Immunogenicity of a rheumatoid arthritis protective sequence when acquired through microchimerism

Sami B. Kanaan[a,1], Oyku Sensoy[a], Zhen Yan[a,2], Vijayakrishna K. Gadi[a,b], Michael L. Richardson[c], and J. Lee Nelson[a,d]

[a]Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, WA 98109; [b]Department of Medicine, Division of Oncology, University of Washington, Seattle, WA 98195; [c]Department of Radiology, University of Washington, Seattle, WA 98195; and [d]Department of Medicine, Division of Rheumatology, University of Washington, Seattle, WA 98195

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HLA class II genes provide the strongest genetic contribution to rheumatoid arthritis (RA). *HLA-DRB1* alleles encoding the sequence DERAA are RA-protective. Paradoxically, RA risk is increased in women with DERAA^{+} children born prior to onset. We developed a sensitive qPCR assay specific for DERAA, and found 53% of DERAA$^{-/-}$ women with RA had microchimerism (Mc; pregnancy-derived allogeneic cells) carrying DERAA (DERAA-Mc) vs. 6% of healthy women. DERAA-Mc quantities correlated with an RA-risk genetic background including DERAA-binding *HLA-DQ* alleles, early RA onset, and aspects of RA severity. CD4^{+} T cells showed stronger response against DERAA^{+} vs. DERAA^{-} allogeneic cell lines in vitro, in line with an immunogenic role of allogeneic DERAA. Results indicate a model where DERAA-Mc activates DERAA-directed T cells that are naturally present in DERAA$^{-/-}$ individuals and can have cross-reactivity against joint antigens. Moreover, we provide an explanation for the enigmatic observation that the same HLA sequence differentially affects RA risk through Mendelian inheritance vs. microchimeric cell acquisition.

microchimerism | rheumatoid arthritis | HLA | noninherited genetic risk | DERAA

The heritability of complex autoimmune diseases remains only partially explained (1); however, a remarkable feature in most autoimmune diseases is an increased prevalence in females (2, 3) and a strong risk association with specific human leukocyte antigen (HLA) class II alleles (4). Rheumatoid arthritis (RA), a chronic autoimmune disease affecting synovial joints, follows this rule, with allelic polymorphisms in the HLA class II region accounting for the highest genetic risk and a female:male ratio of 3:1 (3). The *HLA-DRB1* strongly RA-associated alleles code for the "shared epitope" (SE): a 5-amino acid motif, ^{70}Q(or R)-K(or R)-R-A-A^{74} in the third hypervariable region (HV3) of the DRβ1 molecule, most notably -*DRB1**01:01, *04:01, *04:04, *04:05, *04:08, *10:01, and *14:02 (5). At the same positions, the ^{70}D-E-R-A-A^{74} sequence is RA-protective, encoded by *HLA-DRB1* alleles including *01:03, *04:02, *11:02, *11:03, *13:01, *13:02, and *13:04 (6–11). Of incidental note, *HLA-DRB1**13 (the allele group that contains the most frequent DERAA-encoding HLA alleles) is protective not only against RA but also against systemic lupus erythematosus, psoriasis, and systemic sclerosis (12, 13). Interestingly, the protective benefits of DERAA are reversed when women are exposed to fetal cells expressing DERAA during pregnancy; a 2017 study unexpectedly found that RA risk was increased when the HLA genotype of children born prior to RA onset encoded DERAA but the mother's did not (14).

HLA molecules frequently present self-peptides themselves derived from HLA molecules (15, 16). The HLA-derived DERAA sequence can be presented on a variety of HLA molecules [e.g., peptide DERAAVDTY presented on HLA-B (16)], but is notable for its affinity to DQ heterodimers encoded by *HLA-DQA1* and -*DQB1* alleles that are in very strong linkage disequilibrium with RA risk-associated SE-encoding -*DRB1* alleles (mainly DQ molecules referred to as DQ7, DQ8, and DQ5) (17–25). A model to explain RA protection when DERAA is inherited was initially proposed in 1996 based on presentation of DERAA by particular DQ molecules (17). More recently, elegant studies by van Heemst et al. (25) identified naturally occurring DERAA-directed T cells with the potential to react against both endogenous DERAA (e.g., from synovium-expressed autoantigen vinculin) and microbial-derived DERAA (present in 66% of bacteria and 4% of viruses). Accordingly, protection against autoimmunity of RA would be maintained by thymic negative selection of DERAA-specific autoreactive T cells in DERAA^{+} individuals. Such T cells are not deleted in DERAA$^{-/-}$ individuals and a break in tolerance could occur in the periphery by molecular mimicry after endogenous/exogenous DERAA encounter, facilitated by presence of the SE and DQ molecules that bind DERAA, in linkage disequilibrium with the SE (25).

Pregnancy creates a long-term legacy of microchimerism (Mc), generating a source of acquired alloantigens (26). Mc of fetal origin is unique to women, and Mc with DERAA^{+} HLA (DERAA-Mc) is compelling as an additional source driving molecular mimicry and explaining the paradoxical increase of RA when a child had DERAA in his/her HLA genotype. Mc occurs naturally as a result of fetal–maternal cell exchange (27), with long-term persistence of allogeneic cells including immunologically relevant cell types (28–30). These microchimeric cells express nonshared familial antigens with the potential for significant immunological

Significance

HLA genes confer the strongest genetic autoimmune disease risk. Specific *HLA-DRB1* alleles predispose to rheumatoid arthritis (RA), while others encoding the sequence DERAA are RA-protective. Unexpectedly, having given birth to children with DERAA^{+} *HLA-DRB1* alleles prior to onset was found to increase RA risk in women. We show that microchimerism (Mc; allogeneic cell long-term legacy of pregnancy) carrying DERAA^{+} HLA increases the odds of RA 17-fold and that allogeneic cells carrying DERAA stimulated DERAA$^{-/-}$ T cells in vitro. Microbial-derived DERAA peptides (among others) are known to stimulate DERAA-directed T cells naturally present in DERAA$^{-/-}$ individuals. Our data indicate that Mc with DERAA alloantigens can potentially activate similar adaptive immunity pathways and mechanistically link naturally occurring Mc to an autoimmune disorder.

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[1]To whom correspondence may be addressed. Email: skanaan@fredhutch.org.

[2]Present address: Diagnostic Pathology Medical Group, Inc., Sacramento, CA 95816.

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consequences for their host (31). Associations of Mc have been described with a number of autoimmune diseases (27) and, in risk of RA, Mc strongly depends on the allelic specificity of the acquired alloantigens (26, 32, 33). Nevertheless, a mechanistic basis for Mc involvement in RA or in the pathogenesis of other autoimmune diseases has been lacking.

The current studies address this knowledge gap and investigate the role of DERAA-Mc in RA biology. We first developed a highly sensitive and specific fluorescence-based qPCR assay for DERAA-encoding *HLA-DRB1* alleles and then employed the assay to determine the prevalence and quantities of DERAA-Mc in women with RA and healthy women. We explored the immunogenicity of DERAA, evaluating T cell reactivity in studied individuals in in vitro studies. Further, we examined DERAA-Mc in the context of the women's HLA class II genotype and clinical characteristics in recent-onset RA and assessed measures of RA severity after RA had become chronic many years after onset.

