Published in final edited form as:

Arthritis Rheum. 2012 February ; 64(2): 380–388. doi:10.1002/art.33358.

Microchimerism in the rheumatoid nodules of rheumatoid arthritis patients

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Abstract

Objective—The rheumatoid nodule (RN) is a lesion commonly found on extra-articular areas prone to mechanical trauma. When present with inflammatory symmetrical polyarthritis, it is pathognomonic of rheumatoid arthritis (RA), an autoimmune disease in which naturally acquired microchimerism (Mc) has previously been described and could sometimes contribute to RA risk. Since RA patients harbor Mc in blood, we hypothesized that Mc is also present in RNs, and could play a role in RN formation. Thus, the current study investigated RNs for Mc.

Methods—RNs were tested for Mc by real-time quantitative PCR (qPCR). For 29 female patients, their RNs were tested for a Y-chromosome specific sequence. Further, after human leukocyte antigen (HLA) genotyping patients and family members, RNs from one male and 14 females were tested by HLA-specific qPCR targeting a non-shared HLA allele of the potential Mc source. Results were expressed as genome equivalents of microchimeric cells per 10⁵ patient genome equivalents (gEq/10⁵).

Results—RNs from 21% of female patients contained male DNA (range: <0.5–10.3 gEq/10⁵). By HLA-specific qPCR, 60% of patients were microchimeric (range: 0–18.5 gEq/10⁵). Combined Mc prevalence was 47%. A fetal or maternal source was identified in all patients who tested positive by HLA-specific qPCR. Unexpectedly, a few RNs also contained Mc without evidence for a fetal or maternal source, suggesting alternative sources.

Conclusion—Mc was frequently found in RNs of RA patients. As Mc is genetically disparate, whether Mc in RNs serves as an allogeneic stimulus or allogeneic target warrants further investigation.

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Financial disclosures: None.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting of the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Chan had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Chan, Atkins, Woo, Nelson.

Acquisition of data. Chan, Atkins, Naysmith, van der Westhuizen, Woo, Nelson.

Analysis and interpretation of data. Chan, Nelson.

Supporting information: Additional supporting information may be found in the online version of this article.

Keywords

Microchimerism; Rheumatoid nodule; Rheumatoid arthritis; Shared epitope; Anti-citrullinated protein antibodies

During pregnancy, there is bidirectional trafficking of DNA and cells between mother and fetus that can result in naturally acquired microchimerism (Mc). Both adverse and beneficial effects have been proposed for naturally acquired Mc (1). Rheumatoid arthritis (RA) is a chronic inflammatory disorder in which the etiology and pathogenesis are not fully understood, but specific alleles of the human leukocyte antigen (HLA) have been associated with the strongest risk for disease (2). Previous studies have suggested that Mc may confer either risk to or protection from RA, depending upon several factors including the HLA specificity of the Mc (3–6). Parity is known to be a robust source of Mc (7), and RA risk is significantly reduced in parous versus nulliparous women (3). On the other hand, Mc containing RA risk-associated HLA sequences was found more frequently and at a higher concentrations in women with RA than healthy controls (5, 6).

A unique clinical feature of RA is the rheumatoid nodule (RN), commonly presenting as a non-caseating granulomatous subcutaneous nodule over a pressure point (8). In the setting of an inflammatory symmetrical polyarthritis, the presence of a RN is 97.7% specific for RA (9). The architecture of a RN is also distinct, containing three different layers of cells and acellular material (8). While Mc has been described in the peripheral blood of some RA patients, RNs have not previously been investigated for Mc. The mechanisms driving RN formation are also not fully understood. Immune complexes containing rheumatoid factor (RF) have been postulated to play a role in initiating nodule development (10), but other factors could be involved. Harboring of Mc in blood by RA patients facilitates the distribution of Mc to other sites, as suggested by one previous study that found Mc in synovial cells and non-affected skin (11). Thus, we hypothesized that Mc is present in RNs and that harboring Mc in areas prone to mechanical trauma facilitates RN formation, as trauma and resultant tissue damage lead to immune activation, and alloantigens from Mc could serve as a stimulus or target of alloimmunity. To begin to test this hypothesis, we sought evidence for Mc in RNs.

