Effects of Various Concentrations of Pronase on Flow Cytometric Crossmatching Patients Treated With Rituximab and Donor HLA-Specific Antibodies

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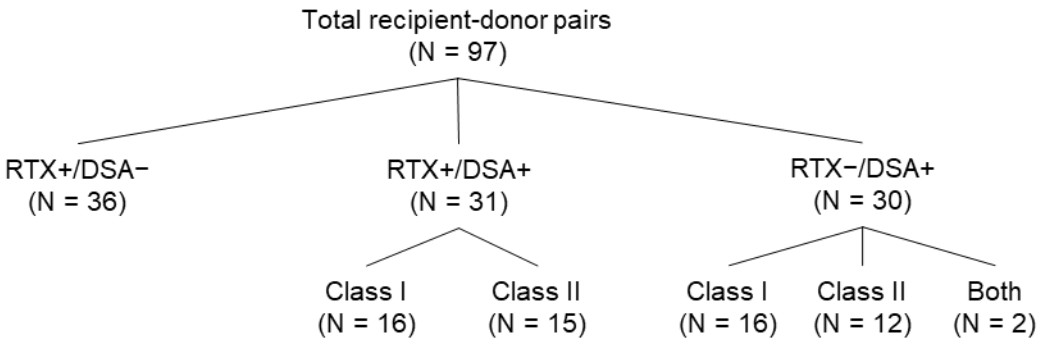
Supplemental Data

Supplemental Data Table S1. Detailed description of cases in the RTX+/DSA+ group that tested positive at 1 mg pronase/mL but negative at 2 mg/mL or 3 mg/mL

No. case	Group	DSA	MFI	T-FCXM* (0 mg/mL)	B-FCXM					
					0 mg/mL	0.5 mg/mL	1 mg/mL	2 mg/mL	3 mg/mL	4 mg/mL
220921-1	RTX+/DSA+	A*02:01	2,987	Neg.	251	109	229	23	56	55
220921-3	RTX−/DSA+	A*02:01	2,604	Pos.	61	0	0	22	12	35
220929-1	RTX+/DSA+	B*51:01	1,866	Neg.	228	255	280	81	62	42
220929-3	RTX−/DSA+	B*51:01	2,573	Pos.	25	95	141	83	119	78
221012-1	RTX+/DSA+	A*26:01	2,755	Neg.	335	118	330	0	10	91
221012-3	RTX−/DSA+	A*26:01	4,325	Neg.	0	0	0	17	30	21
221102-1	RTX+/DSA+	B*37:01	2,642	Neg.	260	162	208	56	0	0
221102-3	RTX−/DSA+	B*37:01	2,676	Neg.	0	0	0	0	0	0
221124-1	RTX+/DSA+	DQB1*05:02	5,226	Neg.	217	268	235	157	111	65
221124-3	RTX−/DSA+	DQB1*05:02	5,226	Neg.	11	3	0	60	44	35
221221-1	RTX+/DSA+	B*67:01	2,159	Neg.	330	256	211	113	63	38
221221-3	RTX−/DSA+	B*67:01	2,842	Neg.	89	30	36	39	44	71
221018-1	RTX+/DSA+	B*44:03	1,784	Pos.	211	362	263	82	101	100
221018-3	RTX−/DSA+	B*44:03	1,953	Pos.	10	147	168	156	156	161
221027-1	RTX+/DSA+	B*59:01, A*24:02	1,736 1,260	Neg.	177	0	186	60	95	78
221027-3	RTX−/DSA+	B*59:01, A*24:02	2,354 2,810	Neg.	0	0	60	168	126	105

*Crossmatches within the RTX−/DSA+ group paired with cases in the RTX+/DSA+ group are shown in parallel with the corresponding RTX+/DSA+ cases.

