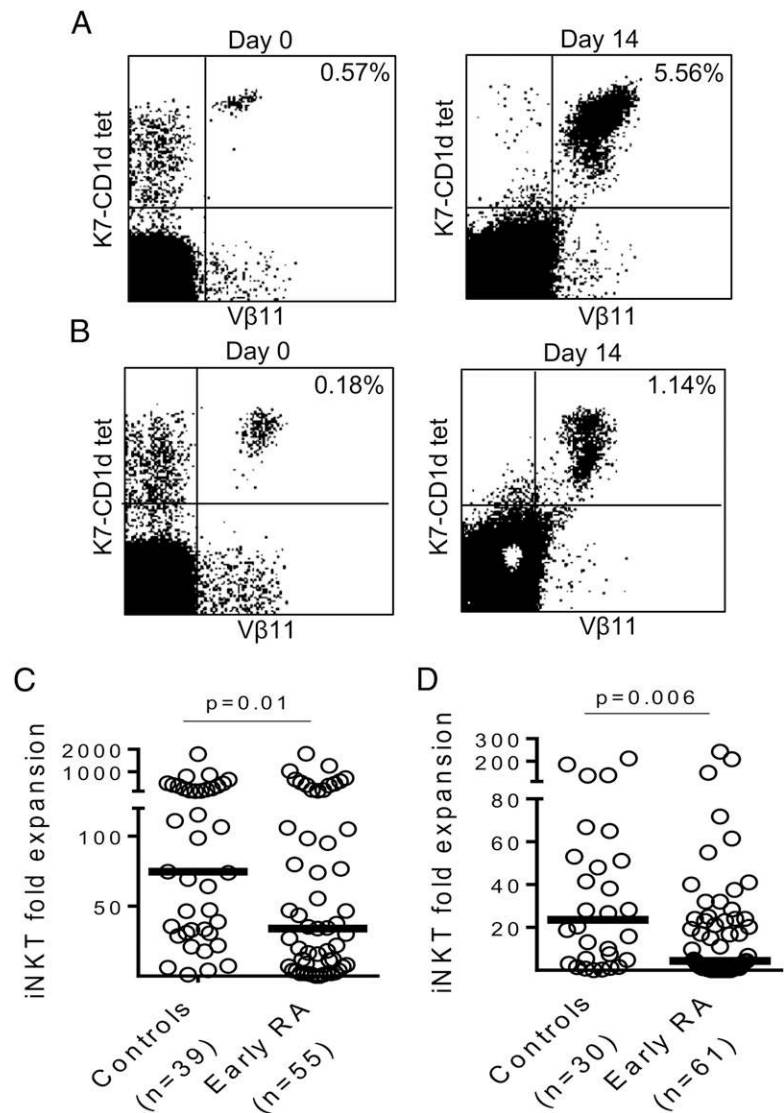


FIGURE 2. In vitro Ag-induced iNKT expansion in early RA patients and controls. Live CD3⁺KRN7000-CD1d tet⁺Vβ11⁺ iNKT cells were stained on day 0 ex vivo and again on day 14 after in vitro culture with KRN7000-pulsed CD1d⁺ monocytes. Plots show percentage of iNKT cells, ex vivo and after in vitro culture, in a control (A) and an early RA patient (B). (C) Peripheral blood T cells were stimulated with monocytes pulsed with either KRN7000 or OCH. Data showing fold expansion (ratio of in vitro grown iNKT/ex vivo frequency) of iNKT cells in response to KRN7000 (C)- or OCH (D)-pulsed CD1d⁺ monocytes in early RA patients and controls. In early RA, expansion in response to both stimuli is reduced.

suggesting a selective loss of high-affinity iNKTs in early RA and an overall shift in the iNKT-TCR affinity repertoire between early RA and controls (Supplemental Fig. 3B). Additionally, we did not find correlations between iNKT-TCR affinity and clonal CD4 coreceptor expression; however, compared with controls, significantly more iNKT cells in early RA were CD4⁺ (Supplemental Fig. 3C). Taken together, these data demonstrate significant qualitative intrinsic changes of the iNKT repertoire in early RA, which are related to a reduction of iNKT clones bearing higher-affinity TCRs.