In this study, we investigated RNs for the presence of Mc using real-time quantitative PCR (qPCR). First, we tested RNs of female patients for male DNA and determined the prevalence and concentration. Male DNA in a female was employed as a marker of Mc, the presence of which most often originates from prior pregnancy with a male fetus. Second, we investigated RNs for Mc by testing them for HLA sequences that the patient did not carry in her or his HLA genotype. This approach involved HLA genotyping patients and their family members and targeting, by qPCR, a non-shared HLA allele that would identify Mc acquired from fetomaternal exchange.

PATIENTS AND METHODS

Subjects and specimens

Thirty-eight RA patients who developed RNs were recruited for this study from multiple solo rheumatology practices in Victoria, British Columbia, Canada. All RA patients met the American College of Rheumatology criteria for RA (9). Thirty patients were female and eight were male. All patients were Caucasian. Chart review was conducted for clinical features, serology and X-ray results. Subcutaneous structures that visually resembled RNs were removed from patients either voluntarily by a plastic surgeon or as standard of care. All nodules were reviewed by a pathologist. In total, 53 RNs were histologically confirmed

and processed as either formalin-fixed or frozen specimens at the Vancouver Island Health Authority in Victoria.

Family members of the patients, including their mothers and children, were recruited to participate by providing buccal swabs. Buccal swabs were obtained from family members of 23 patients, which included 60 children and 11 patients' mothers. All specimens were sent to the Fred Hutchinson Cancer Research Center in Seattle, Washington, USA, for testing. The study was approved by the institutional review boards of both the Vancouver Island Health Authority and the Fred Hutchinson Cancer Research Center.

DNA extraction

Genomic DNA was extracted from RNs and buccal swabs using the QIAamp® DNA Mini Kit (QIAGEN, Valencia, CA).

HLA genotyping

HLA-DRB1, DQA1 and DQB1 genotyping of subjects was performed on their DNA using the LABType® SSO DNA typing system and a LABScan™ 100 flow analyzer (One Lambda, Canoga Park, CA), according to the manufacturer's instructions. Alleles were assigned using the HLA Fusion™ software.

Real-time qPCR

Quantification of male Mc (DYS14 gene) and HLA sequence-specific Mc was performed according to our previously described methods (12–14). All qPCR assays were run by the ABI Prism® 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). A minimum of six aliquots was tested for each sample, and results for all aliquots of one sample were added. Data were analyzed using the 7000 System Sequence Detection Software. A positive result for male- or HLA sequence-specific Mc was defined as amplification of the respective sequence at $C_t < 40$. The concentration of Mc was expressed as the number of microchimeric genomes that would be equivalent to the total amount of microchimeric DNA (referred to as genome equivalents, or gEq) detected in a sample containing the equivalent of 100,000 host genomes (denoted as gEq/10⁵). While all RNs were collected and processed under sterile conditions, due to the sensitivity of qPCR for male DNA, additional criteria were applied that within a single experiment, a sample must test positive in at least two wells and a total concentration of 0.5 or greater was required to consider a sample positive.

RESULTS

Patient characteristics including clinical features and HLA shared epitope status

Tables 1 and 2 provide patient characteristics according to each individual and summarized for the study population overall. A majority of the studied patients were female, 79% versus 21% male. On physical examination, all 38 patients showed symmetric swelling in five or more joints. X-ray evidence of joint erosions was identified in the peripheral joints of 27 patients, absent in eight patients (#4, 12, 15, 16, 17, 45, 51, and 59), and unknown in the remaining three patients (#30, 37, and 57). The median age at RA onset was 42, and the median disease duration was 14 years. Of 30 patients with RF tests available, all had positive results. Of 29 patients with anti-citrullinated protein antibodies (ACPA) tests available, 90% had positive results, 62% had levels >100 U/ml, and just three patients were within the reference range (0–5 U/ml). Patients ranged from 35 to 80 years of age when RNs were biopsied (median 66 years). Regarding the known relationship between development of RNs and methotrexate (MTX) (8), a majority of patients (53%) were first documented to have RN development after MTX treatment, as compared to before treatment (25%) or after

the use of MTX and an anti-tumor necrosis factor biologic (22%). Collectively, the clinical features of these patients were consistent with those usually associated with severe disease.

RA is strongly associated with specific alleles at the HLA-DRB1 locus, the sequences of which encode a five-amino-acid motif at positions 70–74 known as the shared epitope (SE) (15–17). Eighty-nine percent of all patients (87% in females and 100% in males) had at least one SE sequence (QKRAA, QRRAA, or RRRAA), with 13% positive for two SE sequences (25% in males and 10% in females). Table 3 and Supplementary Table S1 provide the distribution of SE motifs and the HLA-DRB1 genotypes of all patients.