Results

Development of DERAA-Specific qPCR Assay. We developed a real-time qPCR assay with specific forward and reverse primers and fluorogenic probe targeting the DERAA-encoding HV3 sequences of the *HLA-DRB1* locus. To assure specificity of the assay, testing was conducted against an extensive panel of DNA from well-characterized human B cell lymphoblastoid cell lines banked at the International Histocompatibility Working Group project. Because the cells are clonally expanded, they are assumed Mc-free and safe for assay validation. The DERAA qPCR assay amplified DNA from *HLA-DRB1* alleles *01:03, *04:02, *11:02, *11:03, *13:01, *13:02, and *13:04 but not other alleles (Fig. 1*A*). DERAA standard curves were generated as previously described (28) and run on background DNA (DERAA$^{-/-}$) at varying concentrations to determine optimal sensitivity. The DERAA qPCR assay could detect a single DNA target in a background of up to 60,000 human cell genome equivalent (gEq) per amplification well (Fig. 1*B*). Because the assays are typically run in 6 replicates per study sample, a practical limit of detection for the assay was determined at ≤1:360,000. In the current study, a median (and interquartile range) of 161,581 [120,349 to 191,456] gEq of sampled DNA from 158 participants was run in DERAA qPCR assays.

DERAA-Mc Is Increased in RA Compared with Healthy Controls and Correlates with Risk-Associated Genetic Background. Subjects derived from a total population of 320 women consisting of 167 who met the 1988 American College of Rheumatology criteria for RA and 153 healthy women with no history of autoimmune disease (*SI Appendix*, Table S1), from which 135 were selected who lacked a DERAA-encoding HLA allele in their genotype (65 healthy controls and 70 patients with an RA onset of ≤2 y). These 135 participants were randomly assigned to an initial cohort (40 controls and 32 RA) and a validation cohort (25 controls and 38 RA). DNA extracted from peripheral blood mononuclear cells (PBMCs) was tested with the DERAA qPCR assay. DERAA-Mc was present in nearly half of the patients and only in 5 to 8% of controls in the initial and replication groups (*SI Appendix*, Fig. S1). It was thus possible to merge, respectively, RA and control data from both initial and replication groups for all subsequent analyses (Fig. 2). The odds ratio (OR) of RA in the presence of DERAA-Mc was 17.1 and the 95% confidence interval was [5.7 to 46.9]. Quantitatively, concentrations of DERAA-Mc were higher in RA patients than in controls. Ranked values of DERAA-Mc were significantly higher among RA women ($P < 0.0001$, Mann–Whitney U) (Fig. 2*A*).

RA-risk alleles, specifically SE-encoding HLA alleles, were present in 80% of RA women and 46% of controls. The immunogenetics of RA indicates risk-associated alleles are not all equivalent, as some combinations of different SE-encoding alleles in a genotype synergistically compound RA risk (34). Such "compound heterozygosity" was extensively studied by Balandraud et al. (35), resulting in a table of OR values translating the risk of RA according to *HLA-DRB1* genotypes. This genetic risk OR score ranges from 0.2 for *DRB1**03/*03, the most protective combination, to 28.2 for *04:01/*10, the most susceptible combination (35). We found that ranked DERAA-Mc concentrations in RA women positively correlated with the risk score (Fig. 2*B*). This HLA risk score analysis was obtained for anti-citrullinated protein antibody (ACPA)-positive RA because of its well-recognized diagnostic and predictive value, alongside the rheumatoid factor (RF) test (36). Considering autoantibody-positive (ACPA and/or RF) RA patients only, our DERAA-Mc data trended toward an increase with the risk score (*SI Appendix*, Fig. S2).

Together, our data showed a striking increase of DERAA-Mc prevalence and quantities in women with RA compared with controls. This is consistent with the unexpected increase of RA risk reported in women for whom children born prior to onset had DERAA-encoding "protective" HLA (14). Moreover, increased DERAA-Mc correlated with an individual's risk-associated HLA genotype. This points to the functional significance of DERAA-Mc in RA pathogenesis, consistent with and extending upon a proposed model of molecular mimicry (25) where DERAA-carrying allogeneic cells may become immunogenic in DERAA$^{-/-}$ subjects and contribute to autoimmunity.

CD4$^+$ T Cells from DERAA$^{-/-}$ Subjects Have a Stronger Response to DERAA$^+$ than DERAA$^-$ Allogeneic Cells. We next explored the potential immunogenicity of allogeneic cells carrying DERAA, which includes DERAA-Mc. We designed alloreactive in vitro cocultures to measure T cell activation against human B lymphoblastoid cell lines (B-LCLs) that mimic 2 different "microchimeric" scenarios: 1 with DERAA-encoding HLA alleles and 1 without (the number of microchimeric cells available from a routine blood sample is insufficient for extended studies). Furthermore, a hallmark of the HLA region is linkage disequilibrium, strongest among class II alleles that are inherited in predictable *DRB1–DQA1–DQB1* haplotypes. The SE alleles are often in linkage disequilibrium with *DQA1**03–*DQB1**03:01 (DQ7.3), *DQA1**03–*DQB1**03:02 (DQ8), and *DQA1**01–*DQB1**05:01 (DQ5.1). These specific *HLA-DQ* alleles code for heterodimers that bind peptides containing the DERAA sequence while other DQ and DR molecules do not (25). We sought to investigate the DERAA-binding *HLA-DQ* alleles. We chose 2 groups of responder PBMCs: 1 with DQ8 alleles (with affinity to DERAA) and 1 with DQ6.2 (*DQA1**01–*DQB1**06:02 in linkage with *DRB1**15) for comparison, the latter chosen because of its reported lack of DERAA affinity (25). Natural Mc has an allele shared with the host by inheritance, usually along with a nonshared allele. Thus, the B-LCL had an allele shared with the responder PBMCs (in 1 case the DQ8 alleles, in the other the DQ6.2) and the nonshared allele was either DRB1*13:01 (DERAA$^+$) or DRB1*14:01 (DERAA$^-$ control) (Fig. 3*A*).

From the participants tested for DERAA-Mc who were candidates for the coculture experiments (either DQ8/x or DQ6.2/x), cryopreserved samples were available for 26: 15 RA (13 DQ8/x and 2 DQ6.2/x) and 11 controls (6 DQ8/x and 5 DQ6.2/x) (Fig. 3*A* and *SI Appendix*, Table S2). Responder PBMCs were cocultured with irradiated stimulator cell lines at a ratio of 10:1. Cocultures were also conducted in blank culture media to measure "baseline" activation, and in media with 0.5% phytohemagglutinin as an activation "positive control."