High-affinity iNKT clones in early RA have an altered functional profile

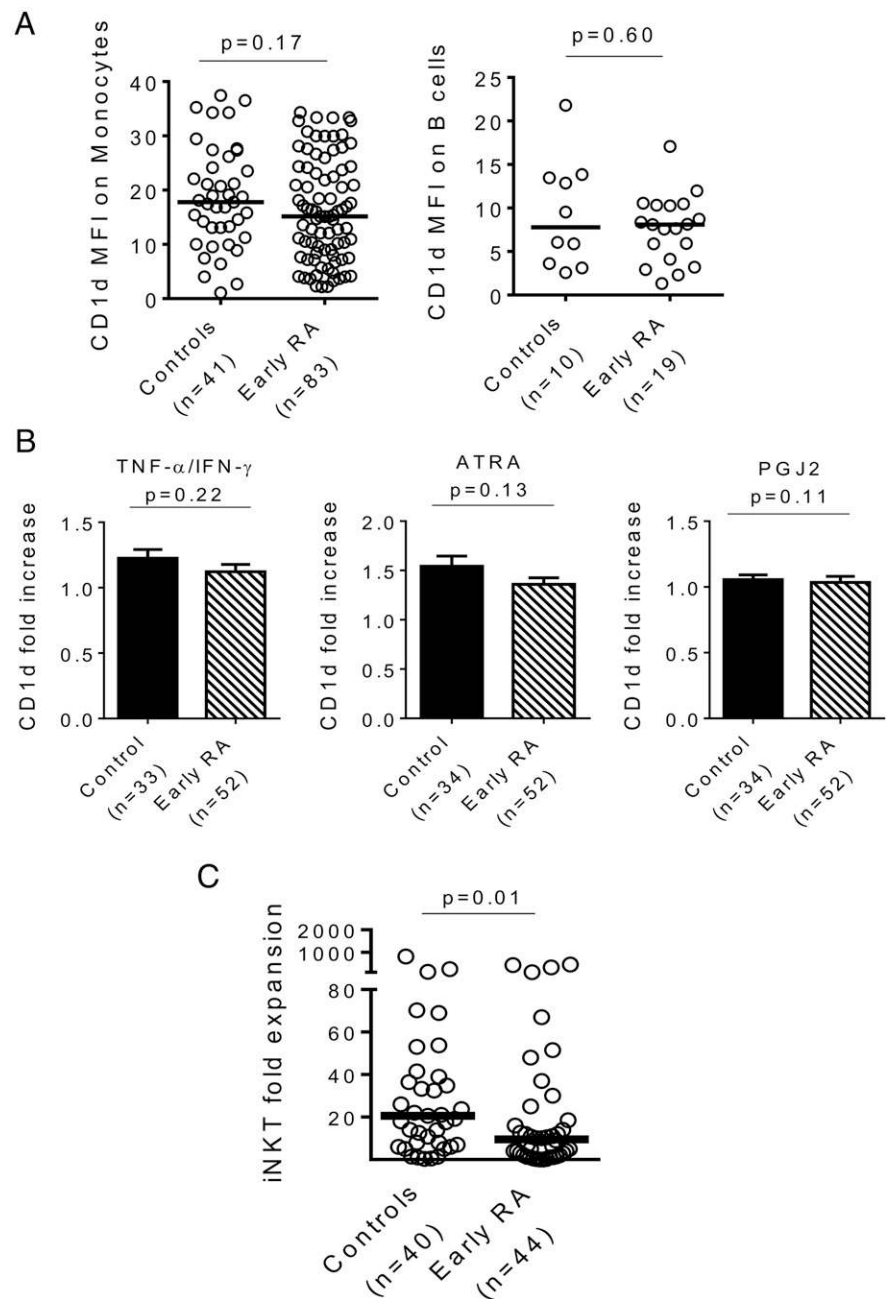
To further characterize the circulating iNKT repertoire in early RA, we compared the CD1d-dependent cytokine secretion patterns of 37 iNKT clones from early RA patients (17 from treatment-naïve and 20 from treated [see Supplemental Table I for treatment statistics]) versus 20 iNKT clones from controls. Based on their cytokine secretion pattern in response to KRN7000-pulsed T2-CD1d, the clones were classified as either Th0-like (IFN-γ⁺ and/or TNF-α⁺, and IL-4⁺ and/or IL-10⁺), Th1-like (IFN-γ⁺ and/or TNF-α⁺ IL-4⁻, IL-10⁻), or Th2-like (IL-4⁺ and/or IL-10⁺ and IFN-γ⁻, TNF-α⁻) (Supplemental Fig. 3D).