Prevalence and concentration of male Mc in the RNs of female RA patients

To address the question of whether Mc is present in RNs, the initial approach was to test RNs of female patients for male DNA by employing real-time qPCR for the Y-chromosome-specific DYS14 gene. The most common source of male DNA in an adult woman is from prior pregnancy with a male fetus. In addition to history of births, history was obtained as to whether the patient had ever had a miscarriage, an older male sibling, a twin or a blood transfusion, all of which represent potential sources of male Mc (14, 18–22).

A total of 33 RNs from 29 female patients were tested for male DNA (Table 4). Six of 33 RNs were positive for male Mc, giving an overall Mc prevalence of 18% among samples tested. Mc prevalence, as determined by the number of patients with a positive result, was 21% (6 of 29 patients; patients #2, 4, 10, 21, 32, and 45). The range of male Mc was 1.8 to 10.3 gEq/10⁵ among those that tested positive. The median total gEq tested was 86,766 (one female patient had an insufficient amount of DNA recovered from her RN for inclusion).

Twenty-three of 29 women tested for male DNA in RNs were parous, 83% of whom had given birth to at least one son (19 of 23). Births preceded RA onset in all but one parous patient (#4), and all births occurred before biopsy (Tables 2 and 4). Among the nulliparous patients, two had at least one older male sibling, while four patients had neither children nor older male siblings. Fourteen patients had at least one miscarriage. Similar to the births, the majority of miscarriages occurred before RA onset (Tables 2 and 4). The sex of the miscarried fetuses was generally unknown.

Eight of 29 women tested had a history of blood transfusion. Whether the products transfused were from male donors was unknown. Indications for blood transfusion included duodenal ulcer (patient #2), tuberculosis (patient #3), bladder repair and post-Caesarean section (patient #4), miscarriage (patients #5 and 11), anemia (patient #6), appendectomy (patient #50, who also received gamma globulins before her RA onset), and post-delivery (patient #58). Patient #28 received plasma transfusion. For five patients, blood transfusions were given prior to RA onset, and for three after (Tables 2 and 4).

Four of six patients with male Mc in their RNs had at least one son (patients #2, 4, 10, and 21). Of the two patients who did not have sons, one had no history of miscarriage or transfusion but had an older brother (patient #45), but the other (patient #32) had no history of miscarriage or transfusion and no older male sibling. The prevalence and concentration of male Mc in the RN were similar between patients with or without a known source from either a male fetus or an older male sibling (prevalence: 5 of 27 RNs or 19% vs. 1 of 6 or 17%; median concentration: <0.5 vs. <0.5 gEq/10⁵).

Identification of Mc by HLA-specific qPCR and determination of Mc source

Of 38 patients recruited for this study, 15 patients were determined to be informative for the detection of naturally acquired HLA-specific Mc (13 for fetal and two for maternal) after HLA genotyping was done on them and their family members. Among these informative

patients were two of six patients we previously determined to be positive for male DNA (patients #2 and 10). Table 5 provides the HLA class II genotypes of four patients and their available family members as examples. Informativeness was defined as any HLA-DRB1, DQA1 or DQB1 allele present in the patient's children and/or mother that was not carried by the patient, for which qPCR could be performed to detect and quantify the presence of the informative allele in the patient's RN. Thus, a distinct advantage of this method was the inclusion of male patients for Mc testing.

Collectively, 9 of 15 patients (60%) were positive for HLA-specific Mc in their RNs based on the sequences we tested (Table 6 and Supplementary Tables S2). Only one of two patients who were positive for male DNA was also positive for HLA-specific Mc (patient #10) as per the sequences tested. Based on HLA genotyping data, the origin of the Mc identified by HLA-specific qPCR was traced to either a fetal (patients #8, 10, 12, 13, 26, 27, and 58) or maternal source (patients #29 and 41). Fetal Mc in the RNs of patients #8 and 10 came from at least two sources. Moreover, we found Mc of the SE sequence QKRAA as well as Mc of DRB1*04 in the RN of patient #13, consistent with the presence of Mc of DRB1*0401 (her children were uniformly DRB1*0401). Unexpectedly, we also found Mc that was not fetal in origin in the RNs of patients #13, 27, and 58, suggesting alternative sources as discussed further below.

