

L'FCXM: Innovazioni Metodologiche ed Interpretati

Antonina Piazza

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SIGNIFICANCE OF THE POSITIVE CROSSMATCH TEST IN KIDNEY TRANSPLANTATION*

RAMON PATEL, M.R.C.P., AND PAUL I. TERASAKI, PH.D.

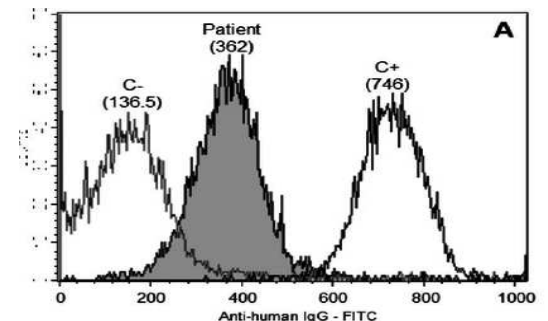
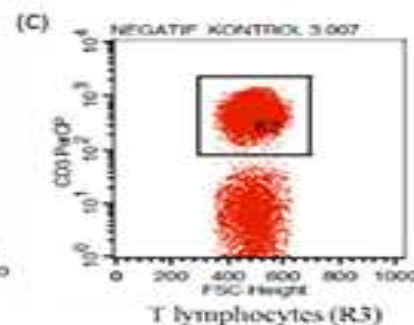
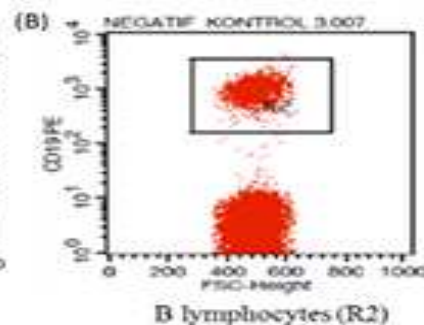
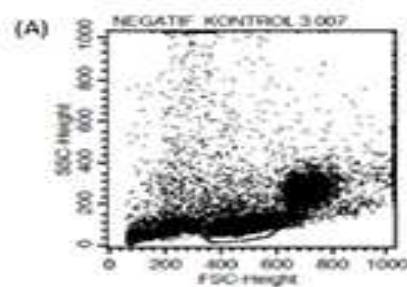
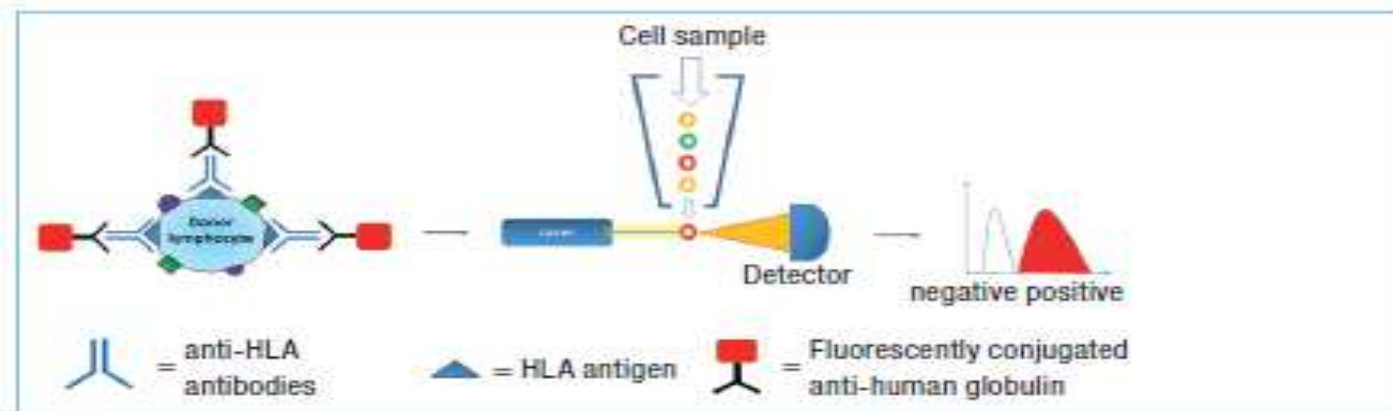
TABLE 2. *Classification of 248 Kidney Transplants Performed in 63 Recipients with and 163 without Preformed Antibodies According to the Duration of Graft Survival.*

GRAFT SURVIVAL	RECIPIENTS WITH ANTIBODIES			RECIPIENTS WITHOUT ANTIBODIES
	POSITIVE CROSSMATCH	NO CROSSMATCH	NEGATIVE CROSSMATCH	
Immediate failures	24 (80.0%)	6 (26.1%)	4 (14.8%)	4 (2.4%)
Failure within < 3 mo	0	6	4	32
Failure after > 3 mo	1	3	7	22
Survival for < 3 mo	2	2	1	6
Survival after > 3 mo	3	6	11	104
Totals	30	23	27	168

(30%)

Crossmatch in Citometria a Flusso

(1980's)



- ✓ FC-XM: elevata sensibilità, maggiore correlazione con anti-HLA DSA rilevati con tecniche in fase solida (Luminex-beads).
- ✓ FC-XM: permette una valutazione del «rischio immunologico» del trapianto.

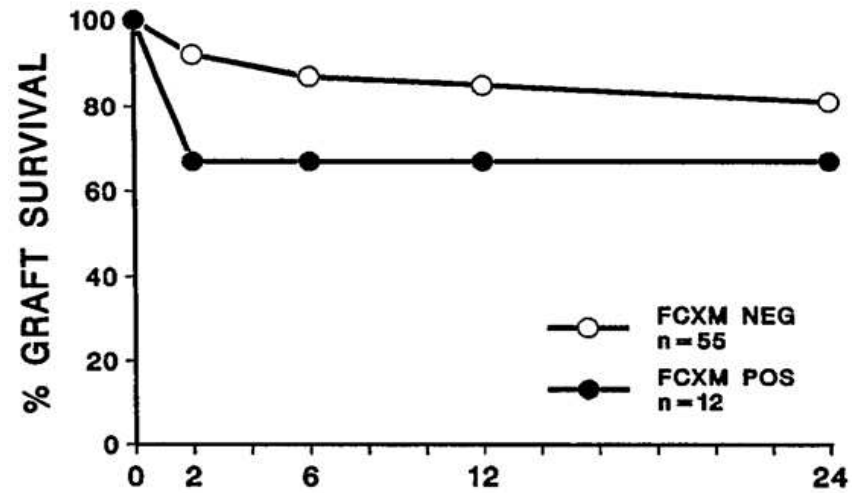
The Flow Cytometric Crossmatch and Early Renal Transplant Loss

Mahoney R.J., Ault K.A., Given S.R., et al.

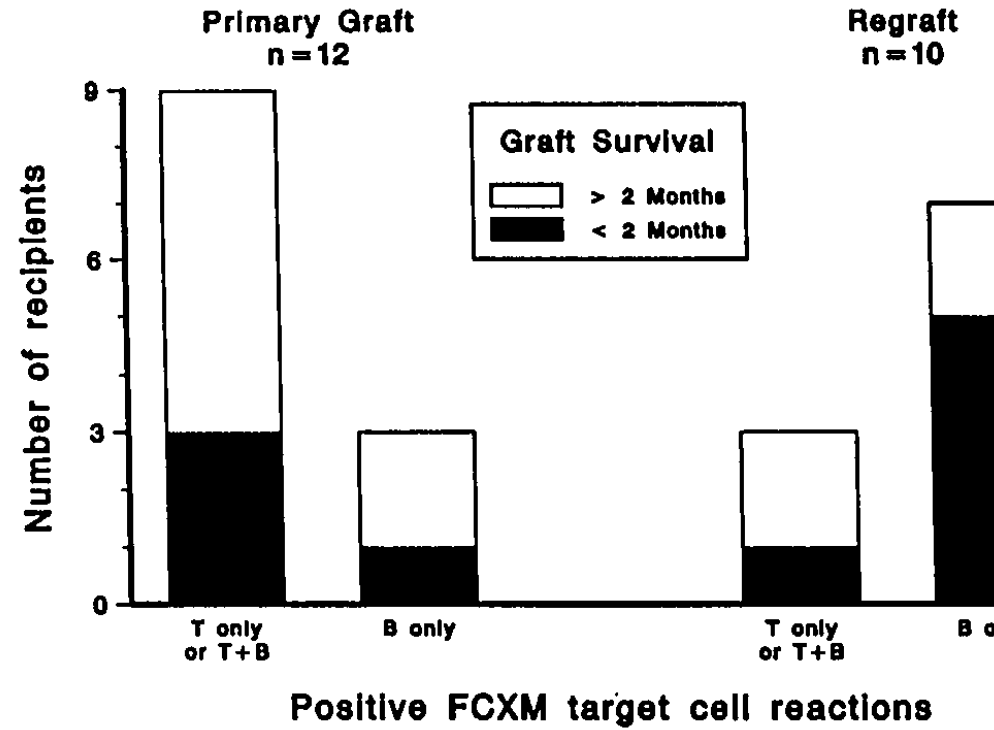
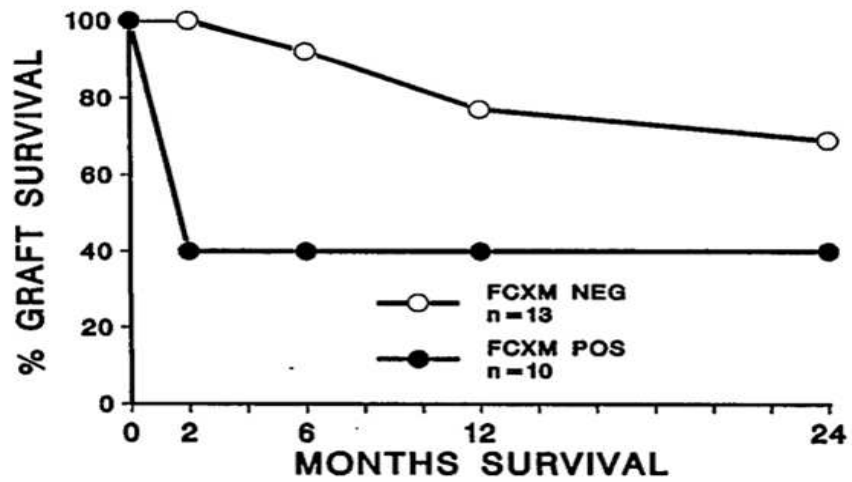
Data from this retrospective study indicate that a positive FCXM is predictive of early renal allograft loss (less than 2 months) in cadaveric kidney donor recipients who had a negative crossmatch by the anti-human globulin complement-dependent cytotoxicity technique.

CAD groups	n	FCXM	n	(%)	<2-Month graft function		Probability ^a	
					n	(%)		
All	90	Pos.	22	(24)	10	(45)	<0.0001	
		Neg.	68	(76)	4	(6)		
1st graft	67	Pos.	12	(18)	4	(33)	<0.03	} 0.4
		Neg.	55	(82)	4	(7)		
Regrafts	23	Pos.	10	(43)	6	(60)	<0.002	
		Neg.	13	(57)	0	(0)		

PRIMARY GRAFTS



REGRAFTS



Multivariate analysis. We examined a number of other variables that might have additional predictive value in addition to the FCXM test for predicting early graft loss. Stepwise logistic regression analysis was performed utilizing the recipient's gender, primary or regraft status, PRA level, mismatched HLA-A-B-DR donor antigens, and the FCXM test. Full set regression analysis of all 5 predictor variables in the 90 cadaveric graft patients indicated that the FCXM test was the best predictor of early graft loss, and that none of the other variables contributed significantly to the predictive value after inclusion of the FCXM result. The difference in the number of HLA-mismatched antigens between FCXM-positive (3.59) and -negative (3.99) cadaveric graft recipients was not statistically significant in early graft loss (rank-sum test, $P=0.17$).

Innovazioni Metodologiche (I)

Utilizzo della pronase (1990's)

HLA. 2017;90:157–164

Pronase Treatment Improves Flow Cytometry Crossmatching Results

M.-J. Apithy, J. Desoutter, A. Gicquel, E. Guiheneuf, P.-F. Westeel, A. Lesage, V. Piot, G. Choukroun, N. Guillaume

Flow cytometry crossmatching (FC-XM) is the most sensitive cell-based method for detecting donor-specific antibodies in clinical organ transplantation.

Unfortunately, background FC-XM reactivity is elevated in assays with B lymphocytes- p because of nonspecific immunoglobulin binding by Fc receptors and B-cell surface immunoglobulins.

The therapeutic Abs used to treat acute rejection or to desensitize patients (such as rituximab anti-CD20), can interfere with XM assays.

In an effort to increase the specificity and sensitivity of FC-XM, researchers have treated cells with pronase (a proteolytic enzyme that can remove Fc receptors from the cell surface).

Pronase treatment can improve the interpretation of B-cell FC-XM

	HLA typing	Sera	DSA class I (MFI)	DSA class II (MFI)	P-XM with pronase	P-XM without pronase	Discrepancies
P17	A2 A24 B27 B51 DR1 DR4 DQ8 DQ5	S1.17	0	DR53 (6800)	T-cells: N B-cells: P	T- and B-cells: N	Without pronase false negative
P18	A1 A23 B8 B44 Cw4 Cw7 DR7 DR12 DQ2 DQ7	S3.18	0	DR7 (4600)	T-cells: N B-cells: P	T- and B-cells: N	Without pronase false negative
P26	A11 A33 B52 B58 Cw7 Cw10 DR4 DR13 DQ8 DQ6 DP2 DP3	S1.26/ S2.26	B52 (3500–6000)	DR53 (8500–11 000)	T- and B-cells: P	T-cells: P B-cells: N	Without pronase false negative
P28	A2 A29 B62 B44 Cw4 Cw16 DR7 DR10 DQ2 DQ5 DP2 DP-	S1.28	0	0	T- and B-cells: N	T-cells: N B-cells: P	Without pronase false positive
P29	A1 A68 B70 B57 Cw10 Cw6 DR17 DR7 DQ2 DQ9 DP1 DP3	S1.29	0	0	T- and B-cells: N	T-cells: N B-cells: P	Without pronase false positive

However, pronase treatment may affect other cell surface molecules (such as MHC), which in turn may affect reactivity in an XM assay.