The majority of iNKT clones from controls (16 of 20 clones, 80%) exhibited a Th0-like cytokine pattern with combined se-

cretion of IFN-γ, TNF-α, and IL-4 ($n = 13$); IFN-γ, TNF-α, and IL-10 ($n = 2$); or TNF-α and IL-4 ($n = 1$) (Fig. 5A). In contrast, Th0-like iNKT clones were significantly less frequent in both treatment-naïve ($p = 0.02$; 7 of 17 clones, 41%) and treated ($p = 0.023$; 8 of 20 clones, 40%) early RA patients. The remaining four clones (4 of 20; 20%) from control subjects were all classified as Th1-like based on their secretion of IFN-γ and/or TNF-α and lack of IL-4 and IL-10. A similar proportion of Th1-like iNKT clones (3 of 20; 15%) was present in treated early RA patients. In contrast, Th1-like iNKT clones were significantly more frequent in treatment-naïve early RA patients (10 of 17; 59%) compared with either treated patients ($p = 0.07$) or controls ($p = 0.02$) (Fig. 5A). Interestingly, a large proportion of iNKT clones from treated patients (9 of 20; 45%) exhibited a Th2-like pattern characterized by the release of IL-4 only ($n = 7$), IL-4 and IL-10 ($n = 1$), or IL-10 only ($n = 1$) compared with clones from controls ($p = 0.001$) or treatment-naïve patients ($p = 0.002$). However, we did not find correlations between the type of treatment and Th2-like pattern, indicating that patients undergoing either DMARD or steroid immunosuppressive drugs had iNKT clones with a Th2-like pattern (Supplemental Table I).

Analysis of the functional profiles of clones bearing high-affinity iNKT-TCRs in these groups revealed that nearly all iNKT clones with a high-affinity iNKT-TCR from controls (12 of

FIGURE 3. Analysis of CD1d expression in monocytes and B cells from early RA patients and controls. **(A)** CD14⁺ monocytes and CD19⁺ B cells were purified from the peripheral blood of early RA patients and controls. Monocytes and B cells were stained with anti-CD1d and an isotype control. CD1d mean fluorescence intensity (ratio of anti-CD1d/isotype) on the cell surface of monocytes and B cells from early RA patients and controls is presented. No difference in CD1d expression was observed. **(B)** CD14⁺ monocytes were cocultured overnight with either PBS, TNF- α /IFN- γ , all-*trans* retinoic acid, or PGJ2. Plots show CD1d expression fold increase, in response to inflammatory stimuli, on monocytes from early RA patients and controls. No difference between controls and early RA patients was observed. **(C)** CD14⁻CD19⁻ peripheral blood T cells were stimulated with KRN7000-CD1d MACSibeads. Live CD3⁺KRN7000-CD1d tet⁺V β 11⁺ iNKT cells were stained after 14 d of *in vitro* iNKT expansion. Data show iNKT fold expansion (ratio of *in vitro* grown iNKT/*ex vivo* frequency) in early RA patients and controls. iNKT expansion is reduced in early RA patients.



14; 85%) exhibited a Th0-like pattern, compared with (5 of 10; 50%) clones in treatment-naïve, and (5 of 15; 33.3%) clones from treated early RA patients. This indicates that a significant proportion of high-affinity iNKT clones in controls exhibited a Th0-like pattern, which was absent in high-affinity iNKT clones in early RA ($p = 0.0004$) (Fig. 5B). In contrast, no differences were observed when we compared the functional cytokine profiles of clones bearing low-affinity iNKT-TCRs between groups (Fig. 5B). Altogether, these data reveal substantial functional alterations of the clonal iNKT repertoire in early RA, and most importantly reveal an altered functional profile for iNKT clones bearing high-affinity iNKT-TCRs in early RA.