DISCUSSION

Our data indicate that almost half of the RNs we studied contained naturally acquired Mc. In rare cases, Mc from alternative sources was also found. Mc has been linked to several autoimmune diseases, including RA (1, 23). Two prior reports studied individuals who lacked RA-associated HLA risk alleles and found a significant increase of Mc carrying RA-associated HLA sequences in the peripheral blood mononuclear cells of RA patients compared to healthy individuals (5, 6). Conversely, acquired Mc could potentially confer benefit against RA if the Mc carries an RA-protective HLA sequence. The latter possibility has not been directly tested but is suggested by another study describing an overall reduction in risk of RA in parous compared to nulliparous women (3). In the current study, we investigated Mc in the RN, a lesion found in 20–25% of seropositive RA patients (8). Due to the ability of Mc to circulate in its host and potentially contribute to immune responses, our working hypothesis was that Mc is also present in RNs and could play a role in RN formation.

Because RNs frequently occur over pressure sites, Ziff suggested that repeated trauma likely contributes to RN development (10). Trauma, and its subsequent damage of tissues resulting in necrosis, could activate the immune system due to the release of activating signals by necrotic cells that activate antigen presenting cells to present antigens to cognate T cells, resulting in T cell activation (24). The presence of Mc expressing foreign antigens in the RN potentially enhances the immune response due to the recruitment of alloreactive T cells that are present at a greater frequency than nominal antigen-specific T cells. Novel to this view is the induction of alloimmunity, with or perhaps without autoimmunity as previously suggested (10), in driving RN formation.

The evolution of the RN into a destructive granulomatous structure has been well characterized largely due to histological and immunohistochemical studies (25–31). Pathology likely reflects the concerted interactions of macrophages and other cells expressing HLA class II, along with T cells, complement, antibodies, and various inflammatory cytokines. Thus, the conventional view suggests a largely Th-1 response in driving RN formation with little involvement of the Th-17 pathway (32–35). In contrast, recent evidence suggests that Th-17 cells are important in the pathogenesis of RA (36–38).

The RN is a unique structure containing three distinct layers, the center layer of which densely harbors palisaded macrophages that express HLA class II, while the outer layer also contains monocytes/macrophages as well as dendritic cells with class II expression (8, 10, 25–27, 29, 31). Thus, depending on the proximity of Mc and cells with antigen presenting capability, presentation of HLA-disparate antigens to T cells could occur robustly.

Our study of RA patients with clinical features typical of those usually associated with severe RA, to our knowledge, is the first documentation of Mc at a site of rheumatoid-specific pathology. A previous study reported Mc in synovial cells from RA-affected synovial tissues of RA patients (11). While results of the prior study are complimentary with the current report, synovitis is less specific for RA as it occurs in other diseases, thus the finding of Mc in RNs is a unique contribution. Moreover, we observed a Mc prevalence of 47% in RNs combining results of male- and HLA-specific qPCR testing, suggesting frequent Mc occurrence at this site. Our study population of RA patients with RNs had a comparable frequency of the RA-associated SE as in other studies, with 89% carrying the SE (39, 40). Whether SE status influences RN development appears controversial. Homozygosity for DRB1*0401 was previously reported as more frequent in RA patients with severe disease who presented with RNs (39). In contrast, another study found that one or more SEs did not significantly increase risk of RN development, at least among Caucasian RA patients (41). Many of our RA patients carried DRB1*0401 but often in heterozygosity with another HLA-DRB1 allele that was not associated with RA (Table 3 and Supplementary Table S1). Thus, other studies are needed to elucidate the role of the SE in RN development. One possibility is that cells bearing the SE enhance the overall immunity that results in nodular pathology by presenting self peptides, or allo-peptides from Mc, to cognate T cells. Since RNs typically develop in areas prone to trauma, tissue damage and associated inflammation can facilitate citrullination of peptides that can then be preferentially presented by SE-bearing HLA molecules to T cells (42, 43), the response of which could result in the lesion that evolves into a RN.