In flow cytometry analyses, CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ T cell populations were identified from alive, singleton events gated from lymphocytes (*SI Appendix*, Fig. S3). To measure T cell activation in responders, we considered 3 markers: carboxyfluorescein succinimidyl ester (CFSE)^{low} (marker of cell division), CD69$^+$ cells (37), and CD38$^+$HLA-DR$^+$ cells (37) (*SI Appendix*, Fig. S4). In a series of measurements on days 1, 3, and 5 of cocultures, only the CFSE^{low} marker showed stability with a steady increase over

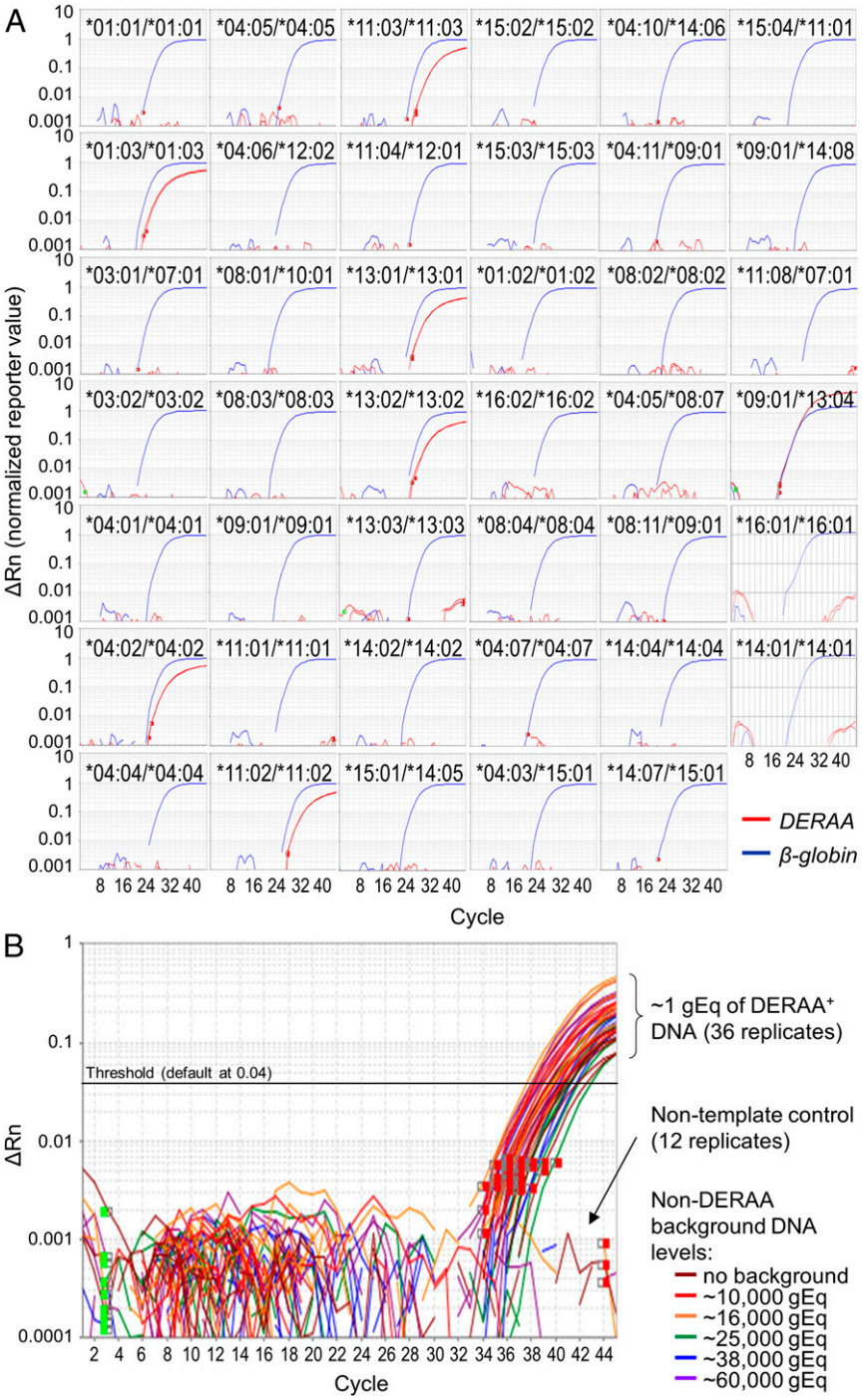


Fig. 1. DERAA qPCR assay is highly sensitive and specific to DERAA-encoding alleles. (*A*) DERAA-specific amplification is shown in red and β-globin–specific amplification, a housekeeping gene on human chromosome 11, is shown in blue. The DERAA assay amplifies *HLA-DRB1* alleles *01:03, *04:02, *11:02, *11:03, *13:01, *13:02, and *13:04 but not other alleles. (*B*) Following a serial dilution procedure, ~1 human cell genome equivalent of DNA from cell line IHW09058 (*13:01/*13:01) was achieved, and mixed with Tris·HCl buffer alone or with 10,000, 16,000, 25,000, 38,000, or 60,000 gEq of non-DERAA background DNA, in 6 replicates for each of the 6 background concentrations. One genome equivalent of DERAA amplified at an acceptable cycle at a threshold (Ct) ranging from 37.7 and 43.0. A nontemplate control (water) was added in duplicate for each of the 6 background concentrations; none amplified (final ΔRn ≤ 0.001 for all 12 nontemplate controls).

time. The cell-surface markers, some known to be early-expressed and short-lived (38), tended to fluctuate with time and were dropped from further analysis (*SI Appendix*, Fig. S5). T cell activation was measured as the fold increase of $CFSE^{low}$: the ratio of $CFSE^{low}$ percentage when mixed with a stimulator and $CFSE^{low}$ percentage at baseline (Fig. 3*B* and *SI Appendix*, Fig. S6).

In paired comparisons, activation of $CD4^+$ T cells was significantly more pronounced when the stimulator cell line's non-shared allele was $DERAA^+$ vs. when it was $DERAA^-$, and this was observed whether the shared allele was DQ8 or DQ6.2 ($P < 0.0001$ and $= 0.03$, respectively, Wilcoxon signed-rank test) (Fig. 3*C* and *SI Appendix*, Fig. S6). We report both RA and control