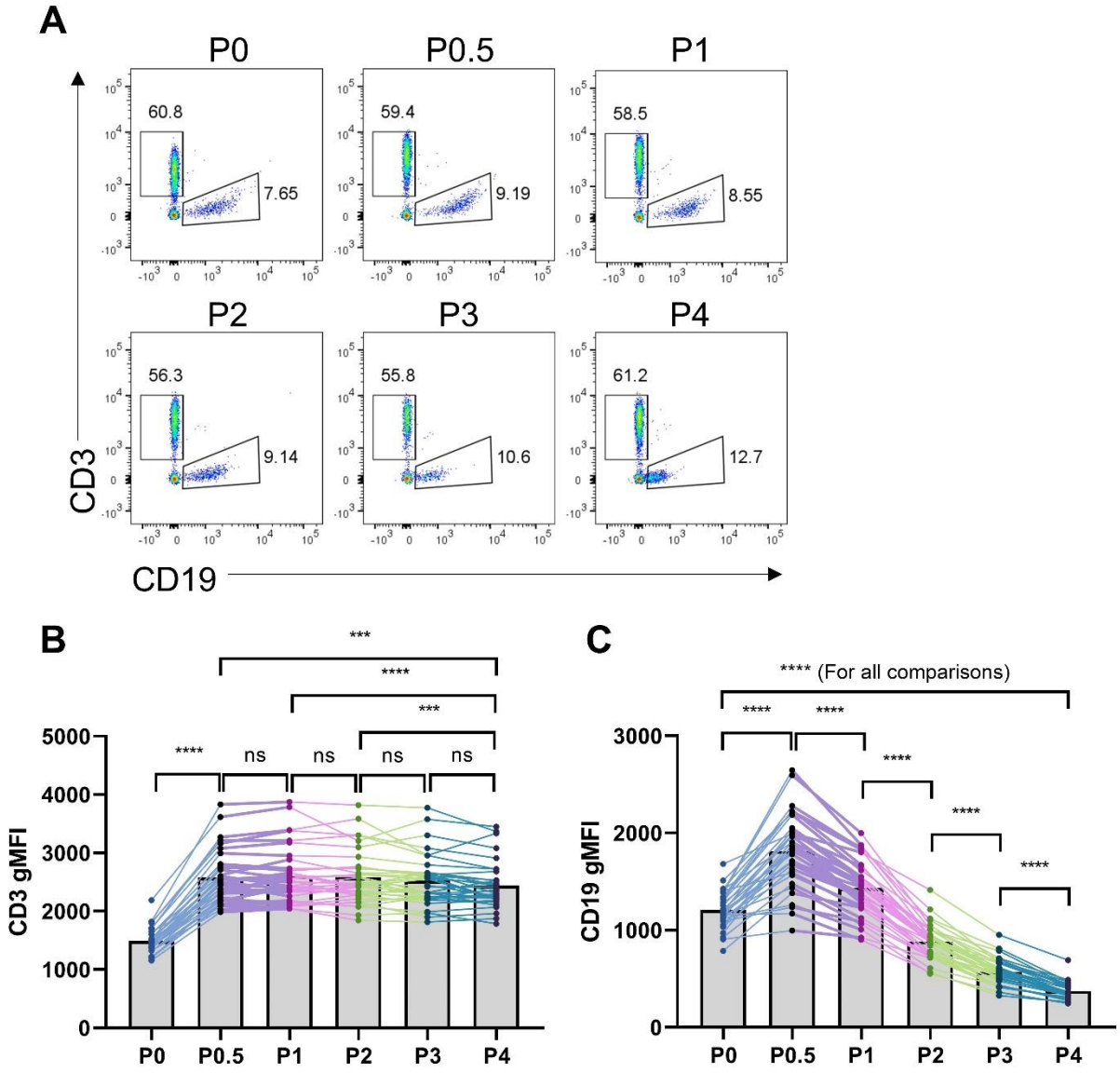
Abbreviations: B-FCXM, B-cell flow cytometry crossmatch; DSA, donor HLA-specific antibody; MFI, mean fluorescent intensity; T-FCXM, T-cell flow cytometry crossmatch; RTX, rituximab; Neg., negative; Pos., positive.