An unexpected observation from our study was the finding of Mc in some patients that was not attributed to fetomaternal exchange. In two patients (#27 and 58), the source of Mc could have been maternal, but mothers were not available for study. A maternal source could also have contributed to Mc in a third patient (#13); however, this patient had more than one source of Mc (DRB1*01, DRB1*07, and DQB1*06 Mc), none of which could have been acquired from pregnancies with her children (Table 5). Results for this patient suggest three unaccounted-for sources of Mc in her RN. Mc is known to result from miscarriage (14, 19, 21), and this patient had a history of two prior miscarriages. Thus, while the origin of Mc in this patient is unknown, a reasonable explanation is from prior miscarriages and from the patient's own mother (who was not available for study). Because a mother continues to harbor Mc from prior pregnancies and some of the mother's cells reach the fetus, the fetus of a subsequent pregnancy could potentially acquire cells from an older sibling (the patient also had an older male sibling). On the other hand, the patient had no history of transfusion, which could otherwise provide a source of Mc (20). It is important to recognize, however, that for those RNs in which we were unable to find HLA-specific Mc as per the sequences tested, this did not rule out the overall possibility that Mc was present and went undetected, as our testing for HLA-specific Mc was not exhaustive. Thus, the prevalence of HLA-specific Mc in RNs (naturally acquired or otherwise) could be higher.

In conclusion, using real-time qPCR to detect male-specific and HLA-specific Mc, we identified Mc in the RNs of RA patients, the presence of which was largely attributable to fetomaternal exchange. While any role that Mc might play in the pathogenesis of RNs is unknown, the presence of genetically disparate DNA/cells at sites that are prone to trauma presents an opportunity for robust immune activation. Further investigation of Mc in RNs is

indicated and includes phenotypic analysis of microchimeric cells (i.e. inflammatory cells vs. primary matrix cells) and evaluation of host immune reactivity to discordant HLA alleles carried by microchimeric cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported by a generous gift from the Lawrence and Sylvia Wong Foundation, by NIH grants AI-45659 and AI-41721, and by a Canadian Institutes of Health Research Fellowship Award to WFNC (SIB-95173). We would like to acknowledge Dr. Paul de Champlain, Dr. Kimberly Northcott, Dr. Milton Baker, and the family doctors of Vancouver Island for patient referrals, and Leeanna Bulinckx of PerCuro Clinical Research for patient counseling. We acknowledge the contributions made to the study by Valerie Cortez, Dawn Stief, and the Experimental Histopathology Shared Resource at the Fred Hutchinson Cancer Research Center. Furthermore, we are grateful for the participation of RA patients and family members.

Abbreviations

ACPA	Anti-Citrullinated Protein Antibodies
gEq	Genome Equivalents
HLA	Human Leukocyte Antigen
Mc	Microchimerism
MTX	Methotrexate
qPCR	Quantitative PCR
RA	Rheumatoid Arthritis
RF	Rheumatoid Factor
RN	Rheumatoid Nodule
SE	Shared Epitope

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Table 1

Clinical features of RA patients with biopsy-proved RNs, and HLA SE status.

ID	Sex	Age at biopsy (years)	Age at RA onset (years)	Disease duration* (years)	SE [§] (+/-)	RF (kU/l)	ACPA (U/ml)
1	M	56	28	28	+	>900	>100
2	F	77	34	43	+	18	20
3	F	49	41	8	+	NA [‡]	>100
4	F	35	26	9	+	35	3
5	F	47	37	10	+	304	11
6	F	46	36	10	+	171	>100
7	M	80	56	24	+	60	<2
8	F	79	75	4	+	850	>100
9	F	66	57	9	+	598	>100
10	F	70; 71	64	7	+	260	19
11	F	68	52	16	+	291	>100
12	F	60	58	2	+	NA	>100
13	F	75	45	30	-	14	>100
14	F	63	55	8	+	225	27
15	F	61	59	2	-	95	>100
16	F	51	36	15	+	22	<2
17	M	57	52	7	+	643	29
21	F	67	59	8	+	51	34
23	F	58	38	20	+	85	>100
24	F	66	61	5	+	259	>100
25	F	59	43	16	+	209	>100
26	F	64	44	20	+	NA	NA
27	F	74; 78	37	41	+	1430	>100
28	F	65	39	26	+	356	NA
29	F	49	40	9	-	47	58
30	M	73	46	27	+	NA	NA
32	F	66	28	38	+	393	>100
37	M	51	41	10	+	524	NA

ID	Sex	Age at biopsy (years)	Age at RA onset (years)	Disease duration* (years)	SE ^{&} (+/-)	RF (kU/l)	ACPA (U/ml)
39	M	78	65	13	+	NA	NA
41	M	69	44	25	+	112.5	NA
42	F	67, 67, 68	58	10	+	222	30
45	F	75, 75	66	9	+	900	>100
50	F	68	40	28	-	900	>100
51	F	50	26	24	+	NA	>100
57	M	53	38	15	+	NA	NA
58	F	73	32	41	+	992	>100
59	F	47	39	8	+	166	NA
60	F	59	35	24	+	NA	NA

* To determine disease duration, the age at RA onset was subtracted from the age at biopsy. For individuals with more than one biopsy, the older age was used to calculate disease duration.