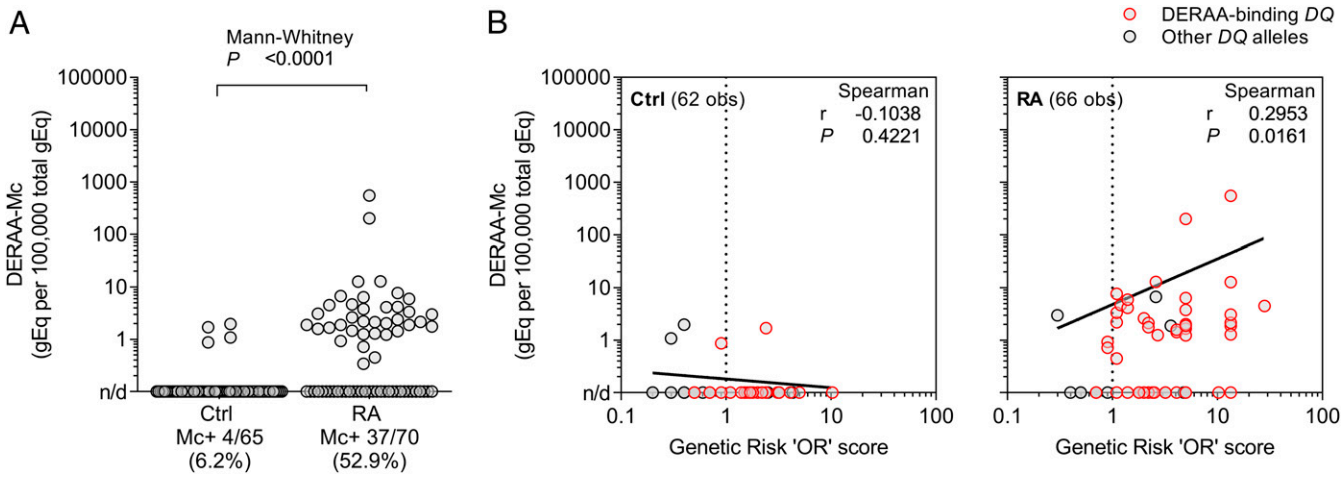


Fig. 2. DERAA-Mc is increased in RA patients and correlates with a risk-associated genetic background. (*A*) DERAA-Mc concentrations are measured in human cell genome equivalents of DERAA-encoding DNA per 100,000 gEq of total cellular DNA and plotted in log scale. (*B*) DERAA-Mc concentrations in correlation with the ORs of RA risk according to *HLA-DRB1* genotypes, when OR computing was available [an OR value was not available for 4 patients and 3 controls (35)]. The presence of DERAA-binding *HLA-DQ* alleles (in red) is distinguished from other *DQ* alleles (in gray). *P* values from the Mann–Whitney *U* and Spearman tests as well as the Spearman *r* are shown. Trend lines are log–log best fit lines. Ctrl, healthy controls; n/d, not detected; obs, observations.

results combined; the observation, however, is similar when analyzed separately (*SI Appendix*, Fig. S6*C*). On the other hand, activation of $CD8^+$ T cells in response to $DERAA^+$ did not differ significantly vs. $DERAA^-$ (Fig. 3*C*); this was expected because responder–stimulator HLA matching was based on class II and not class I.

Of the 26 subjects from the T cell activation studies, 11 healthy controls and 13 RA patients could be evaluated for correlation with their corresponding DERAA-Mc concentrations. No correlation was detected between DERAA-Mc levels and the T cell activation ratio of a $DERAA^+$ stimulation over a $DERAA^-$ stimulation (*SI Appendix*, Fig. S7).

Together, these data showed that, when controlling for the HLA class II genotypes of responders and stimulators, allogeneic cells that carried DERAA-encoding HLA induced a stronger in vitro helper T cell activation in both patients and controls, compared with cells that did not. The data support an immunogenic role of DERAA when carried by allogeneic cells, as also strongly implicated by results of familial HLA genotyping reported by others (14). However, we cannot exclude alternative explanations for the in vitro observations, including, for example, intrinsic differences of the cell lines or other experimental variables known or unknown.

DERAA-Mc Data Are Not Correlated with Clinical Features or Pregnancy History. In our study, DERAA-Mc appeared to be independent of age at RA onset. DERAA-Mc did not correlate with patients' ACPA and RF statuses, nor with patients' swollen joint count (*SI Appendix*, Fig. S8). Some patients were taking disease-modifying antirheumatic drugs (DMARDs) at the time blood was obtained, including hydroxychloroquine, gold, methotrexate, sulfasalazine, and azathioprine; however, use of these drugs appeared unrelated to DERAA-Mc levels (*SI Appendix*, Fig. S8). The most common sources of naturally acquired Mc are fetal-origin Mc (FMc) acquired from pregnancies resulting in birth, miscarriage, or elective termination (27, 39) and maternal Mc (MMc) acquired during fetal life. Most of our study subjects were parous women (*SI Appendix*, Table S1); however, DERAA-Mc results did not appear to correlate with parity or gravidity (*SI Appendix*, Fig. S8).

The DERAA-Mc Observations Differ from Those of Other Mc Specificities. Because DERAA-Mc levels differed sharply between RA patients and controls, we asked if this phenomenon was similar with other types of Mc. One such type is Mc carrying the SE. Indeed, the hypothesis that RA patients who are $SE^{-/-}$ can acquire RA risk through SE-Mc was addressed in a study that found a higher occurrence of Mc-carrying *HLA-DRB1**04 and *01 allele groups (among which a majority although not all alleles encode SE sequences) in RA women who were not *HLA-DRB1**04 and *01 (32). This was subsequently confirmed and extended with direct demonstration of the SE sequences QKRAA and QRRAA as Mc (33). These studies, considered together with the recent report of increased RA risk in SE-negative women for whom a previously born child had an SE allele (14), bring support to the "minigene transfer" hypothesis whereby patients without risk-associated alleles may nevertheless acquire them through Mc (26, 27).

In our SE-Mc studies, we included subjects who participated in the earlier study of Yan et al. (33) as well as additional subjects, for a total of 64 women with RA and 41 healthy women, all of whom lacked an SE-encoding HLA allele in their genotype. DNA from PBMCs of these study subjects was tested for both QKRAA and QRRAA markers and, with the additional participants, results remained similar to previous findings (*SI Appendix*, Fig. S9) (33). We evaluated overall SE-Mc, defined as having either QKRAA-Mc or QRRAA-Mc or both. Therefore, results of both assays were added to generate quantitative values of SE-Mc (*Methods*). SE-Mc detection was significantly increased in patients vs. controls, and ranked values of SE-Mc were significantly higher among RA women (Fig. 4*A*). When positive, Mc exclusively originated from either a QKRAA or a QRRAA marker, except in 3 RA patients and 1 control positive for both markers simultaneously. Bearing in mind the hypothesis of Mc-derived DERAA peptide involvement in RA pathogenesis of SE^+ individuals, we asked if the minigene transfer hypothesis could involve SE^+ microchimeric immune cells reacting against peptides from a $DERAA^+$ host. However, we found no suggestion of a difference in SE-Mc results if a subject had DERAA (*SI Appendix*, Fig. S10).

Another type of Mc is HLA specificities other than DERAA or SE. To identify non-DERAA non-SE Mc ($DERAA^-/SE^-$ Mc), we conducted HLA genotyping for family members of RA and healthy women and were able to identify 25 women with RA and 47 healthy women to target a nonshared marker that was not a DERAA- or an SE-encoding allele. Because familial HLA was known, the origin of $DERAA^-/SE^-$ Mc could be identified as maternal and/or fetal-origin (*SI Appendix*, Fig. S11*A*). DNA from