B

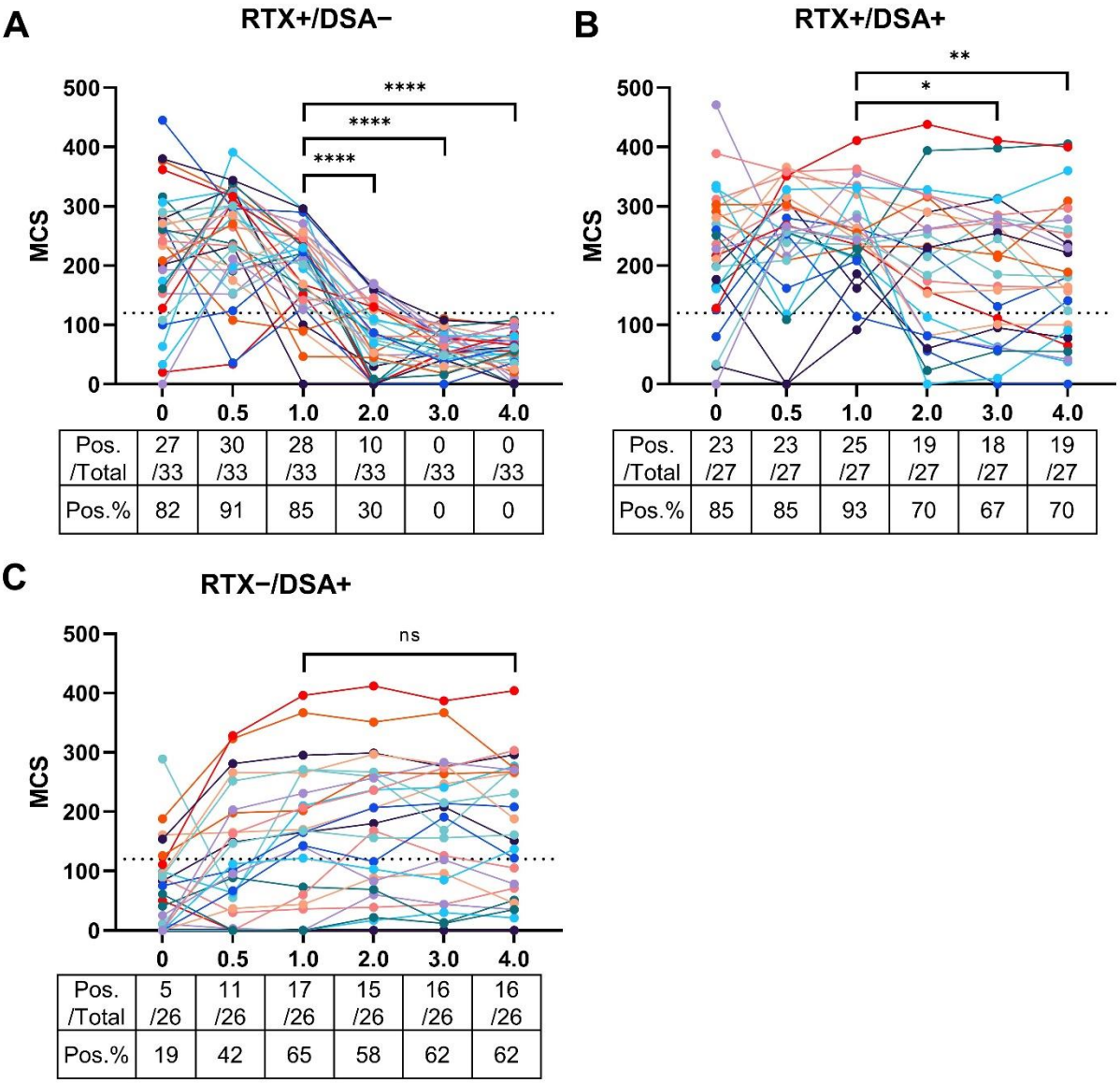
	RTX+/DSA− (N = 36)	RTX+/DSA+ (N = 31)	RTX−/DSA+ (N = 30)	*P*
DSA				
HLA-A	-	02:01, 11:01, 24:02, 26:01	02:01, 11:01, 24:02, 26:01, **33:03**	
MFI, median (min–max)		2,871 (1,260–4,215)	3,443 (1,525–6,626)	0.154
HLA-B	-	**07:02**, 13:02, 15:01, 27:05, 37:01, 40:01, 40:06, 44:03, 51:01, 58:01, 59:01, 67:01	13:02, 15:01, 27:05, **35:01**, 37:01, 40:01, 40:06, 44:03, 51:01, **54:01**, 58:01, 59:01, 67:01	
MFI, median (min–max)		2,854 (1,736–10,325)	2,759 (988–8,287)	0.377
HLA-DRB1	-	01:01, 03:01, 04:06, 07:01, 09:01, 11:01, 12:01, 13:02, 14:54, 15:01	01:01, 03:01, **04:03**, **04:05**, 04:06, 07:01, 09:01, 11:01, 12:01 13:02, 14:54, 15:01	
MFI, median (min–max)		3,104 (1,677–6,719)	2,846 (1,972–3,938)	0.248
HLA-DQ	-	**02:01**, 03:01, 03:02, 03:03, 05:02	03:01, 03:02, 03:03, **05:01**, 05:02	
MFI, median (min–max)		7,210 (1,443–19,033)	5,488 (1,655–21,879)	0.883
RTX				
Dose (mg), mean ± SD	546.6 ± 128.3	491.0 ± 174.8	-	0.192
Days post-RTX, median (min–max)	29 (16–33)	29 (11–42)	-	0.262
Plasmapheresis				
Times (n)	6 (2–8)	6 (0–9)	-	0.433