Impact of pronase on flow cytometric crossmatch outcome

S. J. Hetrick, K. P. Schillinger, A. A. Zachary, A. M. Jackson

We examined the effect of pronase treatment on human leukocyte antigen (HLA) expression and FCXM results. Lymphocytes were tested untreated and after treatment with either 2 mg/mL (6 donors) or 1 mg/mL (6 cell donors) of pronase.

Effect of pronase treatment (2 mg/mL) on human leukocyte antigen expression: reduction of HLA

Subject	Untreated cells			Pronase-treated cells				
	T cells		B cells	T cells		B cells		Class II
	Class I	Class I	Class II	Class I	Change (%)	Class I	Change (%)	
1	267,942 ^b	292,354	77,531	202,053	−25	224,495	−23	66,540
2	332,149	328,955	54,769	234,337	−29	270,846	−18	14,258
3	248,172	250,573	46,979	157,899	−36	189,567	−24	11,476
4	377,885	421,574	108,993	261,534	−31	443,068	+5	51,690
5	372,268	268,678	109,589	282,884	−24	231,472	−14	153,508
6	374,718	173,883	127,405	153,079	−59	133,477	−23	29,560
7	182,987	261,504	447,425	136,813	−25	203,649	−22	25,541
8	371,096	446,859	90,668	237,847	−36	246,723	−45	20,039
9	215,336	185,222	84,707	144,630	−33	124,998	−33	46,806
12	254,754	293,151	110,791	183,742	−28	192,607	−34	78,702
Mean	299,731	292,275	85,616	199,482	−33	226,090	−23	49,812
SD	74,222	88,619	29,268	52,245	±10	89,003	±13	48,216
p value					0.0006		0.012	

^aConcentration of pronase.

^bMolecules of equivalent soluble fluorochrome.

Effect of pronase treatment (2 mg/mL) on flow cytometry crossmatch: reduction of reactivity in 5 (31%) tests of T cells and 15 of 16 (94%) tests of B cells.

Cell/serum	Untreated		Pronase treated		Change (%)	
	T cells	B cells	T cells	B cells	T cells	B cells
1/A	19,399 ^b	32,734	18,621	15,523	−4	−47
1/A-5 ^c	5399	5891	2743	2059	−49	−65
2/B	39,239	64,859	46,294	67,458	+18	+4
2/B-5	13,423	29,648	9505	13,717	−29	−53
3/C	46,084	71,053	50,255	50,740	+9	−29
3/C-5	10,990	22,183	9982	11,311	−9	−50
5/E	2137	50,154	3703	43,510	+73	−14
5/E-5	2038	36,898	2526	29,133	+24	−21
5/E-10	2018	23,509	2026	18,420	0	−21
9/F	1878	20,705	2898	6704	+54	−68
9/G	4470	18,983	5267	12,303	−18	−35
9/G-50	2926	9575	2211	3758	−24	−60
9/H	1705	22,581	2151	6574	+26	−71
12/M-1	14,642	20,133	7903	6302	−46	−69
12/M-5	6857	8271	7757	4487	+13	−46
12/M-10	3984	3243	4167	1904	+5	−42
Mean	11,725	64,082	11,697	20,828	+3	−67
SD	13,321	108,659	14,713	21,155	±33	±10
p value					0.967	0.000

^aConcentration of pronase.

^bMolecules of equivalent soluble fluorochrome.

Effect of pronase treatment (1 mg/mL) on human leukocyte antigen expression: variable among cell preparations and between class I and class II antigens.

Subject	Untreated cells			Pronase-treated cells				
	T cells		B cells	T cells		B cells		Class II
	Class I	Class I	Class II	Class I	Change (%)	Class I	Change (%)	
2	236,330 ^b	227,527	66,887	279,288	+18	284,633	+25	64,882
3	231,937	495,116	60,942	208,189	−10	492,620	−1	65,612
9	348,950	479,431	117,050	307,290	−12	422,210	−12	112,901
10	115,368	303,392	68,142	162,839	+41	341,957	+13	89,948
11	121,023	218,054	47,819	100,898	−17	139,715	−36	46,465
12	254,734	293,151	110,791	175,256	−31	190,755	−35	58,238
Mean	218,000	336,112	78,605	205,627	−2	311,982	−8	73,008
SD	88,173	122,039	28,357	76,797	±26	134,758	±25	24,174
p value					0.673		0.887	

^aConcentration of pronase.

^bMolecules of equivalent soluble fluorochrome.

Effect of pronase treatment (1 mg/mL) on flow cytometry crossmatch: significant increase reactivity in 20 of 23 tests of T cells (87%, $p = 6.0 \times 10^{-5}$).

Cell/serum	Untreated		Pronase treated		Change (%)	
	T cells	B cells	T cells	B cells	T cells	B cells
2/B	28,202 ^b	33,776	44,408	46,123	+57	+57
2/B-5 ^c	16,894	22,672	22,657	19,654	+34	–
2/B-10	10,120	14,933	16,729	13,452	+65	–
3/C	56,492	163,326	63,773	108,350	+13	–
3/C-5	11,601	39,405	15,714	23,608	+35	–
3/C-10	7291	22,525	9790	13,634	+34	–
9/F	1703	24,603	7403	11,417	+335	–
9/G	18,506	51,506	22,186	17,026	+20	–
10/I	8373	93,460	14,589	56,812	+74	–
10/I-5	3573	61,923	7323	39,110	+105	–
10/I-10	2707	42,629	4761	30,055	+76	–
10/J	10,946	57,909	17,670	80,185	+61	+61
10/J-5	4627	28,244	4806	23,101	+4	–
10/J-10	3713	19,445	2707	11,375	–27	–
11/L	26,924	55,200	38,001	60,168	+41	+41
11/L-5	17,005	32,296	17,005	23,885	0	–
11/L-10	11,485	22,772	16,682	23,322	+45	+45
11/K	7464	37,282	8701	30,201	+17	–
11/K-5	2895	15,305	4007	11,595	+38	–
11/K-10	2323	7323	3008	5710	+29	–
12/M	14,642	20,133	16,647	13,032	+14	–
12/M-5	6857	8271	8530	4412	+24	–
12/M-10	3984	3243	5179	2200	+30	–
Mean	12,101	38,182	16,186	29,062	51	–
SD	12,206	343,396	14,798	26,053	±77	±77
p value					0.00006	0.00006

^aConcentration of pronase.

^bMolecules of equivalent soluble fluorochrome.

- ✓ Le osservazioni dell'aumentata reattività di FCXM usando cellule T trattate con pronasi suggeriscono che oltre a ridurre l'espressione di HLA, il trattamento con pronasi può influenzare la conformazione della molecola HLA, distruggendo epitopi allogenici e esponendo epitopi non nativi. (Terasaki e colleghi hanno riportato la presenza anticorpi verso tali epitopi presenti nel siero di individui non sensibilizzati).
- ✓ La maggiore concentrazione di pronasi potrebbe rimuovere le molecole di HLA dalla superficie cellulare, mentre la concentrazione inferiore si limita a modificarle.
- ✓ Inoltre, l'impatto della pronasi può essere maggiore sulle molecole di classe I rispetto a quelle di classe II perché la tasca di legame del peptide è formato dalla piegatura di una singola catena e può essere più suscettibile a qualsiasi alterazione della molecola.
- ✓ Indipendentemente dai meccanismi alla base dell'effetto del trattamento con pronasi, è probabile che l'effetto sia clinicamente rilevante e dovrebbe essere preso in considerazione valutando l'espressione di MHC e la forza del DSA quando si interpretano i risultati di FCXM con cellule trattate con pronasi.

Questi dati indicano che il trattamento con pronasi può causare errati risultati di FCXM

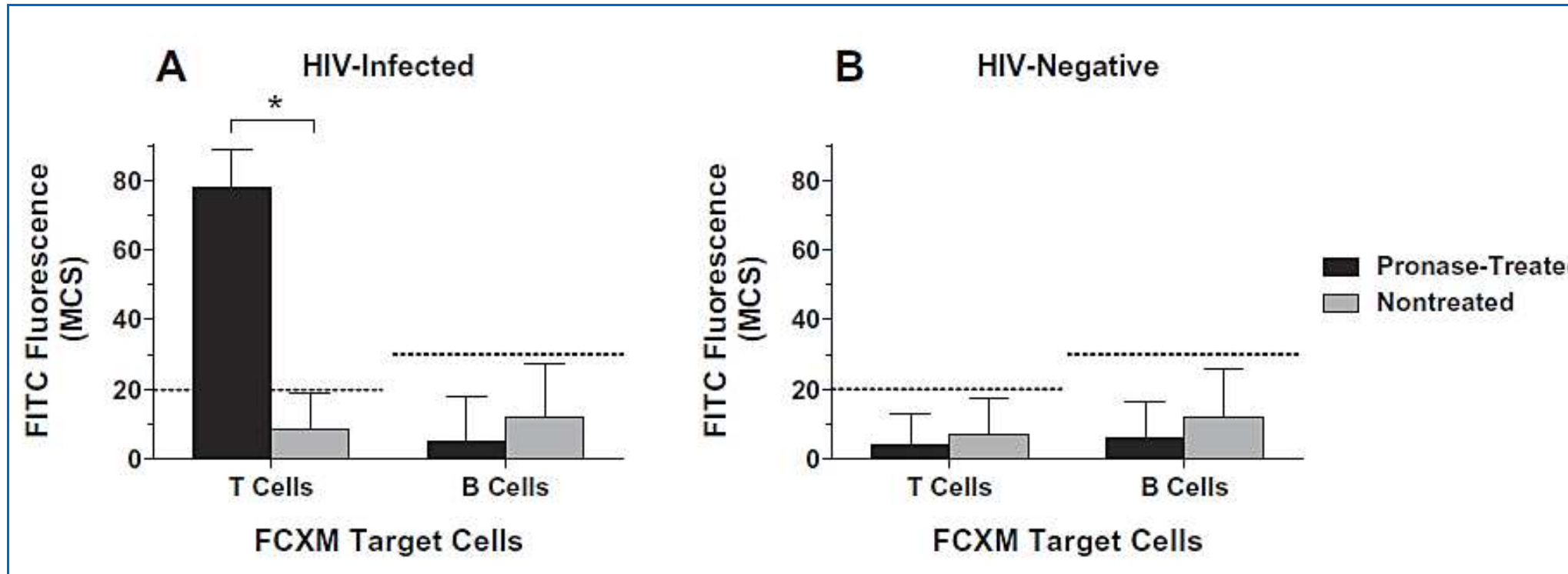
Flow cytometry crossmatch reactivity with pronase-treated T cells induced by n autoantibodies in human immunodeficiency virus-infected patients

K. Szewczyk, K. Barrios, D. Magas , K. Sieg, B. Labuda, M. D. Jendrisak , A. Jaramillo

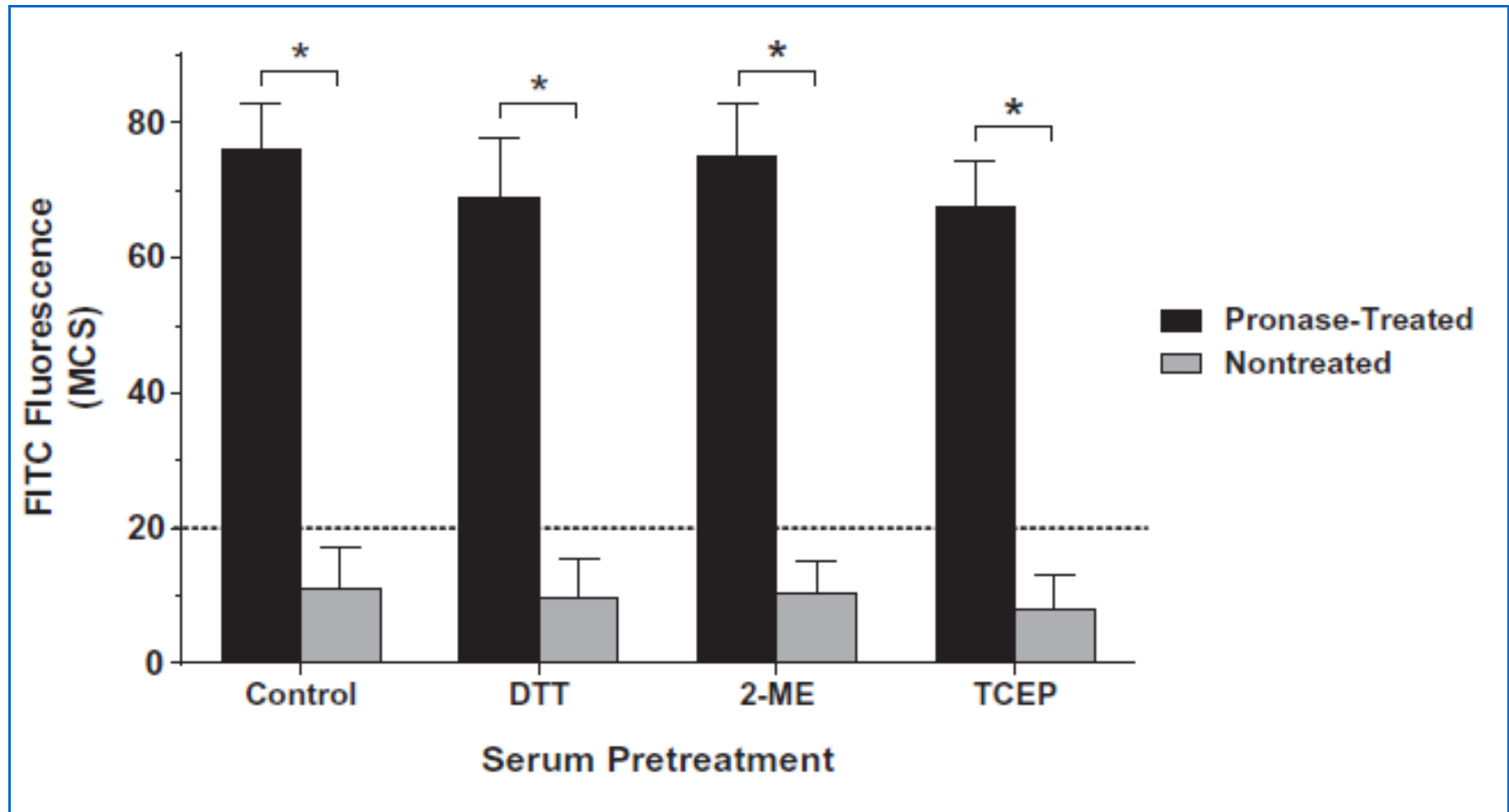
Pronase treatment is used in the flow cytometry crossmatch (FCXM) to prevent nonspecific antibody binding on B cells.