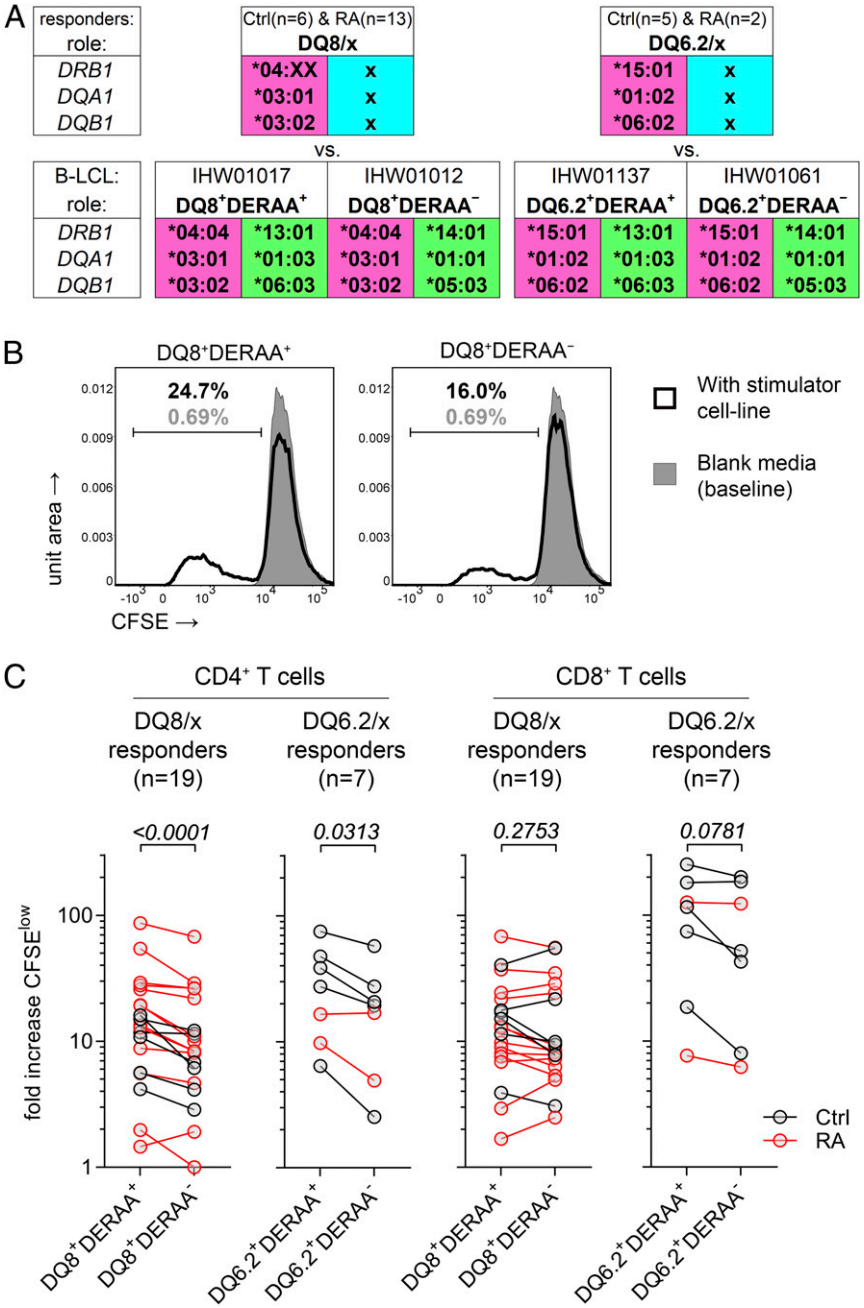
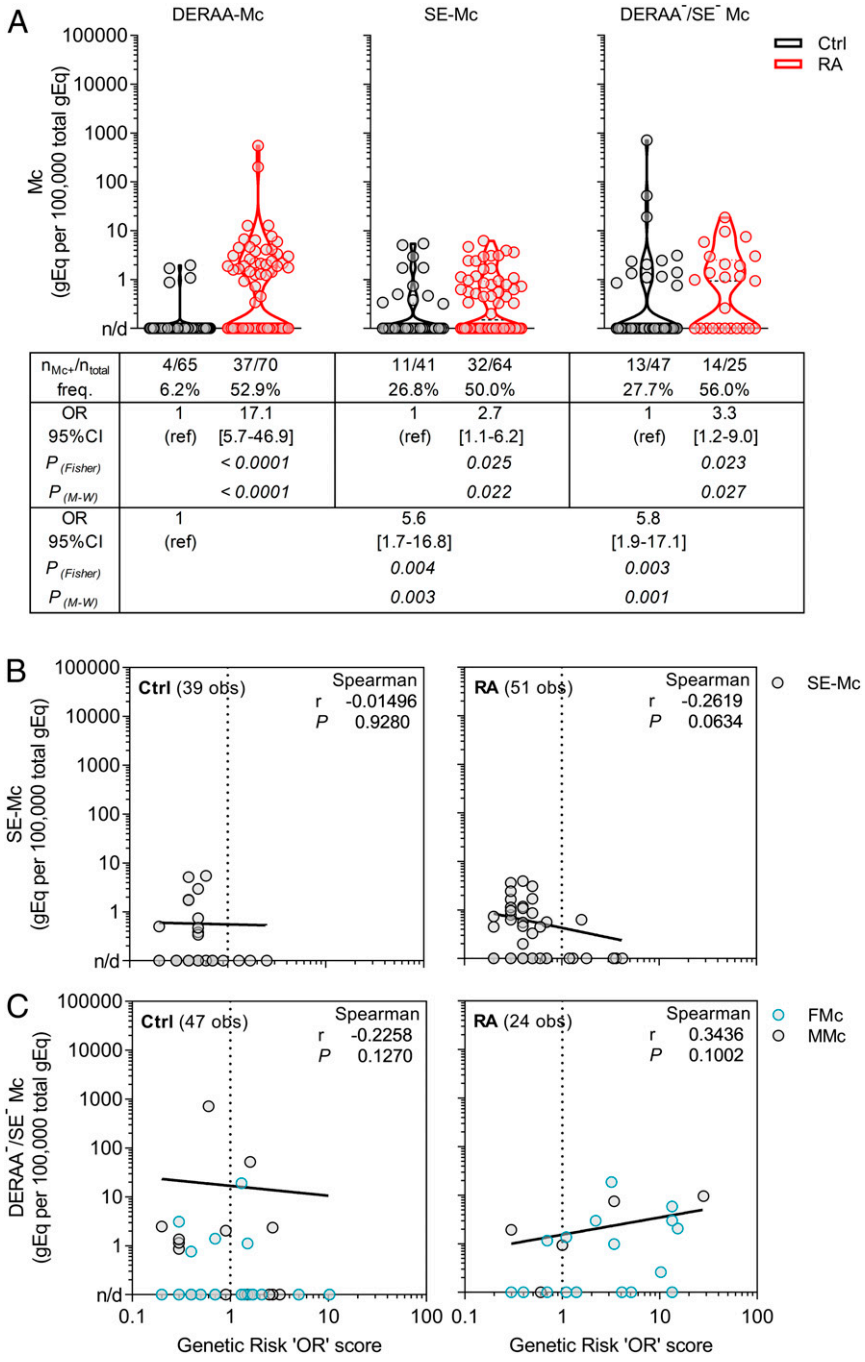