Supplemental Data Fig. S1. Serum samples used for flow cytometry crossmatch (FCXM) testing. (A) All recipient–donor pairs (N=97) were divided into three groups according to rituximab (RTX) use and the presence of donor-specific HLA antibodies (DSAs) in the serum. (B) The table summarizes the HLA specificity and mean fluorescence intensities (MFIs) of the DSAs in each group and provides information related to RTX treatment and plasmapheresis. The HLA specificities of the DSAs included in only one group are shown in bold. *P* calculations were performed using an unpaired t-test for the DSA MFIs or the Mann–Whitney test for the RTX dose, days post-RTX treatment, and the number of times plasmapheresis was performed.



Supplemental Data Fig. S2. Effects of pronase on the CD3 and CD19 expression levels found during flow cytometry crossmatch testing. (A) Representative flow cytometry histograms of CD3 versus CD19 at increasing pronase concentrations (0, 0.5, 1, 2, 3, and 4 mg/mL). The numbers in the histograms represent the percentages of positive cells in each gate relative to the total lymphocytes. (B and C) Summary plots depicting changes in the geometric mean fluorescence intensity (gMFI) for CD3 and CD19 as a function of the pronase concentration for 34 donor lymphocyte samples incubated with negative-control serum. Four of the 38 donor samples were excluded from analysis owing to the absence of

the 0 mg/mL pronase condition. Statistical analysis was performed using repeated-measures ANOVA, followed by Tukey's multiple-comparison test. ***P <0.001; ****P <0.0001. Abbreviation: ns, not significant.



	0	0.5	1.0	2.0	3.0	4.0
Pos. /Total	27 /33	30 /33	28 /33	10 /33	0 /33	0 /33
Pos.%	82	91	85	30	0	0

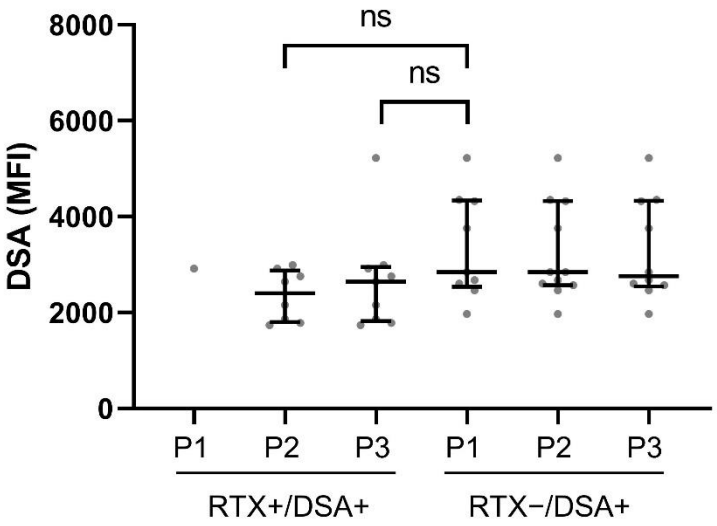
	0	0.5	1.0	2.0	3.0	4.0
Pos. /Total	23 /27	23 /27	25 /27	19 /27	18 /27	19 /27
Pos.%	85	85	93	70	67	70