However, we have observed unexpected positive results with pronase-treated T cells in human immunodeficiency virus (HIV)-infected patients.

In this study, 25 HIV-infected patients without HLA antibodies were tested with pronase-treated and nontreated cells.



FCXM reactivity with pronase-treated T cells in HIV-infected patients. Three-color FCXM tests were performed in HIV-infected and HIV-negative patients with pronase-treated (1 mg/mL) and nontreated T and B cells.



FCXM reactivity with pronase-treated T cells in HIV-infected patients is not abrogated by serum pretreatment with reducing agents.

DTT, dithiothreitol; TCEP, tris(2-carboxyethylphosphine); 2-ME, 2-mercaptoethanol.

Long-term kidney allograft survival in HIV-infected

Patient	Transplanted	Graft status	Graft survival time (Days)	Graft Failure time (Days)	Last creatinine (mg/dL)	T cell FCXM reactivity		B cell FCXM reactivity	
						Pronase- treated	Nontreated	Pronase- treated	Nontreated
1	Yes	Functioning	2248		2.2	+	-	-	-
<p>Recent studies have shown that pronase treatment generally increased the T cell FCXM reactivity.</p> <p>We have also observed a similar effect of pronase treatment on the T cell FCXM results with sera from HIV-infected patients.</p> <p>In conclusion, laboratories using pronase in their FCXM protocol should consider testing HIV-infected patients with nontreated cells to prevent these patients from being inappropriately excluded from receiving an organ because of positive T cell FCXM results with pronase-treated cells.</p>									
20	Yes	Failed		498		+	-	-	-
21	Yes	N/A	N/A	N/A	N/A	+	-	-	-
22	No								
23	No								
24	No								
25	Expired								
Mean ± SD			1193 ± 631	833 ± 294	1.6 ± 0.3				

Pronase independent flow cytometry crossmatching of rituximab treated Patients

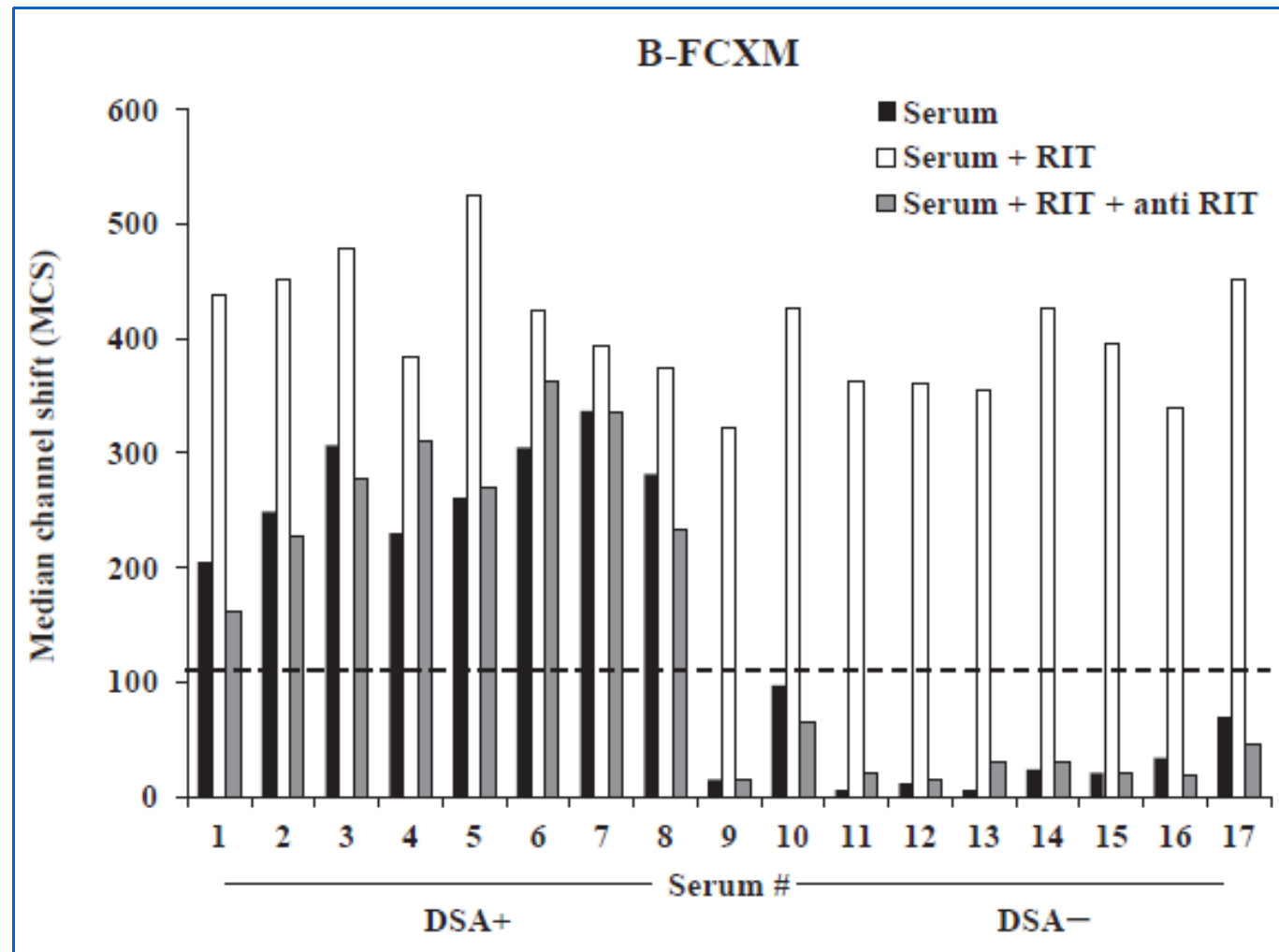
R. S. Liwski, A.L. Greenshields, D. M. Conrad, C. Murphey, R. A. Bray, J. Neumann, H. M. Gebel

It is known that even low amounts of remaining rituximab in serum of patients results in positive B cell cross match results, masking detection of potentially harmful donor human leukocyte antigen (HLA) specific antibodies.

Treatment of donor cells with high concentrations (> 1 mg/mL) of pronase is currently standard procedure for elimination of rituximab (RIT) interference. As already noticed by Bearden, pronase does not completely remove CD20 on B cells even when using high concentrations of pronase (≥ 1 mg/mL).

However, more problematic is the recent finding that pronase can modulate the expression of HLA molecules, which may lead to incorrect cross match results.

The aim of this study was to evaluate an alternative pronase-free FCXM for crossmatching patients treated with rituximab.



Flow cytometric crossmatching donor specific antibody (DSA) negative and positive sera supplemented with rituximab. B-FCXM using DSA- and DSA+ sera.

Crossmatching was done with only, serum supplemented with RIT (10 µg/mL) and serum supplemented with RIT and pre-treated with anti-RIT (10 µg/mL).

Dashed line indicates cut-off for positive crossmatch (MCS: 106).

Using Student T test did not indicate any significant differences between serum alone or serum/RIT/anti-RIT (cell p=.88).

Innovazioni Metodologiche (III)

Ottimizzazione della tecnica dell'FCXM

Human Immunology 79 (2018) 28–38

Rapid optimized flow cytometric crossmatch (FCXM) assays: The Halifax and Halifaster protocols

R. S. Liwski, A.L. Greenshields, D. M. Conrad, C. Murphey, R. A. Bray, J. Neumann, H. M. Gebel

The flow cytometric crossmatch (FCXM) assay, which detects the presence of donor specific HLA antibodies in patient sera, is a cornerstone of HLA compatibility testing.

Since relatively long FCXM assay turnaround times may contribute to transplant delays and increased graft ischemia time, we developed and validated two modified crossmatch procedures, namely the Halifax and Halifaster FCXM protocols.

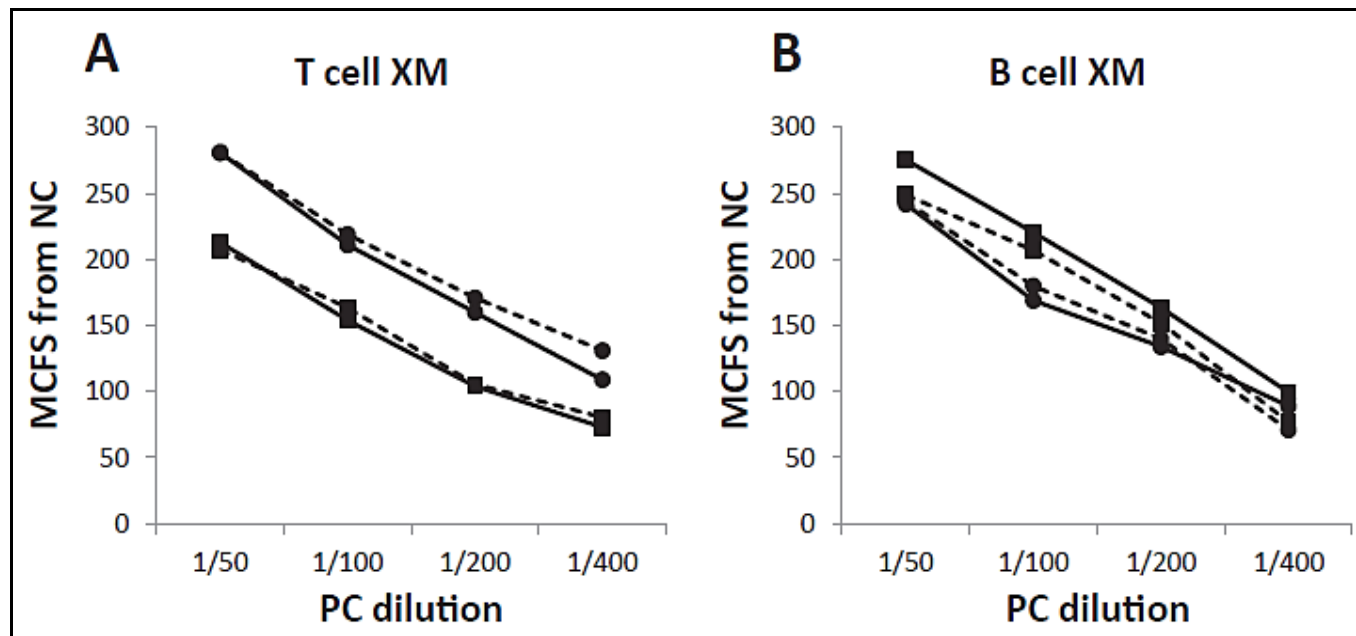
These protocols reduce FCXM assay time >60% and simplify their set-up without compromising quality or sensitivity.

Parameter	Standard FCXM Tube Method	Standard FCXM Tray Method	Halifax FCXM Protocol	Halifaster FCXM Protocol
Assay platform	5-mL, 12 × 75 mm tubes	96-well tray	96-well tray	96-well tray
Serum (μL)	30	30	50	30
Cell isolation method, cell preparation	Lympholyte-H, PBMC	Lympholyte-H, PBMC	Lympholyte-H, PBMC	EasySep™ Direct. Lym
Pronase/DNase treatment time (min)	15/2	15/2	15/2	15/2
Cell preparation time (min)	90	90	90	55
Cell suspension (μL)	30	30	25	15
Cell number	2.5×10^5	2.5×10^5	2.5×10^5	1.5×10^5
First incubation (min)	30	30	20	20
Washes (1st set)	3 × 5 min at 500 × g	3 × 1 min at 500 × g	3 × 1 min at 500 × g	3 × 1 min at 500 × g
Wash buffer (μL)	1000	200	200	200
Antibody cocktail (μL)	100	100	100	50
PBS/CD3/CD19/IgG-FITC	89.75/5/5/0.25	89.75/5/5/0.25	94.75/3/2/0.25	46.88/2/1/0.125
Second incubation (min)	30	30	10	5
Washes (2nd set)	2 × 5 min at 500 × g	2 × 1 min at 500 × g	2 × 1 min at 500 × g	2 × 1 min at 500 × g
Final suspension μL	500	500	400	400
FCXM assay time (min)	85	65	35	30
Total time, including cell preparation (min)	175	155	125	85