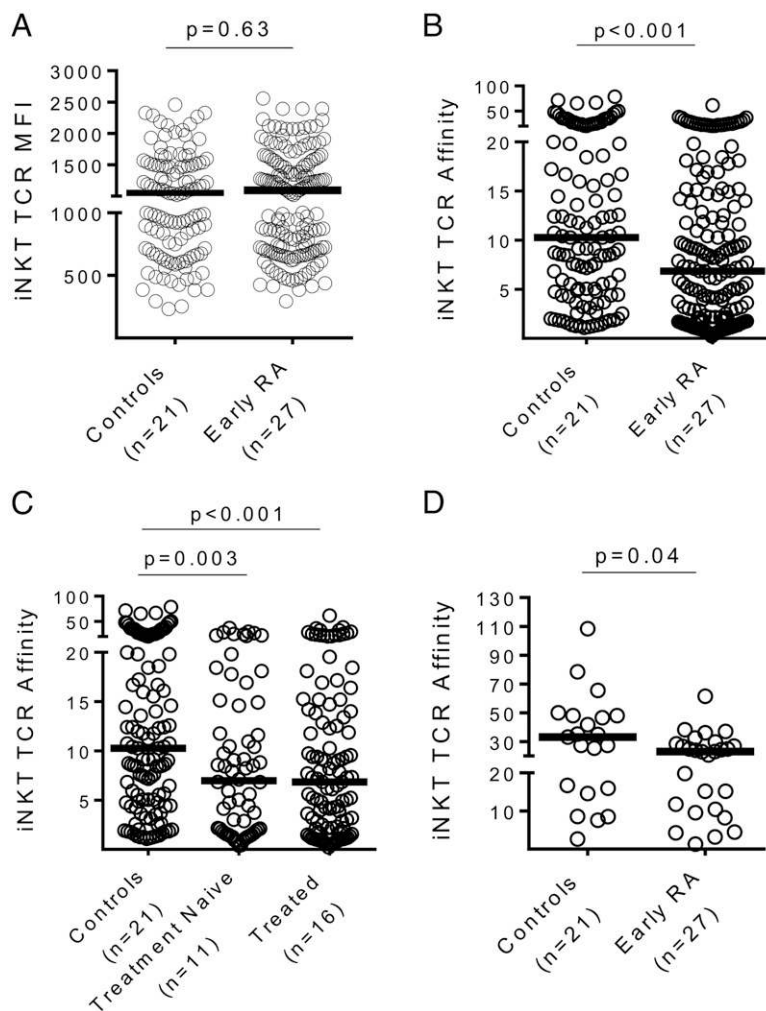
Discussion

CD1d-restricted iNKT cells are potent and versatile regulators of both innate and adaptive immunity. A large body of evidence, including data from preclinical models and phenotypic studies in different human autoimmune diseases, points toward a critical role

for iNKT cells in both the induction and maintenance of immune tolerance (17–21). The results of the current study provide new insights into the structural and functional alterations of human iNKT cells in early RA, and strongly suggest that these changes are directly related to a shift in the circulating iNKT clonal repertoire toward cells bearing lower-affinity TCRs.

Previous studies have found numerical defects and deficient cytokine responses of circulating iNKT cells in RA (8, 9, 14). Our results in a larger RA population confirm these earlier findings, and they corroborate their significance by showing that highly significant changes in circulating iNKTs are already present during the early stages of RA, occur independently of age and gender, and are inversely correlated to DAS28, an objective measure of disease activity in RA. As iNKT cell frequencies decline with age (22, 23), previous non-age-matched studies of iNKT cells in RA were most likely subject to the confounding influence of age (9). Furthermore, circulating iNKT were inversely correlated with the inflammatory markers CRP and ESR in the treatment-naïve early

FIGURE 4. Clonal distribution of iNKT-TCR expression and binding properties in early RA patients and controls. **(A)** Single $V\alpha 24^+/V\beta 11^+$ iNKT clones were sorted and subsequently expanded with PHA. TCR expression was measured by KRN7000-CD1d tetramers or with anti- $V\alpha 24$ Ab. No difference in iNKT-TCR expression (anti- $V\alpha 24$ mean fluorescence intensity) in clones from early RA patients (175 clones) and controls (121 clones) was demonstrated. **(B)** iNKT clones were stained with KRN7000-CD1d and OCH-CD1d tetramers and iNKT-TCR affinity was calculated as the ratio of (OCH-CD1d mean fluorescence intensity/KRN7000-CD1d mean fluorescence intensity). iNKT-TCR affinity was significantly lower in clones from early RA patients (175 clones) compared with controls (121 clones). **(C)** iNKT-TCR affinity is lower in clones from both treatment-naïve (64 clones) and treated (111 clones) early RA patients relative to controls (121 clones). **(D)** iNKT clones of the highest iNKT-TCR affinity from early RA patients (27 clones) are lower than that from controls (21 clones).