Fig. 3. CD4$^+$ T cells from DERAA$^{-/-}$ donors have a stronger response to DERAA$^+$ than DERAA$^-$ allogeneic cells. (*A*) Two groups of responder PBMCs: 1 DQ8 and something else (DQ8/x) and 1 DQ6.2 and something else (DQ6.2/x) are cocultured vs. 2 sets of human B lymphoblastoid cell lines. *HLA-DRB1*, *DQA1*, and *DQB1* genotyping is depicted. One haplotype is shared between responders and stimulators (in pink) and the other is mismatched (blue or green). The "x" in blue is always different from the "green" allele; furthermore, the x in DQ6.2/x is never a DQ5.1, DQ7.3, or DQ8 (reported to be DERAA-binding). *04:XX includes *04:01 ($n = 9$), *04:03 ($n = 2$), *04:04 ($n = 7$), and *04:08 ($n = 1$). (*B*) Representative flow cytometry analysis of singleton-gated alive CD3$^+$CD4$^+$ CFSE-labeled T cells from a DQ8/x study participant cocultured with a B-LCL that has the DQ8 and DERAA in trans (DQ8$^+$DERAA$^+$) or with a DQ8$^+$DERAA$^-$ B-LCL. Cocultures in blank media (baseline) are depicted in gray. The ratio of the 2 percentages of CFSE^{low} (black over gray) is the basis for computing the "fold increase CFSE^{low}." (*C*) Paired data points of the fold increase CFSE^{low} of singleton-gated alive CD3$^+$CD4$^+$ and CD8$^+$ T cells pooled according to the setup described in *A*. *P* values from the Wilcoxon matched-pairs signed-rank test comparing T cell proliferation vs. DERAA$^+$ B-LCL and vs. DERAA$^-$ B-LCL are represented. Healthy controls are in black and RA patients are in red.

PBMCs of these study subjects was tested in assays specific to *HLA-DRB1**15/16, *03, *07, *08, *DRB4**01, *DQA1**01, *03, *DQB1**02, *03, *04, *06, and *B**44 or non-HLA polymorphisms in the *GSTT1*, *TG*, and *ATIII* genes in case HLA of the subject and the Mc source was indistinguishable (28, 40). DERAA$^-$/SE$^-$ Mc was significantly increased in patients vs. controls, and ranked values were significantly higher among RA women (Fig. 4*A*). The trends were similar when considering DERAA$^-$/SE$^-$ that was FMc or that was MMc separately (*SI Appendix*, Fig. S11*B*). In a few cases there was overlap, namely a woman's mother and the woman's child shared the same marker (*SI Appendix*, Fig. S11*A*).

Neither SE-Mc nor DERAA$^-$/SE$^-$ Mc concentrations correlated with the genetic risk OR score in either RA patients or controls (Fig. 4 *B* and *C*). However, there was a tendency toward



n_{Mc+}/n_{total}	4/65	37/70	11/41	32/64	13/47	14/25
freq.	6.2%	52.9%	26.8%	50.0%	27.7%	56.0%
OR	1	17.1	1	2.7	1	3.3
95%CI	(ref)	[5.7-46.9]	(ref)	[1.1-6.2]	(ref)	[1.2-9.0]
$P_{(Fisher)}$		*< 0.0001*		*0.025*		*0.023*
$P_{(M-W)}$		*< 0.0001*		*0.022*		*0.027*
OR	1		5.6		5.8	
95%CI	(ref)		[1.7-16.8]		[1.9-17.1]	
$P_{(Fisher)}$			*0.004*		*0.003*	
$P_{(M-W)}$			*0.003*		*0.001*	

Fig. 4. DERAA and non–DERAA-Mc patterns in women with RA and healthy controls. (*A*) DERAA-Mc, SE-Mc, and non-DERAA non-SE (DERAA$^-$/SE$^-$) Mc, measured in human cell genome equivalents of microchimeric DNA per 100,000 gEq of total cellular DNA, are represented for RA and Ctrl. Frequency (freq.) of detecting Mc, as well as the corresponding ORs, 95% confidence intervals, and *P* values (Fisher's exact test) are reported. Differences of Mc quantities were tested by the Mann–Whitney *U* (M-W) test and *P* values are reported. Violin plots (kernel density) represent probability distribution of Mc data. (*B* and *C*) SE-Mc concentrations (*B*) and DERAA$^-$/SE$^-$ Mc concentrations (*C*) (for which fetal and maternal origins could be distinguished, noted as FMc and MMc) in correlation with the OR values of RA risk according to *HLA-DRB1* genotypes, when OR computing was available (35). *P* values from the Spearman test as well as the Spearman *r* are shown. Trend lines are log–log best fit lines.

lower levels of SE-Mc with increasing RA-risk score ($P = 0.063$) (Fig. 4 B, *Right*) in agreement with the minigene transfer hypothesis (26, 27).

It is worth noting that, among healthy subjects across the 3 groups (of different Mc specificities), both prevalence and quantities of DERAA-Mc were significantly decreased compared with SE-Mc and DERAA$^-$/SE$^-$ Mc [including some non-DERAA markers associated with RA protection (35), e.g., *DRB1**03 and *07] (Fig. 4*A*). Moreover, our results of SE-Mc and DERAA$^-$/SE$^-$ Mc in healthy controls generally agreed with Mc findings from previous studies in the healthy adult population, when testing PBMC samples using HLA and other polymorphism-specific assays (29, 40, 41).

Together, our data indicate that Mc of any kind, consistently prevalent in about half of RA patients, is naturally increased in RA compared with the healthy population. Such consistent patterns

are not observed in healthy subjects where DERAA-Mc is very uncommon, contrasting SE-Mc and DERAA^{-}/SE^{-} Mc that are prevalent in about a quarter of controls.

DERAA-Mc Is Greater in Recent-Onset than in Established RA. Considering the time-dependent effects of parity on disease onset (42, 43), we asked whether DERAA-Mc differed according to time since RA onset. Of the 70 patients with an RA onset between 0.1 and 2 y (median 0.8 y) tested in the DERAA-Mc arm of our study, we obtained a sample to test the same individual for 15 of them after onset. To these, we added 8 additional patients with established RA of onset >2 y for a total of 23 RA patients for which time since onset ranged from 2.2 to 11.3 y (median 7.3 y). DERAA-Mc was positive at early RA, and dropped to undetectable at follow-up in 5 patients, and in 2 cases remained detectable but without increase (Fig. 5*A*); DERAA-Mc was undetectable in early RA and remained undetectable at follow-up for 7 patients. DERAA-Mc was detected in 22% of the RA patients tested at both time points, and a Wilcoxon signed-rank test on 15 paired values determined this decrease was statistically significant ($P = 0.016$) (Fig. 5*A*).

DERAA-Mc–ranked concentrations tended to correlate inversely with the genetic risk OR score of patients when tested after RA was established ($P = 0.05$) (Fig. 5*B*), and DERAA-Mc was not detected when patients were SE$^{+/+}$ (*SI Appendix*, Fig. S12*A*). The SE, which is associated with high genetic RA-risk scores (35), in some studies has been considered a marker of severe disease when on an appropriate background, including cartilage erosion and bone destruction, regardless of therapy (34, 44, 45). DERAA-Mc concentrations were significantly inversely correlated with the joint-space narrowing score, disability score, and pain score of RA patients who were seen at onset and again after RA was established (*SI Appendix*, Fig. S12*B*).