	0	0.5	1.0	2.0	3.0	4.0
Pos. /Total	5 /26	11 /26	17 /26	15 /26	16 /26	16 /26
Pos.%	19	42	65	58	62	62

Supplemental Data Fig. S3. B-cell flow cytometry crossmatch test results obtained after six different pronase treatments. We crossmatched 86 recipient–donor pairs, consisting of 33 RTX+/DSA− (A), 27 RTX+/DSA+ (B), and 26 RTX−/DSA+ (C) samples, with lymphocytes treated using the indicated pronase concentrations. Each color represents the same serum–cell type pair. The dotted lines indicate the median channel shift (MCS) cutoff (120) for positive results. The MCS values for each pronase-concentration group were compared using Friedman's test, followed by Dunn's multiple-comparison test. The median MCSs of the 2, 3, and 4 mg/mL pronase groups were compared with that of the 1 mg/mL pronase group. The table below each graph shows the total number of samples tested (Total), the number of

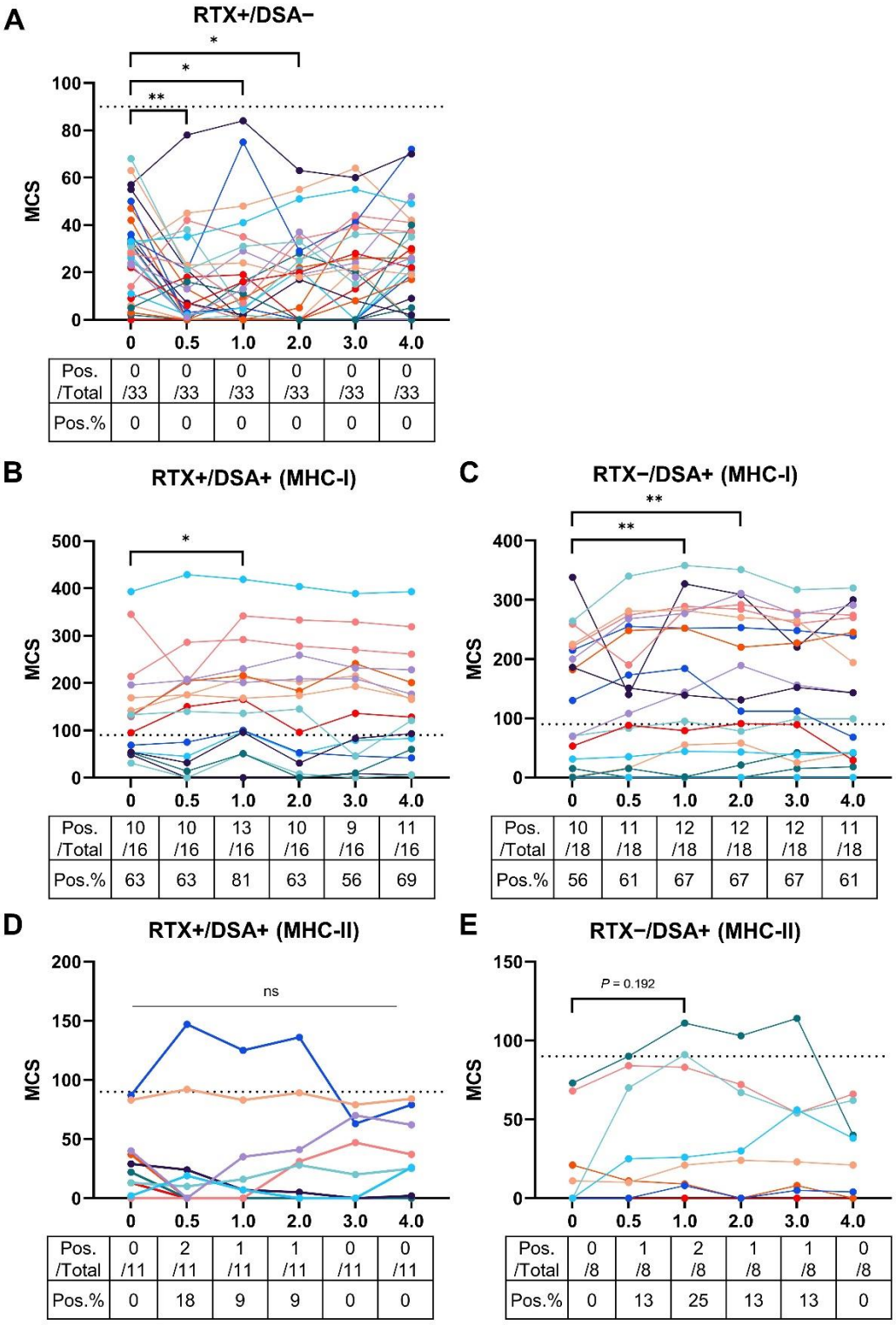
positive samples (Pos.), and the percentage of positive results (Pos. %). *P<0.05; **P<0.01; ****P<0.0001.