[&] Positivity for SE includes both homozygotes and heterozygotes irrespective of SE motif (QKRRRA, QRRRAA or RRRRAA).

[‡] NA denotes not available.

Table 2

Summary of clinical features.

Clinical feature	Range (median)
Age at biopsy (years)	35 – 80 (66)
Age at onset (years)	26 – 75 (42)
Disease duration (years)	2 – 43 (14)
RF (kU/l)	14 – 1430 (242)
ACPA (U/ml)	<2 to >100 (>100)
Time interval between last child birth and RA onset (years)	–3 – 41 (16.5) *
Time interval between last child birth and RN biopsy (years)	6 – 49 (38.5)
Time interval between last miscarriage and RA onset (years)	–2 – 38 (15) *
Time interval between last transfusion and RA onset (years)	–13 – 34 (0.2) *

* A negative value indicates that the birth, miscarriage, or transfusion occurred after RA onset.

Table 3

Frequency of different SE motifs in the study population.

SE motif	Frequency (%)	
	Male (N = 8)	Female (N = 30)
QKRAA, QKRAA	–	–
QKRAA, Q(R)RRAA	25.0 (2)	3.3 (1)
QKRAA, no SE	37.5 (3)	36.7 (11)
QRRRAA, Q(R)RRAA	–	6.7 (2)
QRRRAA, no SE	37.5 (3)	33.3 (10)
RRRAA, RRRAA	–	–
RRRAA, no SE	–	6.7 (2)
None	–	13.3 (4)

Table 4

Results of male-specific Mc testing in relation to history of children and older male siblings, miscarriages, transfusions, and time between birth and RA onset.

ID	gEq/10 ⁵	Sons (N)	Daughters (N)	Older male siblings (N)	Time between birth and RA onset (years)*	Time between miscarriage and RA onset (years)*	Time between transfusion and RA onset (years)*
2	4.60	1	2	1	4 ^{&} , 9, 12	13	-3
3	<0.5	1	1	0	18, 23	21	34
4	10.34	1	3	1	-3 [‡] , 0.1, 3	-2, 1, 4	0.1, 3, 5
5	<0.5	2	2	0	8, 9, 14, 15	13	NA
6 [§]	<0.5	2	1	0	11, 14, 15	NA	28
8 [§]	<0.5	2	3	0	41, 44, 49, 50, 52	NA	NA
9 [§]	<0.5	0	0	1	NA	18, 28 [¶] , 28 [¶]	NA
10 [§]	1.78	4	1	0	38, 40, 42, 43, 44	NA	NA
	<0.5						
11	<0.5	1	0	0	32	29	29
12	<0.5	0	2	2	29, 30	NA	NA
13	<0.5	4	1	1	13, 15, 17, 21, 23	-1, 20	NA
14	<0.5	0	0	0	NA	NA	NA
15 [§]	<0.5	1	0	1	27	32	NA
16	NA	1	2	0	11, 16, 20	NA	NA
21	4.44	1	2	1	34, 35 [‡]	38	NA
23 [§]	<0.5	0	2	0	2, 19	NA	NA
24	<0.5	1	1	1 [‡]	37, 41	NA	NA
25 [§]	<0.5	4	0	0	16, 18, 20, 21	23	NA
26	<0.5	2	1	0	21, 23, 25	NA	NA
27	<0.5	1	2	0	12, 16, 17	15	NA
28	<0.5	2	1	0	17, 20, 21	14, 21, 22	-4
29	<0.5	0	1	0	23	NA	NA
32	2.68	0	0	0	NA	NA	NA
42 [§]	<0.5	0	2	2	33, 35	NA	NA
	<0.5						
	<0.5						
45 [§]	<0.5	0	0	1	NA	NA	NA
	2.45						
50	<0.5	0	0	0	NA	NA	-13
51	<0.5	0	0	0	NA	NA	NA
58	<0.5	2	2	1	0.25, 3, 9, 11	NA	0.25
59 [§]	<0.5	3	0	1	0.04 [‡] , 7	-7 [¶] , 7 [¶]	NA
60	<0.5	2	0	1	12, 14	12	NA

* The year of RA onset was subtracted by the birth year to determine the time interval. Similarly, the year of RA onset was subtracted by the year in which the miscarriage or transfusion occurred. A positive value indicates the birth occurred before the event, and a negative value indicates after.