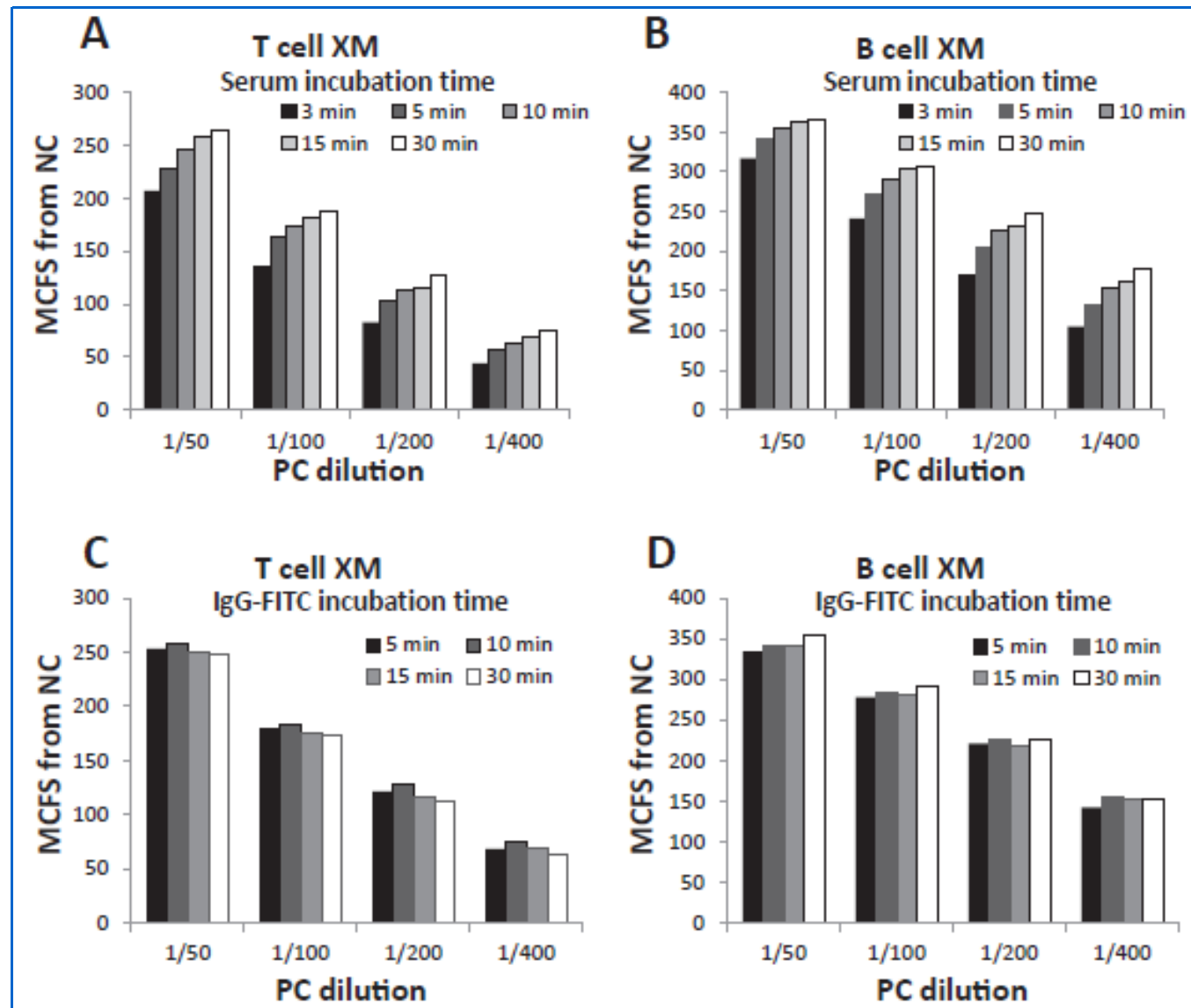
- ✓ Optimization of the FCXM, the Halifax protocol, includes a 96-well tray platform, reduced incubation times, increased serum to cell suspension volume ratio, shortened incubations and incubation temperature.
- ✓ The Halifaster protocol is a further modification, employing methods that improve lymphocyte purity compared to density gradient centrifugation ($96 \pm 2.63\%$ vs $69 \pm 19.06\%$), reduce isolation time (by ~40%) and conserve FCXM assay reagents.

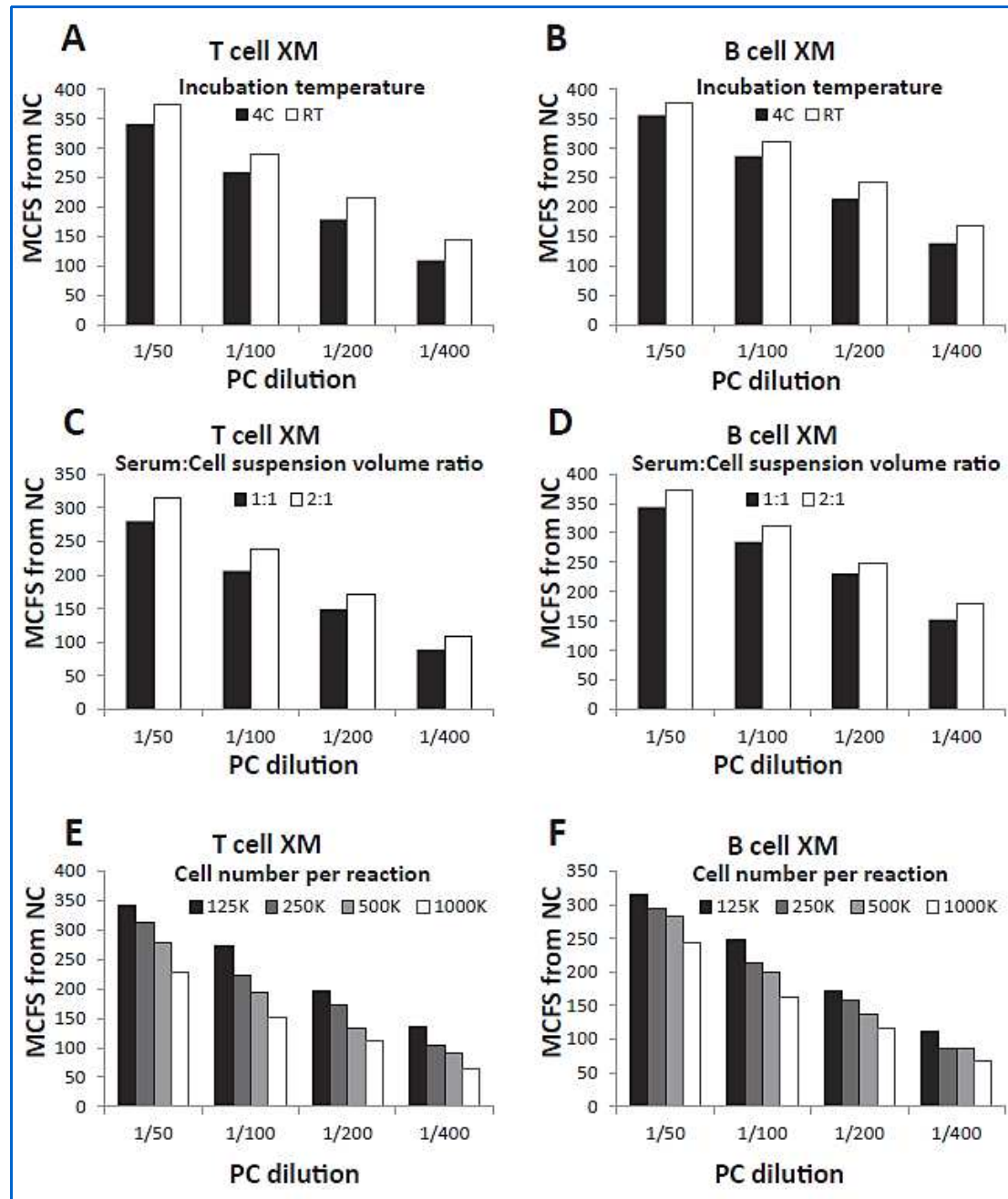
Comparison of FCXM performance using different study protocols.

Parameter	T cell FCXM			B cell FCXM	
	Standard	Halifax	Halifaster	Standard	Halifax
Negative control, mean MCF (SD)	168 (21)	168 (18)	176 (16)	216 (25)	215 (25)
Negative crossmatch patient sera, mean MCF (SD)	161 (26)	158 (25)	170 (24)	213 (32)	203 (34)
3SD cutoff value, MCFS	79	74	71	97	101
Mean crossmatch cutoff, MCF	247	242	247	313	316

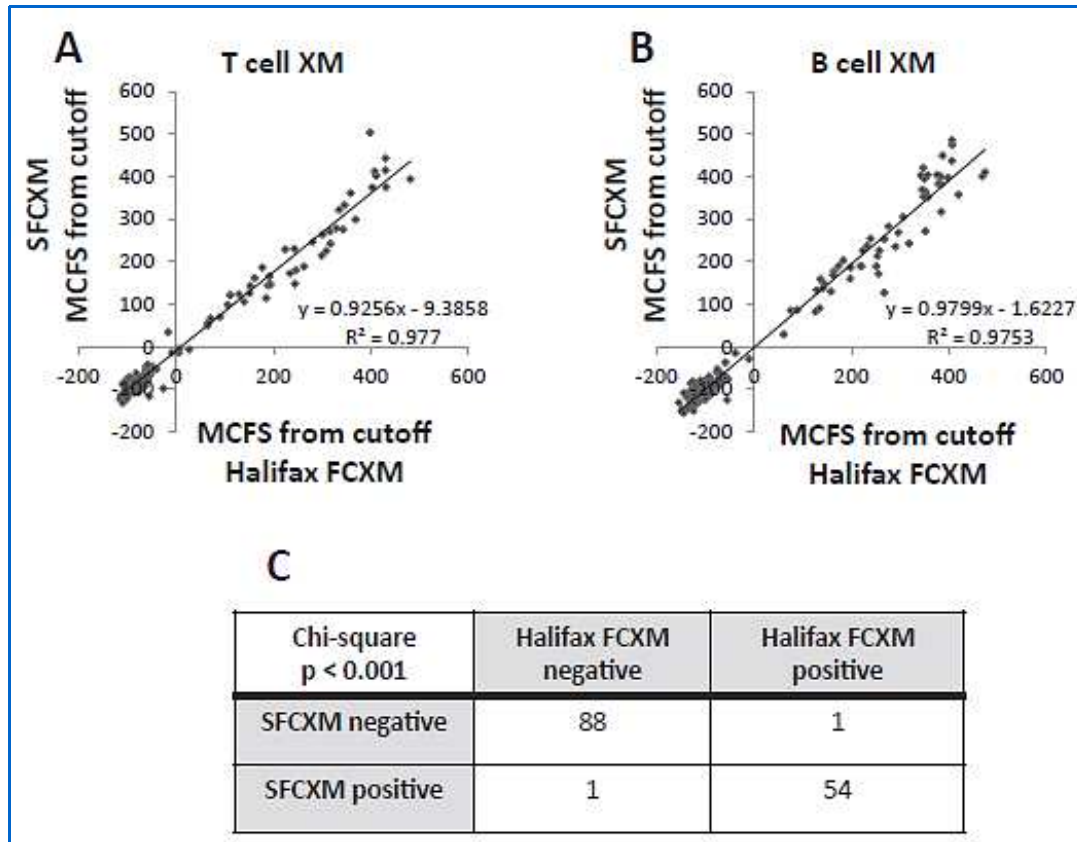


Standard FCXMs performed in (dashed line) or trays (solid line) comparable results. Square (■) and circle (●): independent experiments are shown.

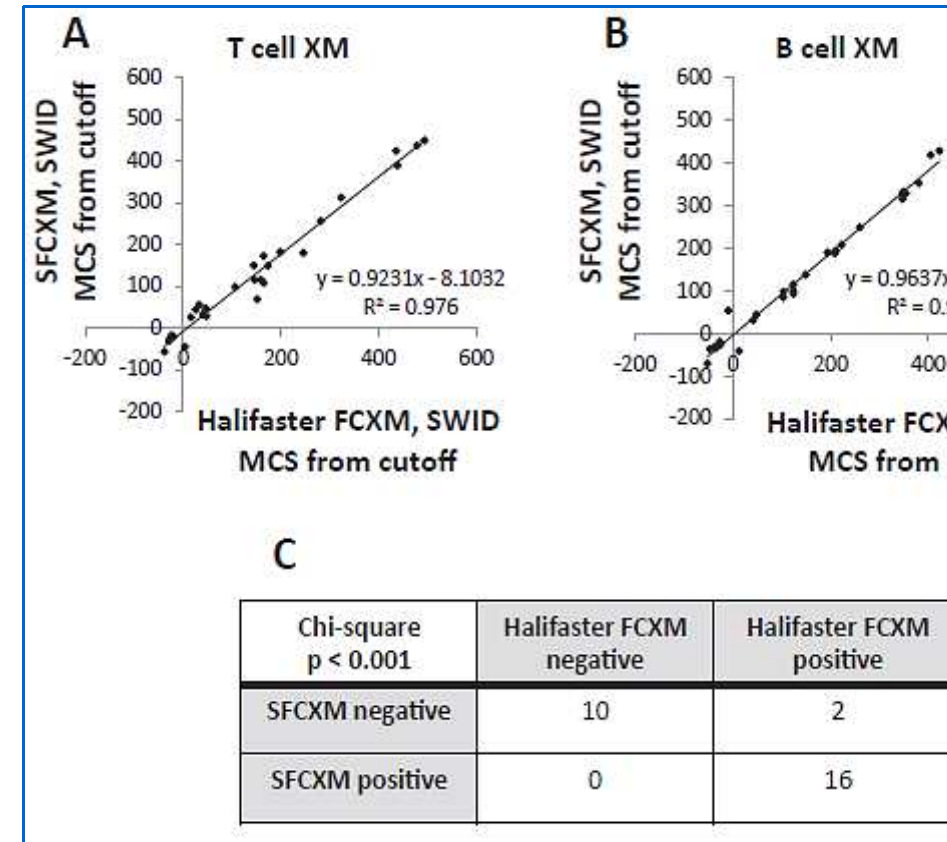
Effects of serum and IgG-FITC incubation time on FCXM results.