Among the 64 RA patients tested in the SE-Mc arm of our study, 13 had an RA onset of ≤2 y and 51 had an RA onset >2 y. However, we found no suggestion of a difference in SE-Mc results if RA was recent vs. established (*SI Appendix*, Fig. S13). As for the RA patients for whom we obtained family members and tested in the DERAA^{-}/SE^{-} Mc arm, such comparison was not possible because all 25 had an RA onset >2 y (*SI Appendix*, Table S1).

Together, these data suggest involvement of DERAA-Mc in RA pathogenesis early on, after which DERAA-Mc levels decrease. This decrease is associated with more severe disease outcome (i.e., joint-space narrowing, disability, pain), reflecting a stronger chronic inflammation that could be naturally more efficient against allogeneic DERAA-Mc.

Discussion

In the current study, we report a striking difference of Mc with HLA alleles encoding the DRβ1 HV3 sequence DERAA in RA patients vs. healthy controls. The presence of DERAA-Mc increased the odds of RA ~17 times. Moreover, DERAA-Mc was greater in recent-onset RA vs. established RA, and this later decrease correlated with a more severe outcome, especially hand and wrist radiograph joint-space narrowing and standardized measures of disability and pain, and correlated with a higher genetic RA-risk score as well. Together, these observations strongly implicate DERAA-Mc in RA pathogenesis. That naturally acquired Mc has functional consequences is further supported by the consistent observation that CD4^{+} T cells had a significantly greater response against DERAA^{+} than DERAA^{-} allogeneic cells in alloreactive in vitro cocultures in RA patients and controls.

Prevalence of DERAA-Mc was ~50% in RA. For comparison, we extended studies to evaluate naturally acquired Mc encoding for HLA specificities other than DERAA. Mc encoding the RA risk-associated SE as well as Mc encoding neither DERAA nor the SE were also detectable in ~50% of patients. However, the difference between RA patients and controls was much less for Mc with specificities other than for DERAA-Mc and did not correlate with RA-risk genotypes. Our interpretation of these results is that DERAA-Mc carries greater immunogenicity and potency as a contributor to autoimmunity in RA, and hence is rare in healthy individuals.

Our results add strength to the report by Cruz et al. (14), who unexpectedly found increased RA risk among women who had children prior to disease onset for whom the paternally inherited HLA allele encoded DERAA. At the same time, this report presented a paradox: Why would HLA alleles that are protective in a person's own genotype be associated with RA risk when acquired as Mc? Our studies offer an explanation for this paradox. In an individual who has DERAA in their genotype, autoreactive T cells are deleted in the thymus. However, in a DERAA$^{-/-}$ individual, naturally occurring DERAA-directed T cells remain present as previously described (25). In these subjects, DERAA peptides from Mc [self-presented on microchimeric cells

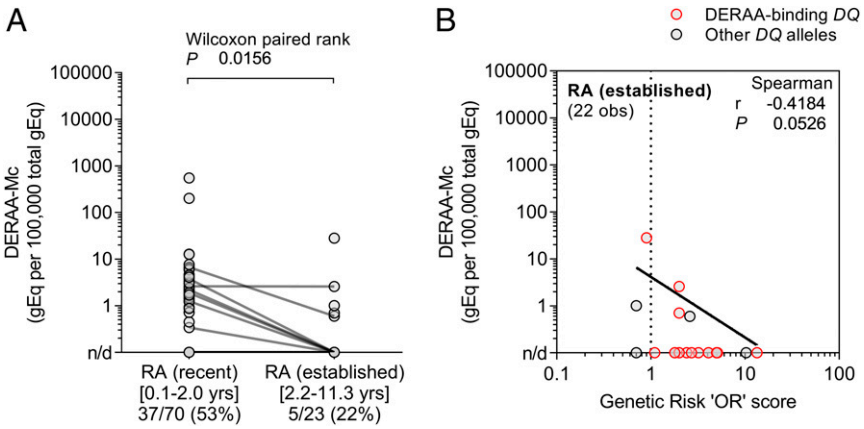


Fig. 5. DERAA-Mc is greater in RA closer to onset than years later when RA is established. (*A*) DERAA-Mc concentrations are measured in human cell genome equivalents of DERAA-encoding DNA per 100,000 gEq of total cellular DNA. Wilcoxon signed-rank test was conducted on $n = 15$ paired values (RA patients who had a first visit and a follow-up sample). The range of time since onset of RA is shown in years for each group. Frequency of detecting DERAA-Mc is shown. (*B*) DERAA-Mc concentrations in correlation with the ORs of RA risk according to *HLA-DRB1* genotypes, when OR computing was available (35). The presence of DERAA-binding *HLA-DQ* alleles (in red) is differentiated from other *DQ* alleles (in gray). *P* value from the Spearman test and Spearman *r* are shown. Trend lines are log–log best fit lines.

or presented by host antigen-presenting cells (16)] would activate DERAA-directed T cells that are part of a repertoire recognizing both endogenous and exogenous antigens containing the DERAA sequence (25). This immune activation can be promoted by a variety of HLA specificities, including by DERAA-binding *HLA-DQ* alleles (25). When this initial immune activation becomes chronic, the risk of emergence of RA through molecular mimicry increases, after which RA chronicity (years later) may become independent of the presence of DERAA-Mc. Our explanation is also consistent with the apparent incongruity that another study reported reduced RA risk when the noninherited maternal allele encoded DERAA (46), since exposure to the noninherited maternal HLA occurs during fetal development and would be expected to result in thymic negative selection and/or induction of fetal regulatory T cells promoting tolerance of maternal DERAA (47). Together, our data indicate a model where microchimeric cells are "stimulators" triggering the autoimmune reaction rather than the "effectors" responding against the host.

Our study has a number of limitations. We do not know the origin of DERAA-Mc that was detected in our study subjects. To achieve this goal, comprehensive family studies would be needed, as there are multiple different potential sources of Mc. Common sources of naturally acquired Mc are cells of fetal origin acquired by women who had births and maternal Mc acquired during fetal life. Additionally, Mc is not uncommon after a miscarriage or an elective termination (39), and can be acquired from a twin (whether recognized or undetected), or potentially acquired from an older sibling, transferred by the mother to the fetus in a later pregnancy (31). Blood transfusion can sometimes result in persisting Mc (48) and 1 study described increased RA risk with history of blood transfusion (49), although another with older patients found no effect (50). Reports of Mc from transfusion, however, are of multiply transfused trauma patients and transfusion is unlikely to confound results. Also, healthy controls were somewhat younger than patients, and though comprehensive studies on the impact of age on Mc are lacking, it is unlikely to confound our results (*SI Appendix*, Fig. S14). In the coculture experiments, the T cell response was tested against whole allogeneic cells as an approach to model cellular Mc. While patients and controls responded to cell lines with DERAA alloantigens, demonstrating direct immunogenicity of DERAA-Mc will require future studies.