RA cohort, but not in patients taking immunosuppressive drugs, suggesting that DMARDs and/or steroid effects on iNKT numbers may not be solely dependent on their role in reducing systemic inflammation. These results also suggest that the levels of iNKTs are an indicator of response to treatment that could be investigated as a disease response biomarker. In addition, these data highlight how it is critically important to study iNKT phenotypes at the early stages of an autoimmune disease in which confounding secondary effects of inflammation and treatment may be less prominent. Together these findings are consistent with a regulatory role of iNKT cells in RA. Indeed, they indicate that these T cells may exert important protective functions against autoimmune inflammation in RA.

In different animal models of autoimmune inflammation, either CD1d-mediated iNKT activation or adoptive iNKT cell transfer provides highly effective protection against pathology (2, 24). The regulatory function of iNKT cells in these models is dependent on the interaction between the iNKT cells' semi-invariant iNKT-TCR and CD1d proteins expressed on the surface of APCs. Hence, either inherent defects of iNKT-TCR-mediated iNKT cell activation or defective CD1d Ag presentation functions of APC could have underlain the observed alterations of the iNKT repertoire in early RA. When comparing early RA and control monocytes, no significant differences were found between the two groups in either CD1d surface expression *ex vivo* or CD1d upregulation *in vitro* in response to diverse stimulatory conditions, strongly arguing against a major defect of Ag presentation in early RA. Conversely, the results from different carefully designed *in vitro*

iNKT stimulations using fixed iNKT:APC ratios, different lipid Ags (KRN7000 versus OCH), or synthetic APCs (CD1dMACSibeads) demonstrated that iNKT cells from early RA patients are intrinsically defective in their response to CD1d.

A recent study in systemic lupus erythematosus (SLE) has described defects in B cell CD1d Ag presentation, which correlated with iNKT defects in SLE patients (25). Consistent with a previous report (9), we did not observe changes in CD1d expression on B cells from early RA patients (Fig. 3). Hence, different mechanisms may underlie the observed iNKT deficiencies in RA and SLE. Furthermore, several studies have defined iNKT subsets according to CD4 coreceptor expression. Although $CD4^+$ iNKTs were shown to exhibit a Th2-type cytokine pattern (14), double-negative and CD8 iNKT cells exhibited a Th1/Th0-type cytokine pattern (26). Our data indicate that a significantly higher proportion of circulating iNKTs in early RA patients was $CD4^+$ compared with controls (Supplemental Fig. 3C). However, we did not find a correlation between iNKT-TCR affinity or iNKT function with CD4 expression, which is in agreement with our earlier study (10). Nevertheless, these findings demonstrate further intrinsic qualitative clonal differences within the iNKT repertoire in early RA.

Human and mouse iNKT repertoires have long been known to contain a significant proportion of autoreactive clones, that is, clones proliferating and/or secreting cytokines in response to CD1d-expressing target cells *in vitro*. Such autoreactive CD1d recognition by iNKT cells has been proposed to be required for their thymic selection, and, more importantly, for their regulatory