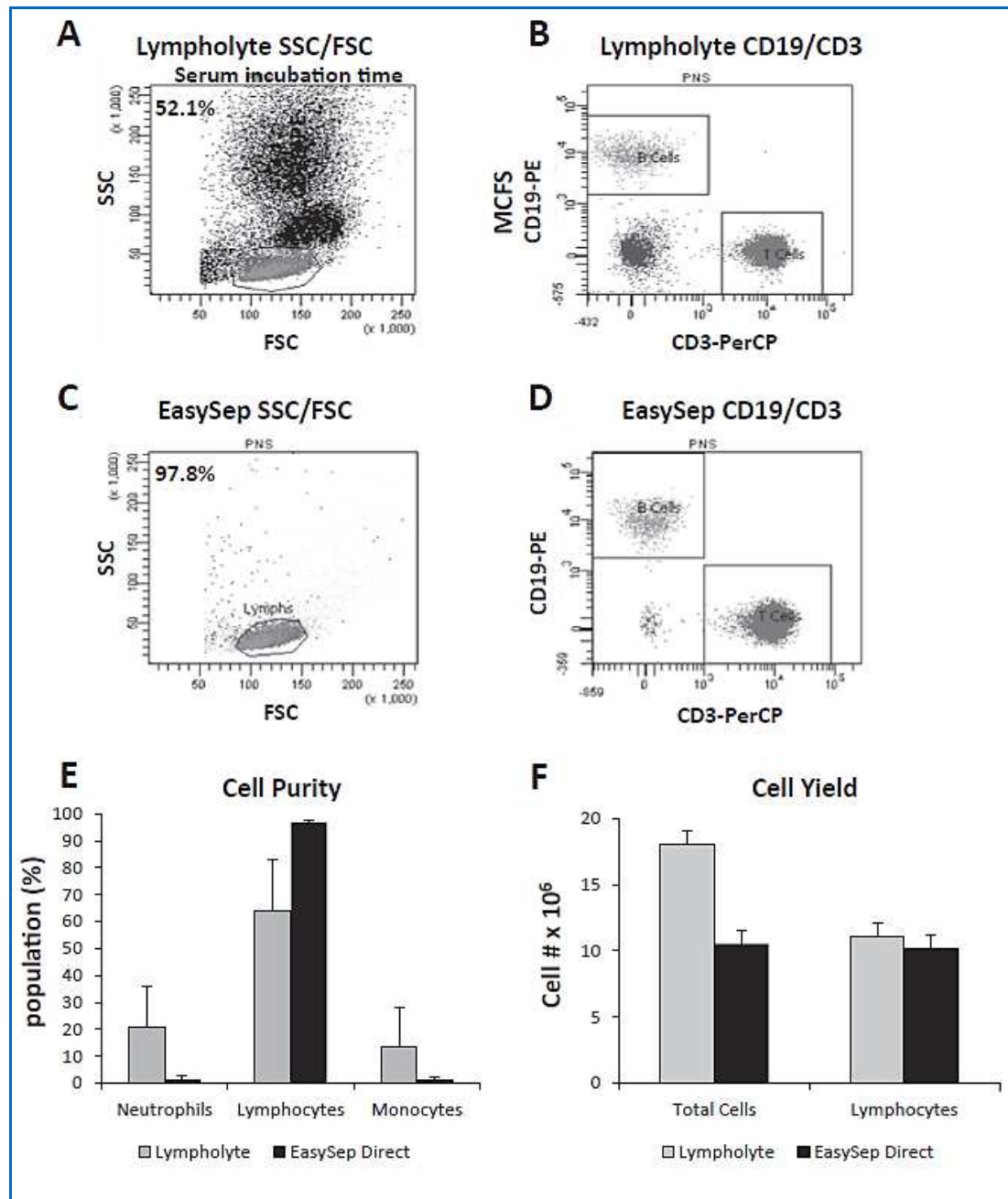
Effects of incubation temperature, to cell suspension volume ratio and number on FCXM results.



The Halifax and standard FCXM protocols show excellent results concordance. Patient sera predicted by the SAB assay to give a negative FCXM (n=90) or a positive FCXM (n=54) against selected donor cells were used.



The Halifaster and standard FCXM protocols show excellent results concordance. Patient sera predicted by the SAB assay to give a negative FCXM (n=10) or a positive FCXM (n=18) against selected donor cells were used.



When compared with Lympholyte, EasySep Direct lymphocyte isolation improves lymphocyte purity while maintaining lymphocyte yield.

Cost comparison for the standard, Halifax, and Halifaster FCXM protocols.

Parameter	Standard FCXM Tube Method	Standard FCXM Tray Method	Halifax FCXM Protocol	Halifaster FCXM Protocol	Halifaster FCXM Protocol
<i>Cell Isolation</i>					
Cell isolation reagent type	Lympholyte-H	Lympholyte-H	Lympholyte-H	EasySep™ Direct Whole blood Sample	EasySep™ Direct Buffy Coat sample
Cell isolation reagent* cost					
Live donor, 10 mL of blood	\$5	\$5	\$5	\$35	\$17.5
Deceased donor, 20 mL of blood	\$10	\$10	\$10	\$70	\$35
Cell preparation time	90 min	90 min	90 min	55 min	60 min
Technologist time isolation cost					
Live donor, regular shift time	\$52.5	\$52.5	\$52.5	\$32	\$35
Deceased donor, regular shift time	\$105	\$105	\$105	\$64	\$70
Deceased donor, on call time	\$99	\$76	\$41	\$35	\$35
Total FCXM assay cost					
Live donor	\$95	\$84	\$42	\$30	\$30
Deceased donor	\$214	\$190	\$95	\$66	\$66
<i>Total cost***</i>					
Total cost (cell isolation + FCXM)					
Live donor	\$153	\$141	\$100	\$97	\$82
Deceased donor	\$329	\$305	\$210	\$200	\$171

Several labs in the United States, United Kingdom, Brazil and other countries have since either adopted the Halifax or Halifaster protocols or are in the process of doing so.

We predict that broader application of these protocols will improve pre-transplant risk assessment, facilitate transplant allocation, and improve patient care.

The Impact of Lymphocyte Purity on Flow Cytometry Crossmatch (FCXM) Assay

It's Not Purely Theoretical

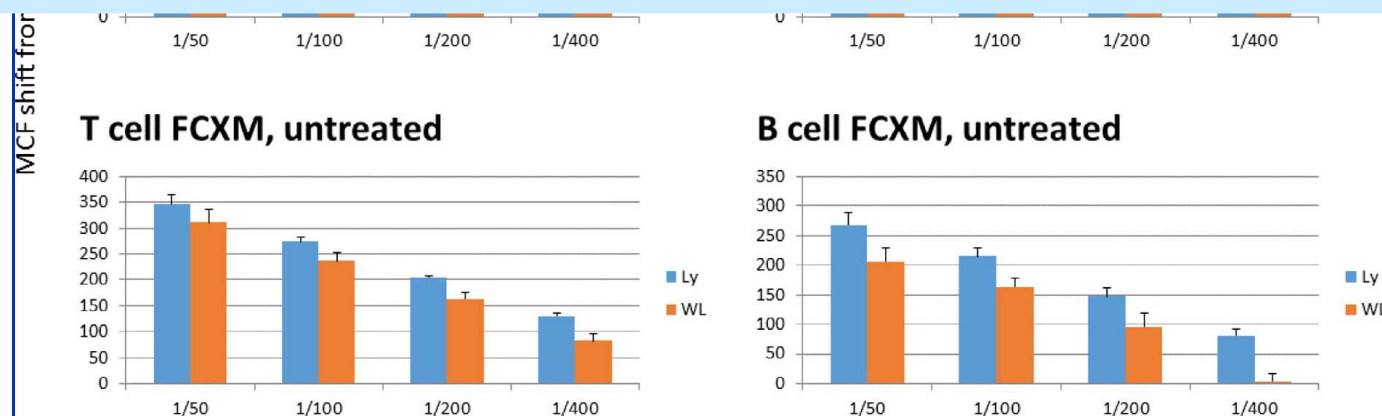
R. S. Liwski, G. Adams, G. Peladeau, K. Heinsteins.

Enriched lymphocytes (Ly; >97%), neutrophils (Nu; >98%) and monocytes (Mo; >92%) were isolated from volunteer donors using subset specific EasySep Direct kits (STEMCELL). Whole leukocyte (WL) cell preps were obtained by mixing enriched Ly, Nu and Mo in equal proportion (approx. 33% each) to mimic poor quality cell preps.

Ly and WL cell preps were treated with pronase (2.35 kunitz units/ml) or control (PBS) and used as targets in FCXM against negative control (NC) and several dilutions of positive control (PC) sera.

Lymphocyte purity has significant impact on FCXM results. Weak DSA could be missed if low purity lymphocyte preps are used in FCXM assay.

Use of highly enriched lymphocytes will improve detection of DSA and reduce variability of FCXM results.



Variable HLA expression on deceased donor lymphocytes: Not all crossmatches are created equal

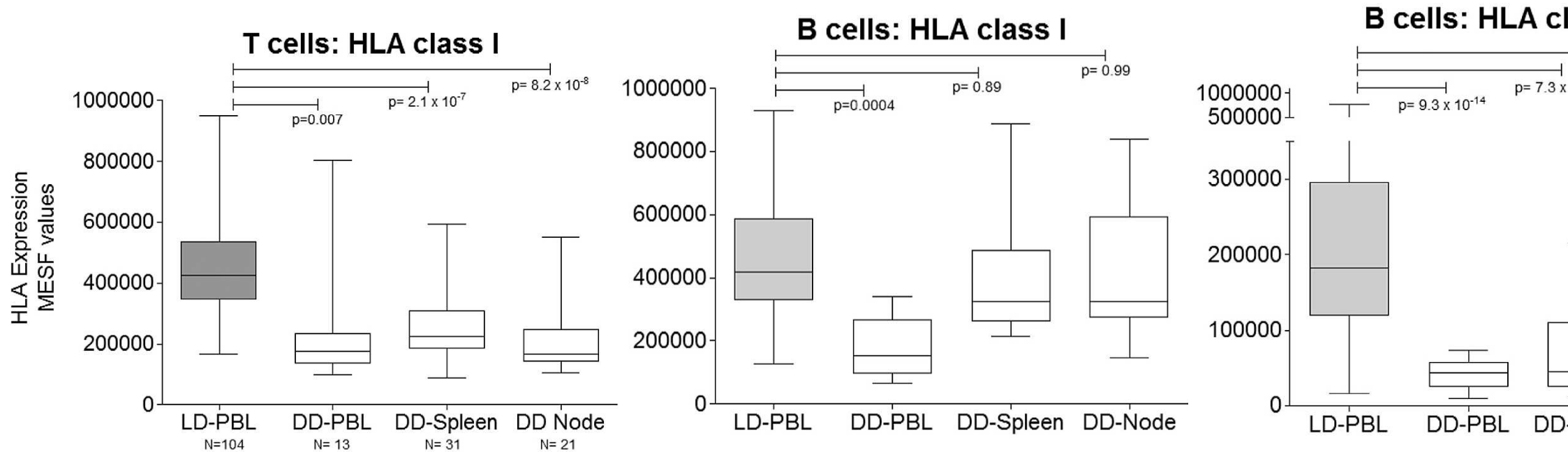
J. L. Badders, J. A. Jones, M. E. Jeresano, K. P. Schillinger, A. M. Jackson.

Flow cytometric crossmatch tests are used to detect donor-specific antibody and determine eligibility for transplantation.

Crossmatch sensitivity is dependent on lymphocyte quality, to include HLA expression on the cell surface.

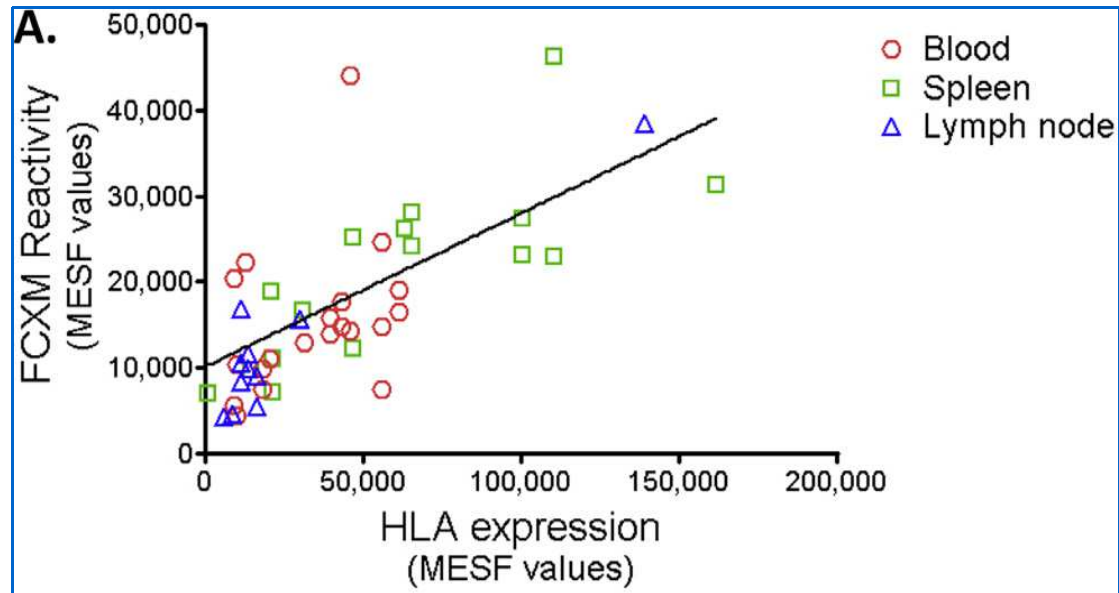
The impact of HLA expression variability on crossmatch reactivity was examined using lymphocytes isolated from different donor types: deceased donor (DD) versus living donor (LD) and tissue sources (blood, spleen, or lymph nodes).

Variable HLA expression on lymphocytes isolated from different donor types and donor tissues



HLA expression, normalized to MESF values, measured at the time of crossmatch for 169 consecutive donor samples fluorescently conjugated antibodies and flow cytometry.