& Values highlighted in gray indicate the birth of a son.

[‡] Patient #4 had a birth of fraternal twins, the female offspring of which was stillborn. Patient #21 had a birth of female twins, one of which was stillborn. Patient #59 had a birth of male twins. The older male sibling of patient #24 was her twin brother.

[§] Mc concentration in patients #6, 8, 9, 15, 25, and 59 was determined as the total concentration in 2 RNs excised on the same day. For patient #23, the total concentration in 4 RNs is shown. For patients #10, 42, and 45, two or more RNs were removed but each at a different time point.

[¶] Patient #9 had 2 miscarriages in her 20's but the specific ages were unknown. To calculate time between miscarriage and RA onset, we used the year equal to when she was 29 for both miscarriages to determine the minimum amount of time. Patient #59 had a miscarriage after RA onset, but the time between miscarriage and RA onset could not be determined due to a lack of information. She also had several miscarriages before her first child (born when she was 32), but the exact number and the ages at which they occurred were unknown. To calculate time between these miscarriages and RA onset, we subtracted the year of RA onset by the year equal to when she had her first child to determine the minimum time.

Table 5

Determination of informative HLA sequences for quantifying fetal or maternal HLA-specific Mc in RNs.

ID	Sex	DRB1(i)*	DRB1(ii)*	DQA1(i)*	DQA1(ii)*	DQB1(i)*	DQB1(ii)*
8A ^{&}	F	0101	0408	0101	0303	0501	0301
8C1 ^{&}	M	0101	0301 [‡]	0101	0501	0501	0201
8C2	M	0101	0801	0101	0401	0501	0402
8C3	F	0101	0801	0101	0401	0501	0402
8C4	F	0301	0408	0501	0303	0201	0301
8C5	F	0101	0301	0101	0501	0501	0201
13A	F	1303	1101	0505	0505	0301	0301
13C1	M	1303	0401	0505	0303	0301	0301
13C2	M	1303	0401	0505	0303	0301	0301
13C3	M	1303	0401	0505	0303	0301	0301
13C5	F	1303	0401	0505	0303	0301	0301
29A	F	0301	1101	0501	0505	0201	0301
29M ^{&}	F	0301	1501	0501	0102	0201	0602
41A	M	0401	0701	0301	0201	0302	0303
41M	F	0401	0301	0301	0501	0302	0201

* HLA class II genotypes are presented as DRB1, DQA1 and DQB1 from left to right. (i) and (ii) denote the two haplotypes.

[&]The RA patient is designated "A". Each child of the patient is designated "C". Thus, C1 denotes the oldest child, C2 the second child etc. Mother of the patient is designated "M".

[‡] Informative HLA sequences (from the patient's perspective) are highlighted in gray. Note that 8C2 and 8C3 carry DQA1*0401 that the patient lacks. However, an assay was not available to test for this sequence.

Table 6

Quantification of HLA-specific Mc in RNs.

ID	HLA sequence tested	Source of Mc	gEq/10 ⁵
8	DRB1*03	Fetal	1.02
8	DRB1*08	Fetal	3.52
8	DQA1*05	Fetal	2.48
8	DQB1*02	Fetal	2.50
8	DQB1*04	Fetal	0.80
8	DRB1*07	Non-fetal/non-maternal	0
13	DRB1*04	Fetal	2.70
13	DQA1*03	Fetal	5.42
13	QKRAA ^{&}	Fetal	4.10
13	DRB1*01	Non-fetal/non-maternal	1.58
13	DRB1*03	Non-fetal/non-maternal	0
13	DRB1*07	Non-fetal/non-maternal	6.80
13	DRB1*15	Non-fetal/non-maternal	0
13	DQB1*06	Non-fetal/non-maternal	3.38
29	DRB1*15	Maternal	1.70
29	DQA1*01	Maternal	5.46
29	DQB1*06	Maternal	2.56
29	DRB1*08	Non-fetal/non-maternal	0
41	DRB1*03	Maternal	0
41	DQA1*05	Maternal	1.58
41	DQB1*02	Maternal	6.42

[&] SE-specific Mc.