In conclusion, here we present multiple lines of evidence that implicate DERAA-Mc in the autoimmune disease RA. While Mc has been associated with a number of different autoimmune diseases (27, 31), little information has been forthcoming to begin to understand the mechanism(s) by which Mc might contribute to susceptibility, chronicity, or protection from an autoimmune disease. Our results provide an explanation for the enigmatic observation that the same HLA sequence can differentially affect RA risk through Mendelian inheritance vs. pregnancy-derived acquisition and, when considered along with other studies, point to the functional significance of Mc and to a potential mechanism by which naturally acquired allogeneic cells contribute to the pathogenesis of an autoimmune disease.

Methods

Study Subjects and Specimens. Participants in this study were women who met the American College of Rheumatology criteria for RA (51) and healthy women with no history of autoimmune disease. The study included a DERAA-Mc analysis, SE-Mc analysis, and DERAA$^-$/SE$^-$ Mc analysis, in which 143, 105, and 72 subjects participated, respectively. Medical records and questionnaires were obtained and reviewed for clinical and demographic information. Characteristics, including age at draw, age at RA onset, ancestral background, parity, gravidity, autoantibody status (ACPA and RF), DMARD usage, and SE and DERAA genotypes are summarized in *SI Appendix*, Table S1, and details including RA evaluation, physical examinations, radiographic measurements, and self-administered health assessment are detailed in *SI Appendix, Supplementary Methods*. All study participants provided informed consent, and approval for the study was obtained from the institutional review board of the Fred Hutchinson Cancer Research Center.

Cryopreservation, DNA Extraction, and Genotyping. PBMCs were isolated from whole blood (in acid citrate dextrose A Vacutainer tubes) by density-gradient centrifugation and cryopreserved in 7% dimethyl sulfoxide and stored in liquid nitrogen. Genomic DNA was extracted using a QIAamp DNA Blood Mini Kit (Qiagen) and resuspended in Tris·HCl (pH 8.5). All subjects were genotyped for *HLA-DRB1*, *DQA1*, and *DQB1* loci. DNA-based typing was conducted with sequence-specific oligonucleotide probe panels or, alternatively, *DRB1*, *DQA1*, and *DQB1* strip detection (Dynal; RELI SSO) was used for initial determination of allelic groups followed by identification of specific alleles by sequencing (Applied Biosystems). To identify noninherited, nonshared polymorphisms targeted in the DERAA$^-$/SE$^-$ Mc studies, HLA genotyping was conducted on DNA from whole blood and/or buccal swabs obtained from participants' family members. When the nontransmitted HLA could not be distinguished from both HLA alleles of the participant, genotyping was carried out for 3 other loci: *GSTT1*, *TG*, and *ATIII*, as previously described (28).

Polymorphism-Specific Real-Time qPCR Assays for Mc Detection and Quantification. Each DNA sample from participants' PBMCs was assayed for Mc by selecting the appropriate assay from a panel of HLA-specific qPCR (28, 29, 33, 40) or non-HLA polymorphism-specific qPCR (28, 41) assays we have developed for this purpose. A new assay that targeted *HLA-DRB1* alleles that code for the DERAA sequence on HV3 was also used. The newly developed assay followed the same design, with rigorous specificity and sensitivity validation steps (Fig. 1) as described previously (40). Real-time qPCR reactions were carried out on an ABI Prism 7700 and on a QuantStudio 5 Real-Time PCR System (Applied Biosystems), as previously described (40). Mc concentrations were calculated according to the number of gEq of microchimeric cells proportional to the number of gEq of total PBMCs tested. SE-Mc was considered only if a participant had results for both QKRAA-Mc and QRRAA-Mc (33), and SE-Mc concentrations were computed as (Mc gEq_{QKRAA} + Mc gEq_{QRRAA}) ÷ (total gEq_{QKRAA} + total gEq_{QRRAA}). The *HLA-DRB1* alleles of the DERAA$^-$/SE$^-$ Mc sources (for whom FMc and MMc sources were identified) were confirmed non-DERAA (non*01:03, *04:02, *11:02, *11:03, *13:01, *13:02, or *13:04) and non-SE (non*01:01, *01:02, *04:01, *04:04, *04:05, *04:08, *10:01, or *14:02) for all of the subjects tested by HLA-specific or non-HLA polymorphism-specific assays.

Human B Lymphoblastoid Cell Lines. Epstein–Barr virus-transformed B-LCLs were used in our studies. Genomic DNA extracted from selected cell lines was utilized in the validation process of the DERAA qPCR assay, and cryopreserved selected samples were used in the T cell activation assays. The cell lines were previously collected and studied under International Histocompatibility Workshops and Conferences and obtained directly from the International Histocompatibility Working Group (https://www.fredhutch.org/en/research/institutes-networks-ircs/international-histocompatibility-working-group.html).

T Cell Activation. Responder PBMCs and stimulator B-LCLs were kept cryopreserved until the day of the coculture experiment (day 0). Cells were thawed and suspended in R10 culture medium (1× RPMI-1640, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 10% heat-inactivated FBS). B-LCLs received 6,000 cGy of radiation from a cesium-137 source, and were then counted. Responder PBMCs were stained for 20 min at 37 °C with carboxyfluorescein succinimidyl ester (BioLegend) at 2.5 μM, and then counted. At day 0, PBMCs were incubated at 37 °C, 5% CO_2 in R10 with each B-LCL at a 10:1 ratio, in R10 alone (blank media for baseline activation), or in R10 + 0.5% phytohemagglutinin (activation positive control). Each incubation was done in 4 replicates. T cell activation was measured as the ratio of the "marker" percentage when mixed with a stimulator divided by the marker percentage at baseline, namely the fold increase of that marker.

Flow Cytometry. Flow cytometry analyses were conducted at days 1, 3, and 5 for the first 10 participants and at day 5 for the remaining 16 participants. Cells were stained 15 to 20 min at room temperature in the dark, first with LIVE/DEAD Aqua Fluorescent Fixable Dead Cell Stain (Life Technologies), followed by staining with a 6-color mixture (CD4-BUV395, CD69-APC, CD8-PerCP-Cy5.5, HLADR-Alexa Fluor 700, CD38-BV786 [BD Biosciences], and CD3-PE-Texas red [Beckman Coulter]). Reagents were individually titrated to achieve optimal staining concentrations. Percentage population was averaged from up to 4 replicates in each case. After thorough quality control, a mean of 3.8 out of 4 replicates were retained in the analyses.

Statistical Analysis. Categorical variables were reported as counts and percentages, and comparisons were performed using Fisher's exact test. ORs were calculated, and the 95% confidence intervals were computed using the Baptista–Pike method. Continuous variables, including Mc quantities and T cell activation quantification, were analyzed without assumption of normal distribution of data; the number of comparisons in the T cell activation studies was always fewer than 30. As for Mc, it occurs by definition at low concentrations and approximates a Poisson distribution (data distribution skewed to the right, often with an excess of zeros and occasional large outlying values). Therefore, nonparametric tests were used, including the Mann–Whitney *U* rank test (for 2-group comparisons), Kruskal–Wallis test (for ≥3-group comparisons), Wilcoxon matched-pairs signed-rank test (for paired comparisons), and Spearman rank test (for correlations). Log–log best fit lines were used to illustrate trends. Analyses were performed using GraphPad Prism 7.

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