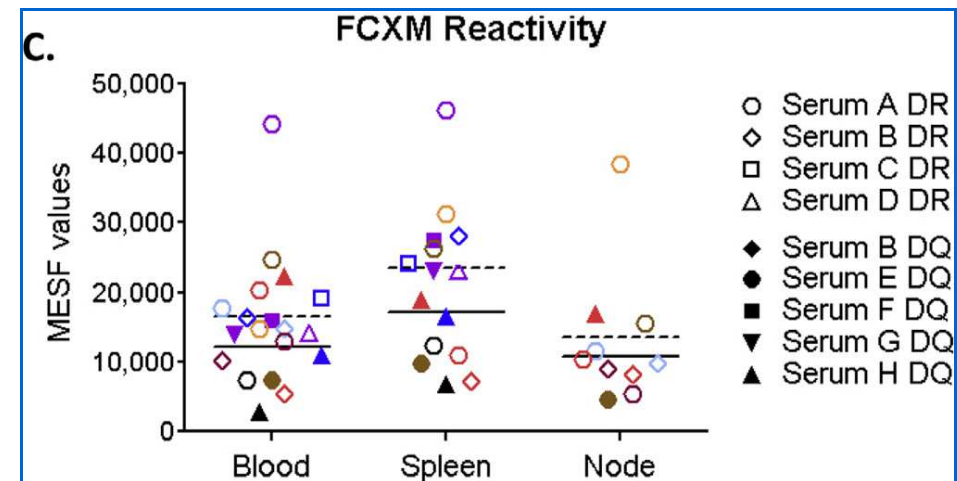
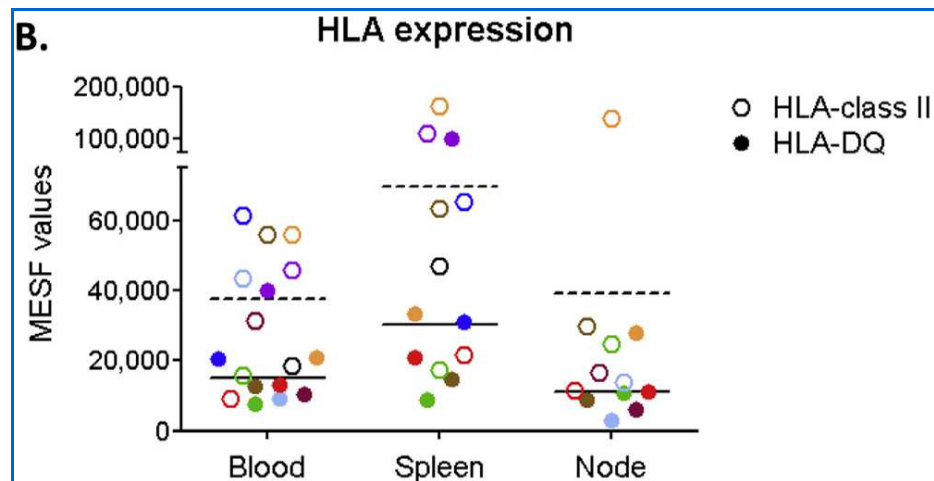
Tissue-specific HLA class II variability impacts B cell crossmatch reactivity.



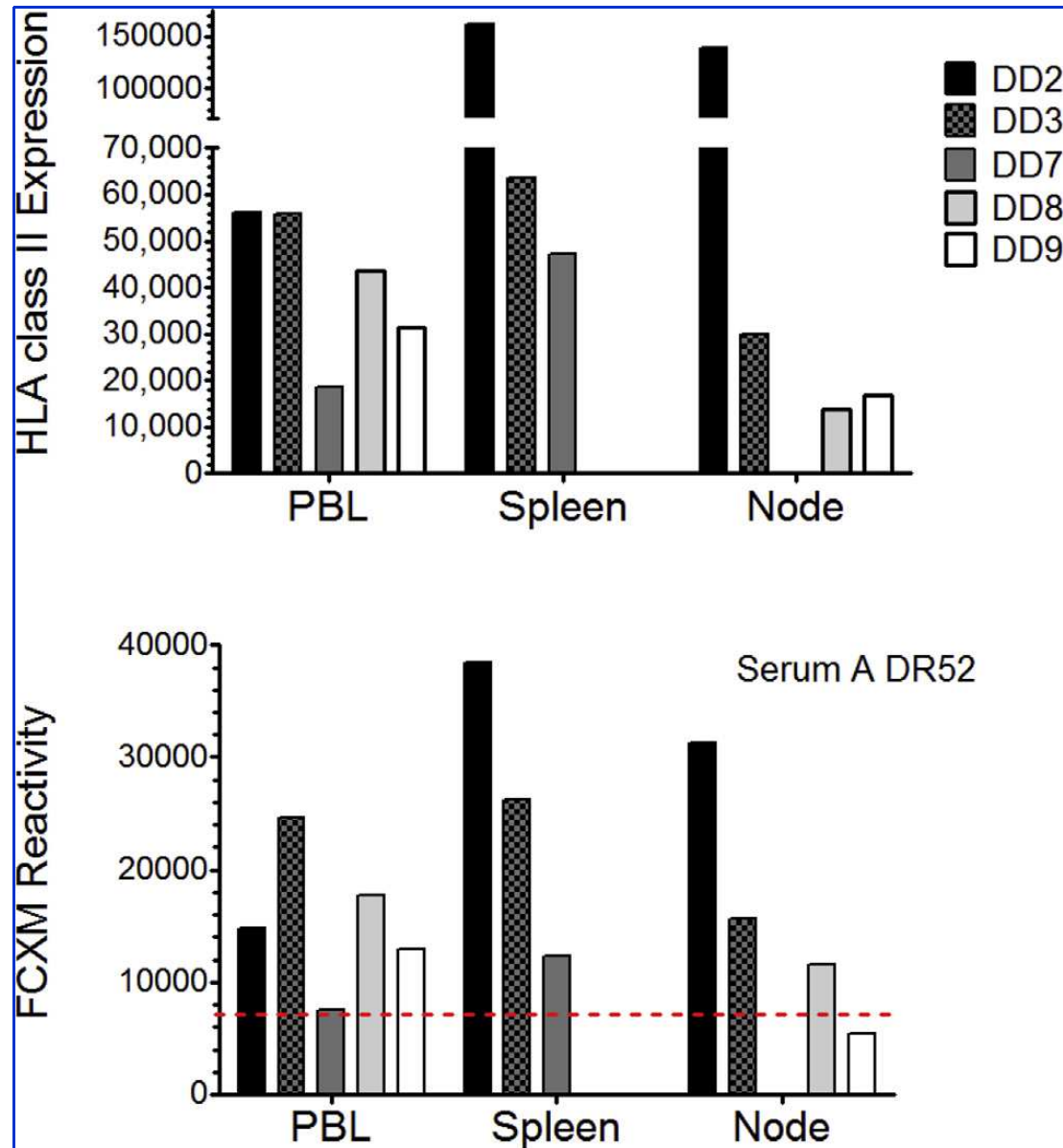
(A) Linear regression ($R^2 = 0.60$, $p = 0.0005$) analysis of FCXM reactivity (C) versus HLA expression (B).

(B) HLA class II (open circles) and HLADQ (closed circles) expression, normalized to MESF values, for B cells isolated from nine deceased donors (individually color coded) using different tissue sources.

(C) The same B cells were tested in flow cytometric crossmatch (FCXM) tests using eight HLA class II specific sera. Dashed and solid lines represent mean HLA class II and HLA-DQ expression and crossmatch reactivity, respectively.



Donor-specific and tissue-specific HLA class II variability impacts B cell crossmatch outcome

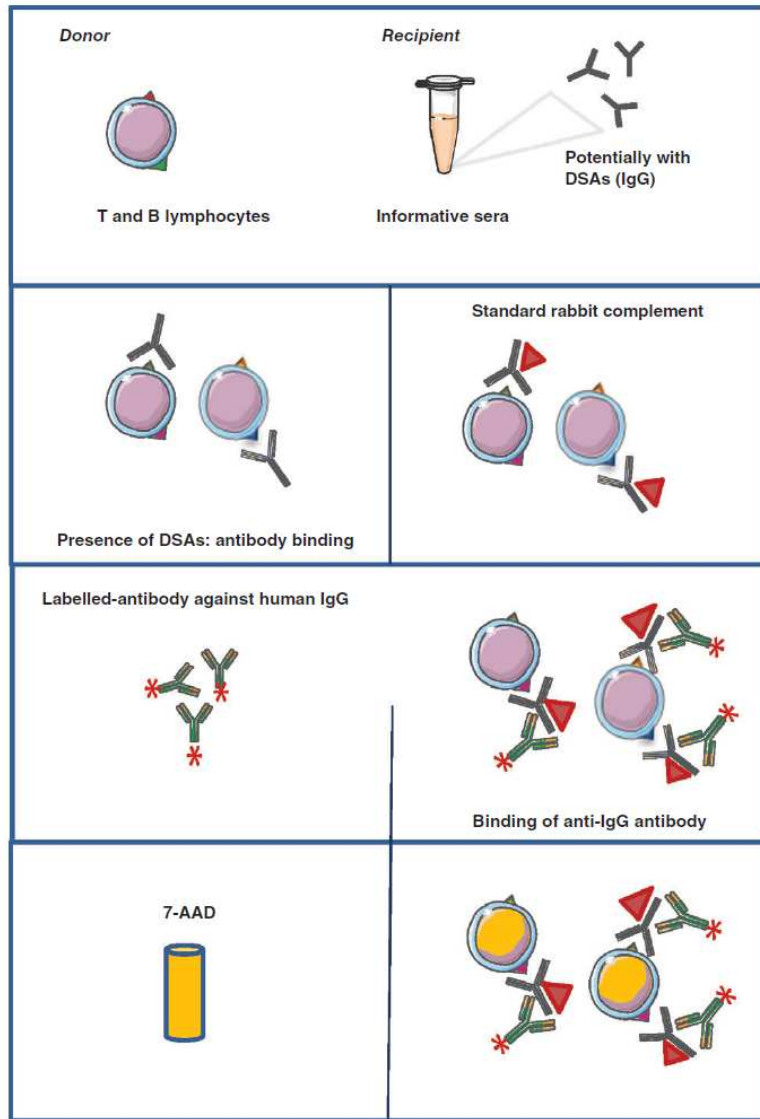


HLA class II expression, normalized MESF values, for B cells isolated from different tissue sources from five donors heterozygous for HLA-DR52.

Flow cytometric crossmatch (FCXM) reactivity using the same B cells and serum reactive with all HLA-DR52 alleles. Dashed line represents a 7000 MESF threshold for a positive B cell FCXM.

Innovazioni Metodologiche (III)

FCXM citotossico



IMPROVED FC-XM BY MEANS OF A FUNCTIONAL ASSAY
By using a fluorescent intercalator (7-AAD) FC-XM becomes a complement dependent cytotoxicity assay. No clear evidence of a higher sensitivity for FCtox assay XM compared with CDC.

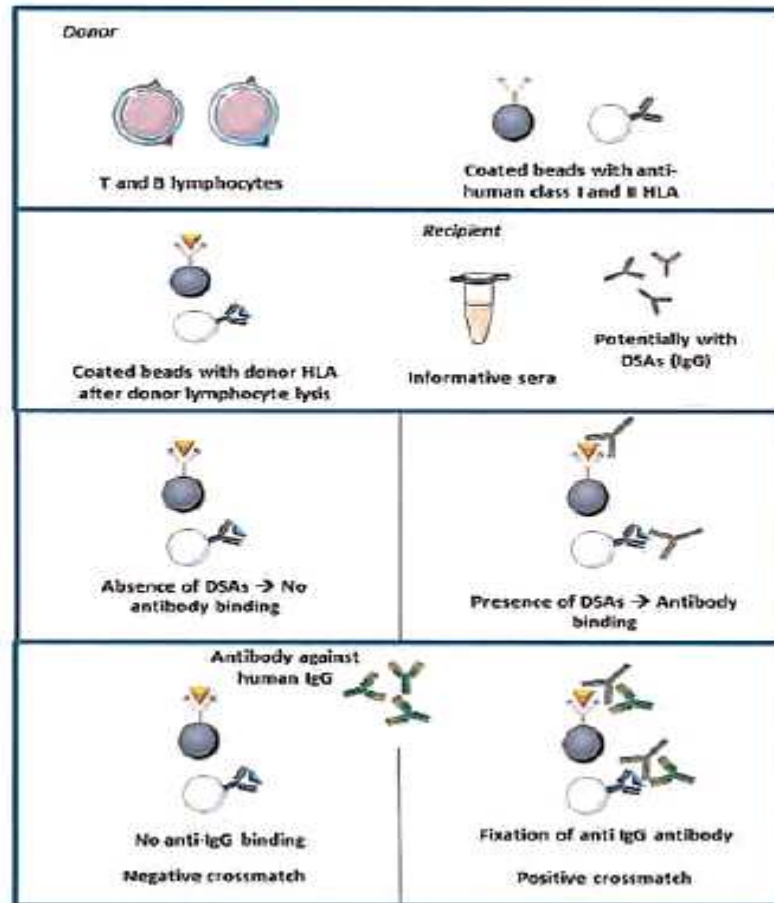
Another approach consists of directly detecting C4d fixing alloantibodies on T- and B-cells by using fluorochrome-conjugated C4d Ab after incubation with complement source. Jain et al tested this assay in a small series of clinical cases. In another study, AlMahri et al showed higher sensitivity by staining the C3d fraction.

These types of assays need to be further investigated in larger cohorts.

Innovazioni Metodologiche ^(IV)

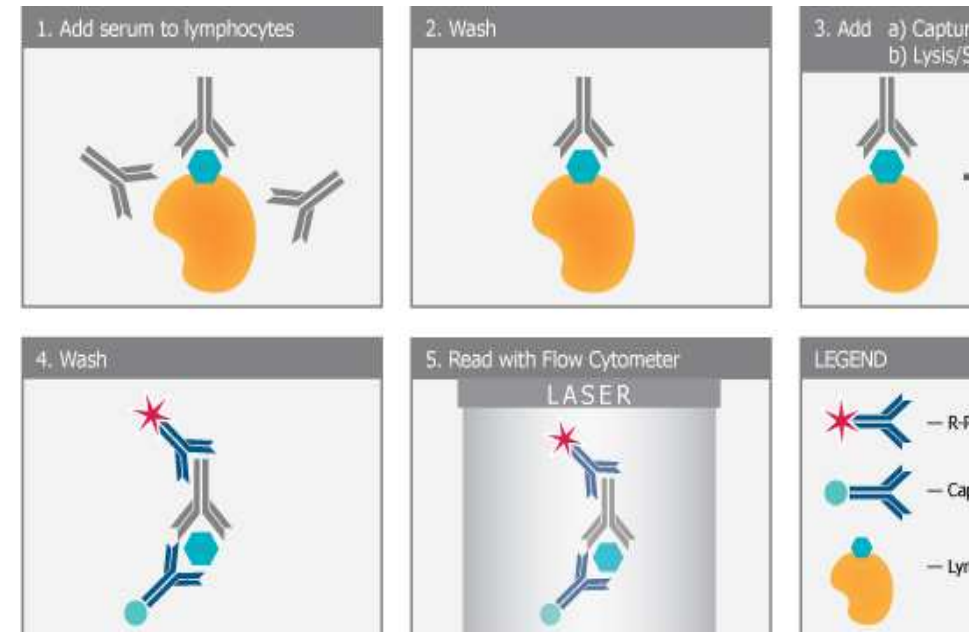
Crossmatch citofluorimetrico specifico per le sole molecole HLA del donatore

Luminex-XM donatore-specifico



Gli antigeni HLA del donatore, ottenuti da lisato dai linfociti del donatore, vengono catturati mediante beads rivestite con anticorpi anti-HLA di classe I e II e successivamente incubati con il siero in esame. Il test ha dimostrato di non reagire con i sieri contenenti solo anticorpi DQ e in parte DP.