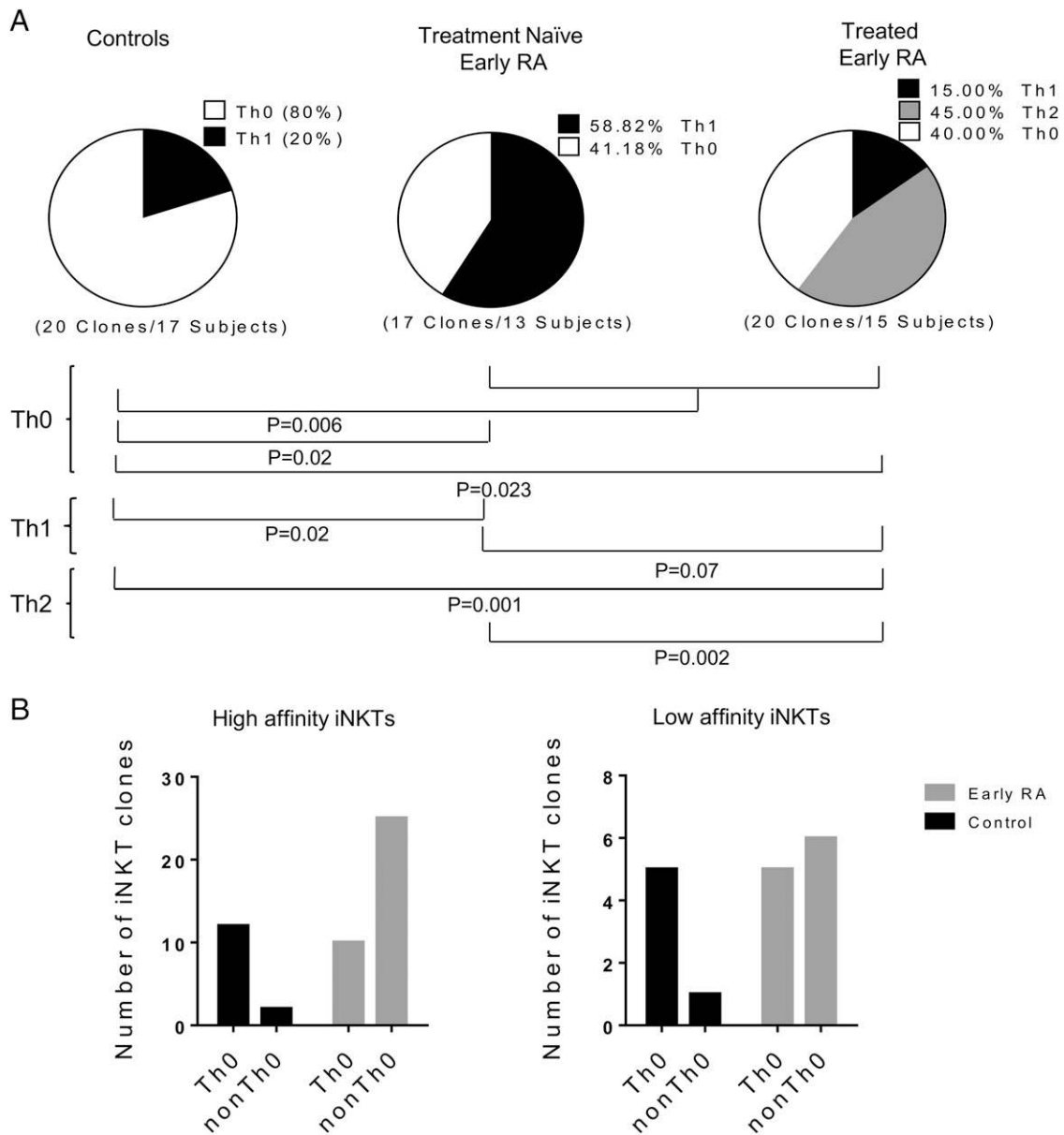


FIGURE 5. Th phenotype of iNKT cells in early RA patients and controls. iNKT cells were cocultured overnight with KRN7000-pulsed (200 μ g/ml) T2-CD1d. Culture supernatants were collected, and IFN- γ , TNF- α , IL-4, and IL-10 were measured by Luminex array. **(A)** The observed proportions of Th1, Th2, and Th0 cytokine patterns in iNKT clones from treatment-naïve and treated early RA patients and controls significantly differ. **(B)** The number of high-affinity iNKT clones and their Th0 versus non-Th0 proportions are significantly ($p = 0.0004$) skewed toward non-Th0 in early RA patients ($n = 21$) compared with controls ($n = 11$) (left panel). The number of low-affinity iNKT clones and their Th0 versus non-Th0 proportions was not significantly different in early RA patients ($n = 11$) compared with controls ($n = 6$) (right panel). Fisher’s exact test was used to determine p values of differences between proportions.

roles in tolerance (27–29). We have recently shown that the only variable part of the human iNKT-TCR, that is, the CDR3 β loop, is a key structural determinant for iNKT cell autoreactivity (10). Depending on its structure, the CDR3 β loop can provide additional critical binding energy to the interaction between the iNKT-TCR and CD1d, independent of the CD1d-bound ligand. Based on this, we have proposed an Ag-independent model to explain the clonal distribution of iNKT cell autoreactivity (10) whereby iNKT cells bearing higher-affinity iNKT-TCRs for CD1d would intrinsically have higher autoreactive potential than low-affinity clones. The model predicts that iNKTs bearing higher-affinity TCRs, rather than lower-affinity TCRs, would be preferentially activated during sterile inflammation to exert their tolerogenic functions. In contrast, the whole iNKT repertoire could be

recruited during more threatening scenarios, for example, bacterial infection, through TLR-mediated changes in CD1d expression and β -glucosylceramide production in APC, and facilitation of iNKT activation by cytokines (27–29).