Abbreviations: RTX, rituximab; DSA, donor HLA-specific antibodies; ns, not significant.



Supplemental Data Fig. S4. Comparison of the mean fluorescence intensities (MFIs) of DSAs present in RTX+/DSA+ and RTX−/DSA+ sera. Some of the crossmatched pairs analyzed in the main figure (Fig. 2B) contained two different DSAs, in which case a simple sum was used to represent the total MFI. To avoid errors in data interpretation caused by simple sums, we analyzed only those cases with a single DSA. FCXM-negative cases that contained a single DSA were divided into RTX+/DSA+ and RTX−/DSA+ groups, which were subdivided into 1, 2, and 3 mg/mL pronase groups (P1, P2, and P3, respectively). The DSA MFIs in the RTX+/DSA+ group that were negative at 2 and 3 mg/mL did not significantly differ from those in the RTX−/DSA+ group that were negative at 1 mg/mL. The horizontal lines and error bars represent the median and interquartile range of the columns.

Abbreviations: RTX, rituximab; DSA, donor HLA-specific antibodies; ns, not significant.



Supplemental Data Fig. S5. T-cell flow cytometry crossmatch test results obtained after six different pronase treatments. We crossmatched 86 donor–recipient pairs, consisting of 33 RTX+/DSA− (A), 27 RTX+/DSA+ (B and D), and 26 RTX−/DSA+ (C and E) samples, with lymphocytes treated using the indicated pronase concentrations. The crossmatching results

for RTX+/DSA+ and RTX−/DSA+ sera were plotted in separate graphs based on the MHC class of the DSAs present in each serum sample. The RTX+/DSA− group (A) showed similar results compared with those found via unpaired analysis (Fig. 3A). RTX+/DSA+ and RTX−/DSA+ groups expressing MHC class I DSAs (B and C, respectively) showed a small (but statistically significant) increase in MCS values at 1 or 2 mg/mL pronase compared with those observed at 0 mg/mL pronase. Nine positive T-cell FCXM results were found with two recipient–donor pairs in the RTX+/DSA+ (MHC class II DSA) group (D) and two recipient–donor pairs in the RTX−/DSA+ (MHC class II DSA) group (E). Each color represents the same serum–cell type pair. The dotted lines indicate the median channel shift (MCS) cutoff (90) for positive results. The MCS values for each pronase-concentration group were compared using Friedman's test, followed by Dunn's multiple-comparison test. The median MCS of each column was compared with that of the control column (0 mg/mL pronase). The table below each graph shows the total number of samples tested (Total), the number of positive samples (Pos.), and the percentage of positive results (Pos. %). *P<0.05; **P<0.01.

Abbreviations: RTX, rituximab; DSA, donor HLA-specific antibodies; ns, not significant.