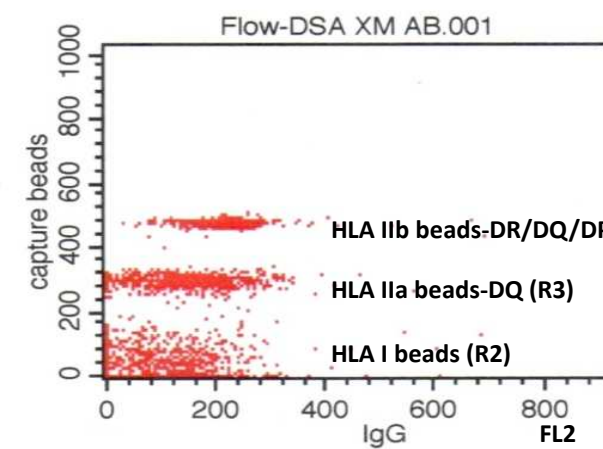
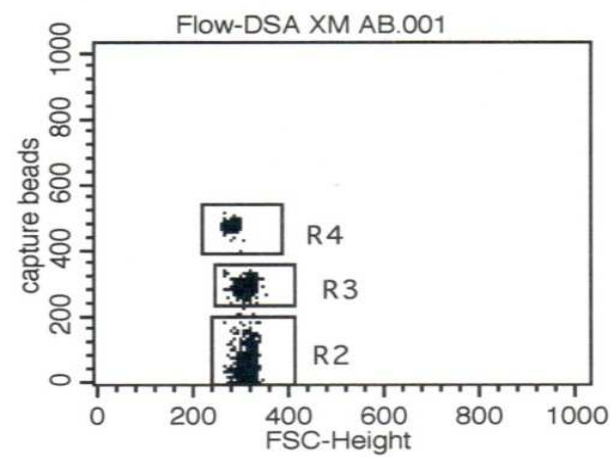
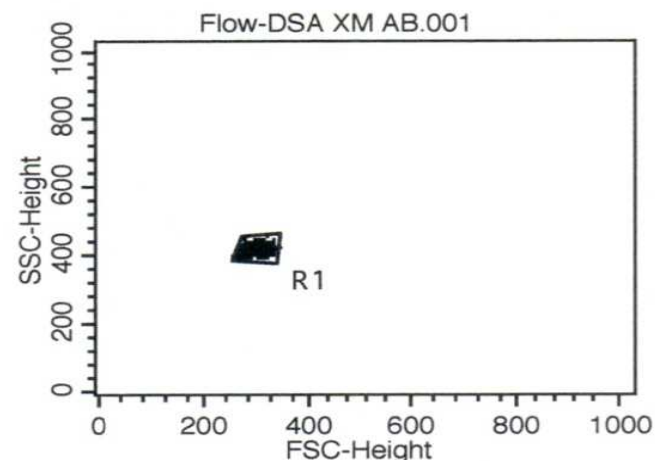
FlowDSA XM



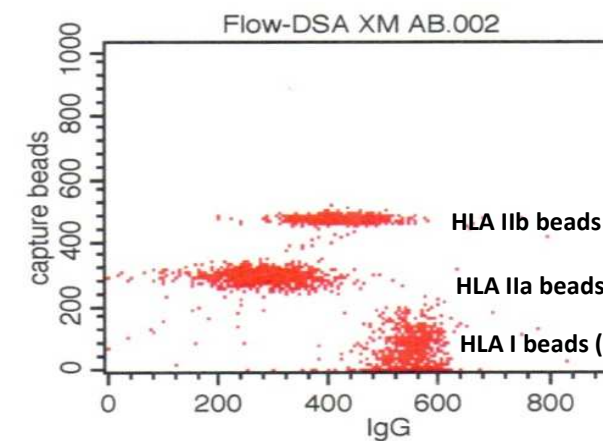
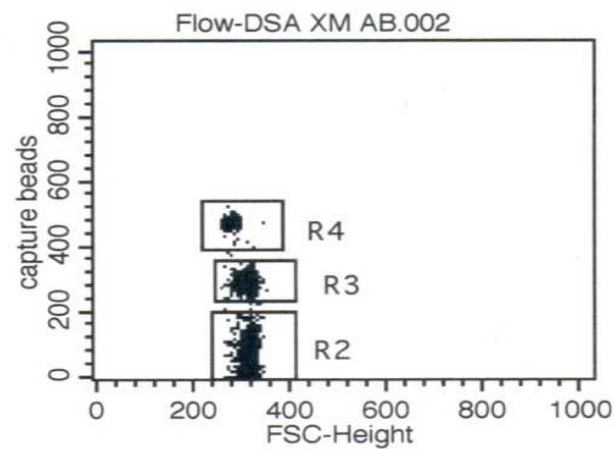
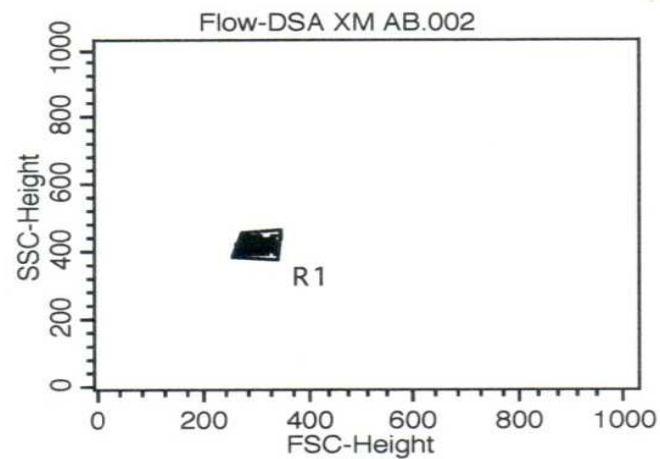
Nuova tecnica citofluorimetrica che combina la sensibilità della FC-XM alla specificità delle LSA beads evidenziando solo anticorpi anti-HLA "specifici" per le molecole HLA di classe I e II "proprie" del donatore.

Analisi del FlowDSA-XM al citofluorimetro

Siero negativo



Siero positivo



FCXM e FlowDSA-XM: Analisi di risultati ottenuti con sieri positivi per soli DSA-classe I

ID	FC-XM			FlowDSA-XM		
	T cells (cutoff >40)	B cells (cutoff >60)	Class I (A/B/C) (cutoff>68)	Class IIa (DQ) (cutoff>33)	Class IIb (all) (cutoff>22)	HLA Class I antibodies target cell (M)
	Result (delta*)	Result (delta*)	Result (delta*)	Result (delta*)	Result (delta*)	
88023	Pos (+85)	Pos (+55)	Pos (+390)	Neg (-36)	Neg (-25)	B35(21650)
79184	Pos (+111)	Pos (+94)	Pos (+505)	Neg (-7)	Neg (-33)	B27 (18200), B51(12080)
87969	Pos (+27)	Pos (+15)	Pos (+370)	Neg (-18)	Neg (-26)	B51(15300)
64033	Pos (+10)	Neg (-2)	Pos (+79)	Neg (-35)	Neg (-27)	Cw4(7800)
75366	Neg (-2)	Neg (-26)	Pos (+44)	Neg (-1)	Neg (-27)	A29(2780), B44(2531),
69468	Neg (-20)	Neg (-9)	Pos (+71)	Neg (-26)	Neg (-28)	A29(1700, Cw16(13546)

FCXM e FlowDSA-XM: Analisi di risultati ottenuti con sieri positivi per soli DSA-classe II

ID	FC-XM			FlowDSA-XM		
	T cells (cutoff >40)	B cells (cutoff >60)	Class I (A/B/C) (cutoff>68)	Class IIa (DQ) (cutoff>33)	Class IIb (all) (cutoff>22)	HLA Class I antibodies target cell (M)
	Result (delta*)	Result (delta*)	Result (delta*)	Result (delta*)	Result (delta*)	
83811	Neg (-19)	Pos (+10)	Neg (-15)	Pos (+110)	Neg (-16)	DQ5(20200)
84048	Neg (-23)	Pos (+55)	Neg (-69)	Pos (+57)	Pos (+288)	DR1(19176), DQ5(18580)
86154	Neg (-11)	Pos (+24)	Neg (-43)	Neg (-9)	Pos (+14)	DR1(11537)
85700	Neg(-20)	Pos (+9)	Neg (-39)	Neg (-37)	Neg? (-1)	DRB3*03:01(7310)
70000LS	Neg(-35)	Neg (-44)	Neg (-48)	Neg (-22)	Neg? (-1)	DR13(4166)

FCXM e FlowDSA-XM: Analisi di risultati ottenuti con sieri positivi per DSA-classe I e II

ID	FC-XM			FlowDSA-XM			HLA Class I antibodies target cell (MFI)
	T cells (cutoff >40)	B cells (cutoff >60)	Class I (A/B/C) (cutoff>68)	Class IIa (DQ) (cutoff>33)	Class IIb (all) (cutoff>22)		
	Result (delta*)	Result (delta*)	Result (delta*)	Result (delta*)	Result (delta*)		
84051	Pos (+72)	Pos (+92)	Pos (+507)	Neg (-28)	Pos (+36)	A2(16445), B18(10600)	
85135	Pos (+17)	Pos(+37)	Pos (+107)	Neg(-39)	Pos (+16)	A23(3600), B35(5700), B35(5200), Cw4(2300)	
83919	Pos ?? (+2)	Pos (+66)	Pos (+223)	Pos (+53)	Neg(-15)	DRB1*13:02(6500), DQ6(17900)	
88733S	Pos (+105)	Pos (+73)	Pos (+496)	Neg ?(-2)	Neg (-27)	A29(20285), A31(20565), B44(20990), B51(20250), Cw16(8420), DQ7(3240)	
69397LS	Pos (+106)	Pos(+107)	Pos (+486)	Neg (-23)	Neg (-17)	B39(6620), B51(21530), Cw12(13400), Cw4(3210)	
78565	Neg (-16)	Pos (+25)	Pos (+116)	Pos (+196)	Neg (-22)	A(3410), DQ6(20320)	
89034	Neg (-21)	Pos (+59)	Neg (-45)	Neg (-42)	Pos (+46)	A3(2300), B35(1928), DQ6(17900)	

L'analisi comparativa dei risultati preliminari ottenuti con le due tecniche ha mostrato:

- FlowDSA-XM è risultato essere una tecnica più sensibile rispetto all'FC-XM, mostrando maggiori valori di positività.
- FlowDSA-XM fornisce risultati "specifici" per anti-HLA DSA di classe I e/o II; inoltre per la specificità DR/DQ dei DSA presenti nei sieri ha fornito risultati positivi di corrispondenti alla specificità DR/DQ dei DSA presenti nei sieri.
- Conferma di risultati negativi del crossmatch, con entrambe le tecniche, in presenza di DSA a basso MFI.

Innovazioni Metodologiche ^(V)

HLA. 2019;93:436–444.

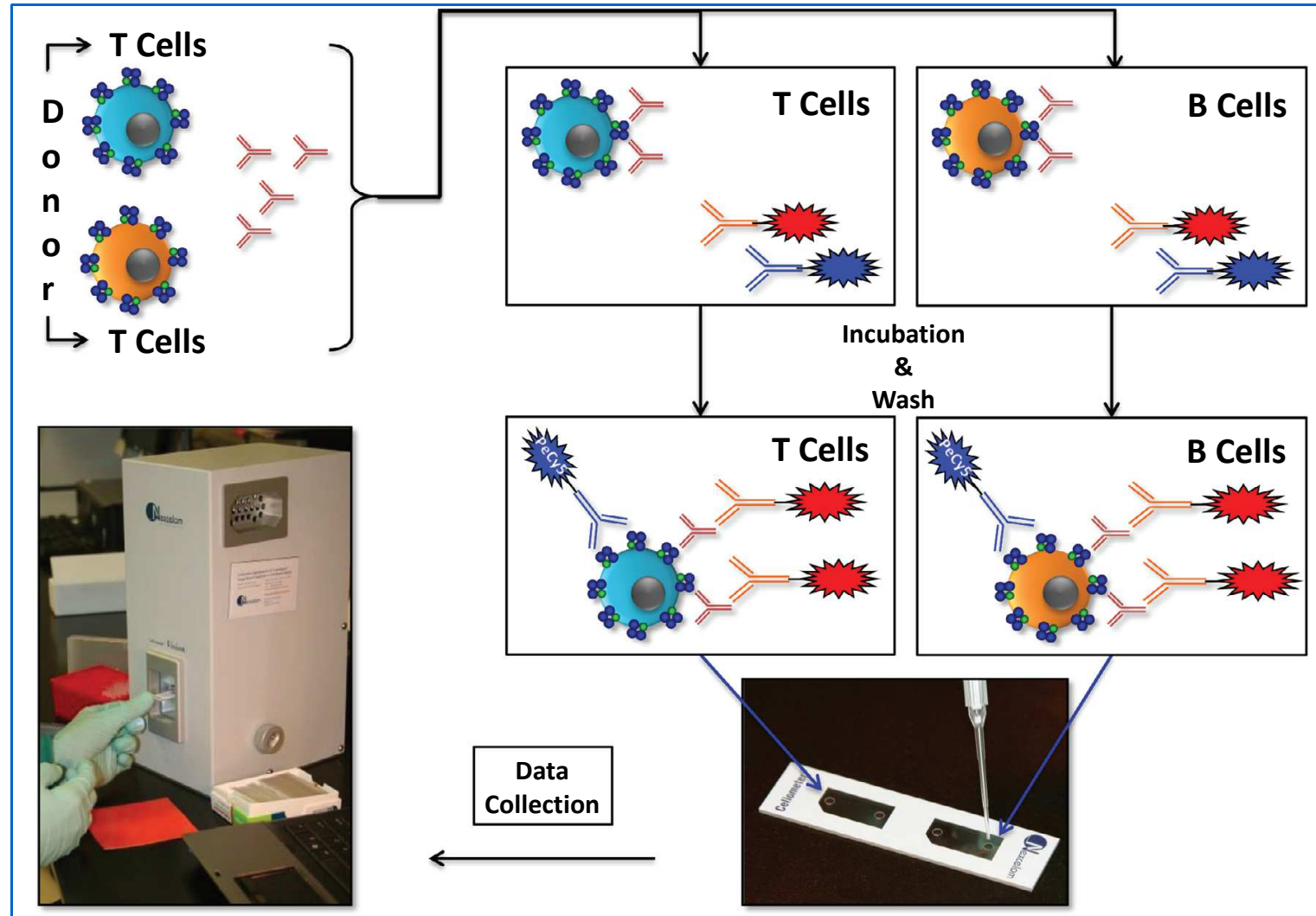
Image cytometry as an alternative to flow cytometry for the transplant histocompatibility crossmatch assay

D.S. Ramon, T. Franks, A. Jaramillo, B.D. Paradis

While in many transplant programs the complement-dependent cytotoxicity crossmatch remains in use, when available, the flow cytometry crossmatch (FCXM) is the method of choice because of its superior sensitivity and specificity.