Our analysis of the circulating iNKT clonal repertoire demonstrated a significant selective loss of iNKT cells bearing higher-affinity iNKT-TCRs in early RA patients, which would be consistent with a loss of autoreactive clones. In line with these results, iNKTs from early RA patients virtually failed to expand in vitro to the partial iNKT agonist OCH, whereas they were still able to mount a substantial response to the full iNKT agonist KRN7000 (Fig. 2) (10). Furthermore, by stimulating whole PBMCs with KRN7000, previous studies have shown increased IFN- γ secretion by iNKTs in RA (14). Similarly, our functional analyses of the

iNKT clonal repertoire in response to KRN7000-pulsed CD1d-expressing lymphoblasts showed a significant increase of Th1-like iNKTs, paralleled by a decrease of Th0-like iNKTs, in treatment-naïve early RA patients. In addition, our study revealed a marked increase in the proportion of Th2-like iNKT clones in treated early RA patients, which was paralleled by a loss of Th1-like iNKTs (Fig. 5). It is therefore intriguing to speculate that treatment, by DMARDs and steroids (Supplemental Table I), exerts direct effects on the iNKT repertoire by skewing cytokine secretion profiles from a pathogenic Th1 to a protective Th2 pattern. An alternative explanation is that iNKTs with a Th2 pattern accumulate in inflamed joints of treatment-naïve patients, but, upon immunosuppressive drug treatment, they return to the circulation. Dissection of the mechanisms behind these observed functional changes requires further study. Compared with both treatment-naïve and treated early RA patients, the great majority of iNKT clones from age- and gender-matched controls exhibited a Th0-like cytokine pattern. Furthermore, high-affinity iNKTs in early RA exhibited a significantly different cytokine secretion profile compared with high-affinity iNKT clones in controls, as high-affinity iNKT clones in early RA were characterized by a loss of the Th0-type cytokine profile (Fig. 5B). In contrast, the cytokine secretion patterns of low-affinity iNKTs from early RA patients and controls were not significantly different. In line with the defects in proliferation and iNKT-TCR affinity differences that we identify, these changes in cytokine profile confirmed the presence of intrinsic iNKT cell defects, specifically within high-affinity iNKTs, in early RA patients.

Taken together, the results from this study may suggest that higher- and lower-affinity iNKT subsets have different *in vivo* functions during autoimmune inflammation and that the higher-affinity iNKT subset may play key roles in promoting immune tolerance. In addition to the current study, we have found similar qualitative iNKT defects in people with type 1 diabetes (A. Tocheva, S. Mansour, J. Sanderson, E. Eren, T. Elliott, R. Holt, and S. Gadola, manuscript in preparation). Overall, this suggests that selective targeting of higher-affinity iNKTs may be required to successfully restore the tolerogenic functions of iNKT cells in autoimmune conditions.

A limitation of this study was its cross-sectional design and the comparison of iNKT repertoires in inflammation versus health, as there is evidence that aberrant T cell responses in autoimmune conditions, such as ankylosing spondylitis, psoriatic arthritis, and inflammatory bowel disease, could be a secondary phenomenon to inflammation due to excessive innate immune activation (30). However, the original hypothesis of this study, that is, that high- and low-affinity iNKT cells might be differentially activated during autoimmune inflammation, is supported by our findings. Further investigations into the observed qualitative defects of the iNKT repertoire in early RA, including synovial fluid iNKTs from the joints of RA patients, would provide crucial insights as to whether these iNKT changes are a cause or a consequence of autoimmune inflammation in early RA.

In conclusion, significant quantitative and intrinsic qualitative defects of the iNKT clonal repertoire, in particular higher-affinity iNKT cells, are present in early RA and may contribute to immune dysregulation and autoimmune inflammation. These results may inform future iNKT-targeting therapies aiming to restore immune tolerance in RA and other autoimmune diseases in humans. In future, it will be important to understand the contribution of the high- and low-affinity iNKT subsets to the initiation and to the chronic phases of autoimmune inflammation.

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Disclosures

The authors have no financial conflicts of interest.

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