Unfortunately, the maintenance and cost of a flow cytometer is a considerable limitation for small histocompatibility laboratories.

Therefore, in this study, we evaluated the use of the Cellometer Vision CBA image cytometer (Nexcelom Bioscience LLC, Lawrence, Massachusetts) as an alternative instrument to perform the crossmatch assay.



Two-color image cytometry crossmatch protocol. The 2-color T cell and B cell image cytometry crossmatch (IXM) protocol was adapted from the 3-color flow cytometry crossmatch (FCXM) pr

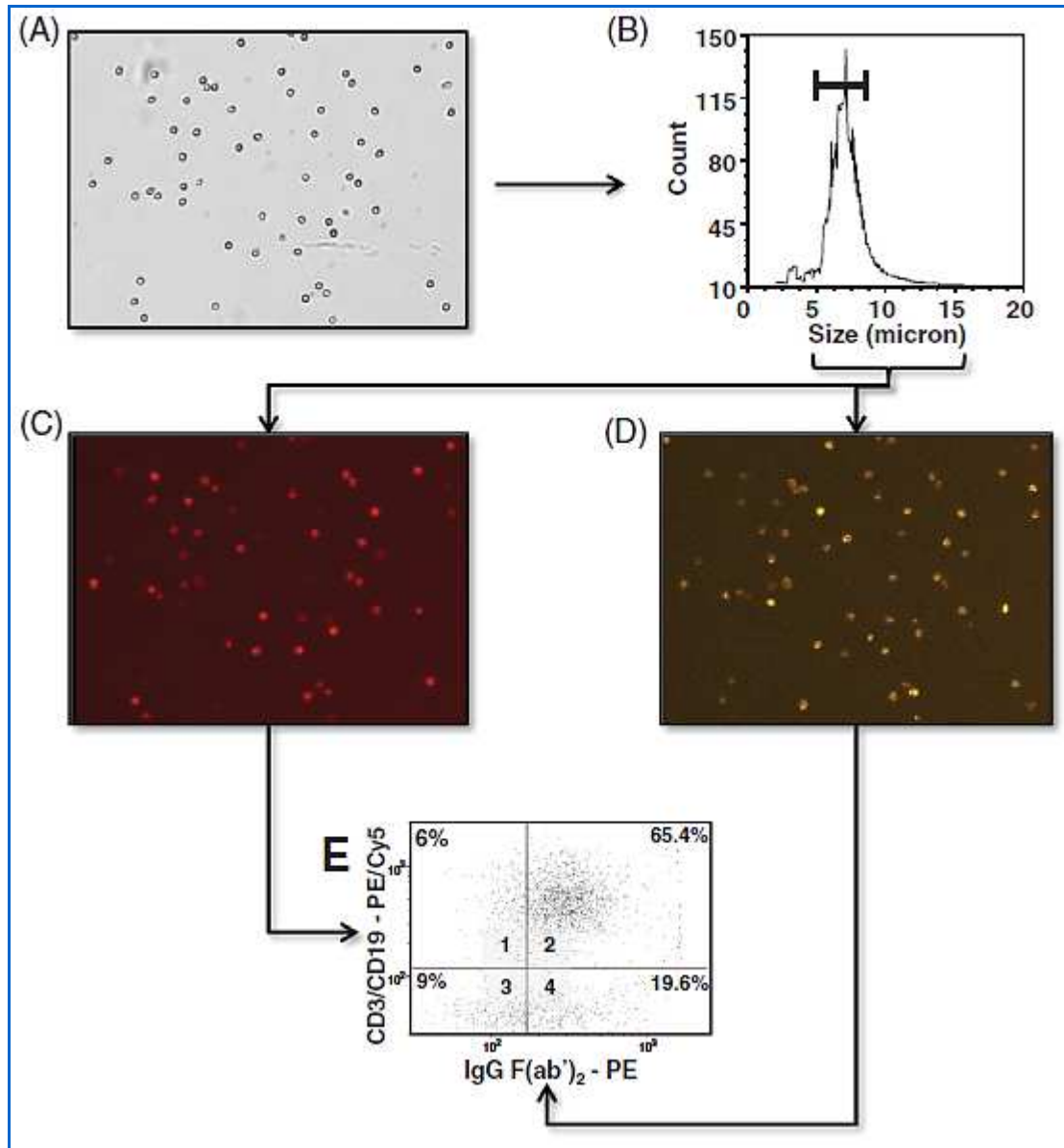


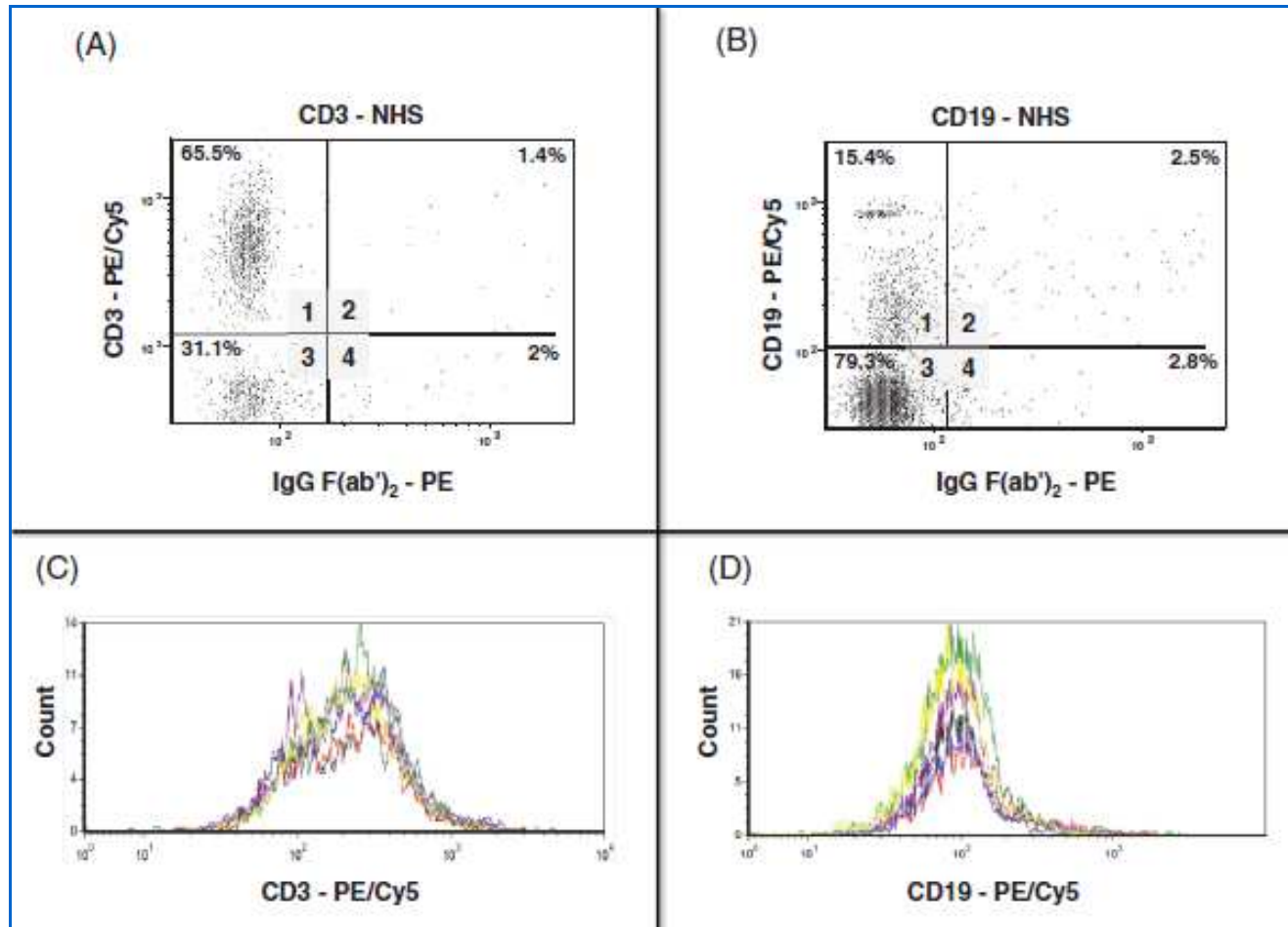
Image collection and analysis. Bright field (A), and FL1 (C), and FL2 (D) images were from each cell.

Figure 2A, B show a pure and uniform cell size distribution.

The fluorescence optic module VB-595-5 (32/605 ± 22 nm) collected the emission light from the PE-conjugated anti-human IgG F(ab)₂ (Figure 2C).

The fluorescence optic module VB-695-5 (32/695 ± 30 nm) collected the emission light from the PE/Cy5-conjugated anti-CD3 or anti-CD19 mAbs to detect T cells or B cells, respectively (Figure 2D).

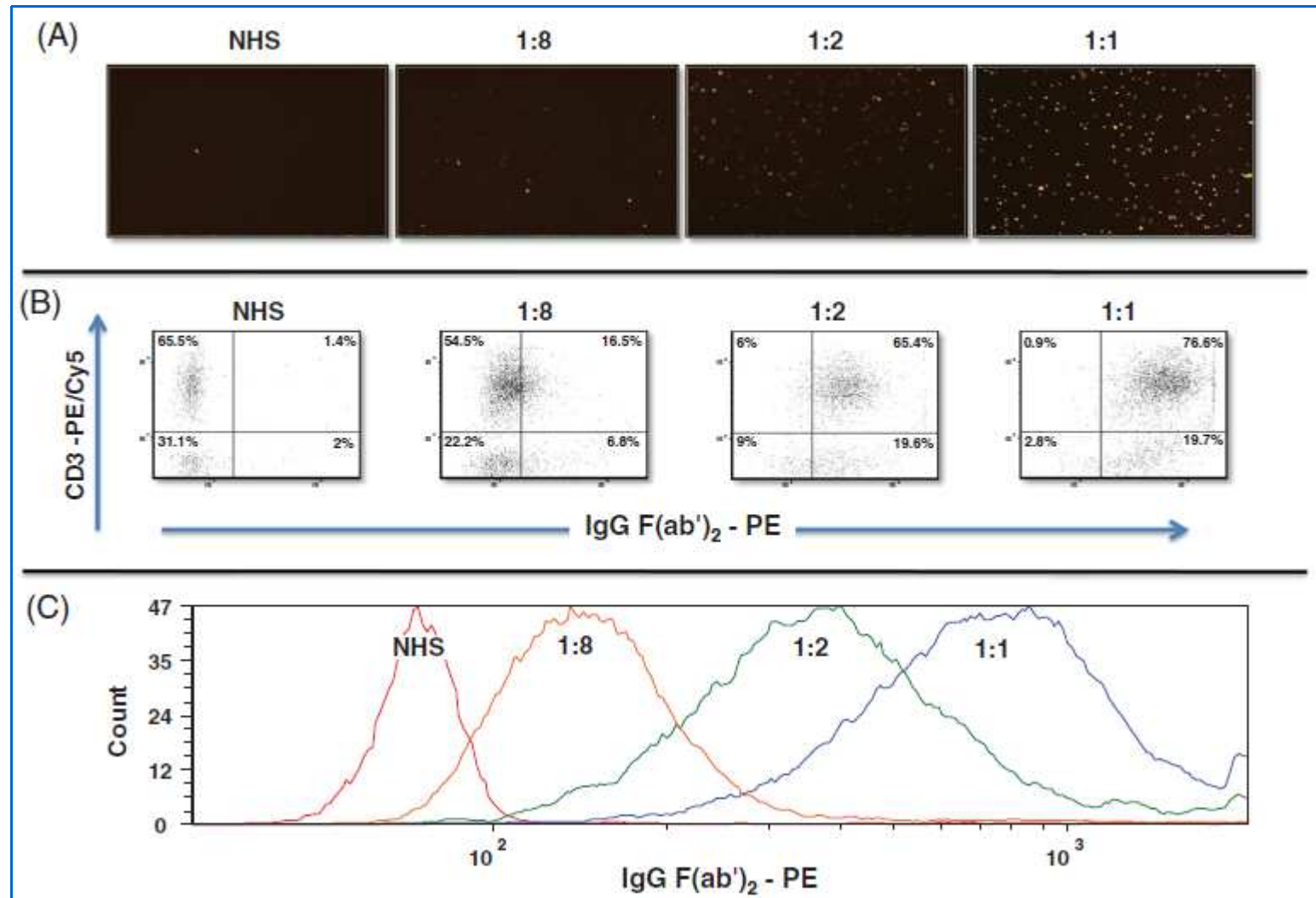
The fluorescent images were segmented and exported to be analyzed by FCS Express 4 cytometry software.



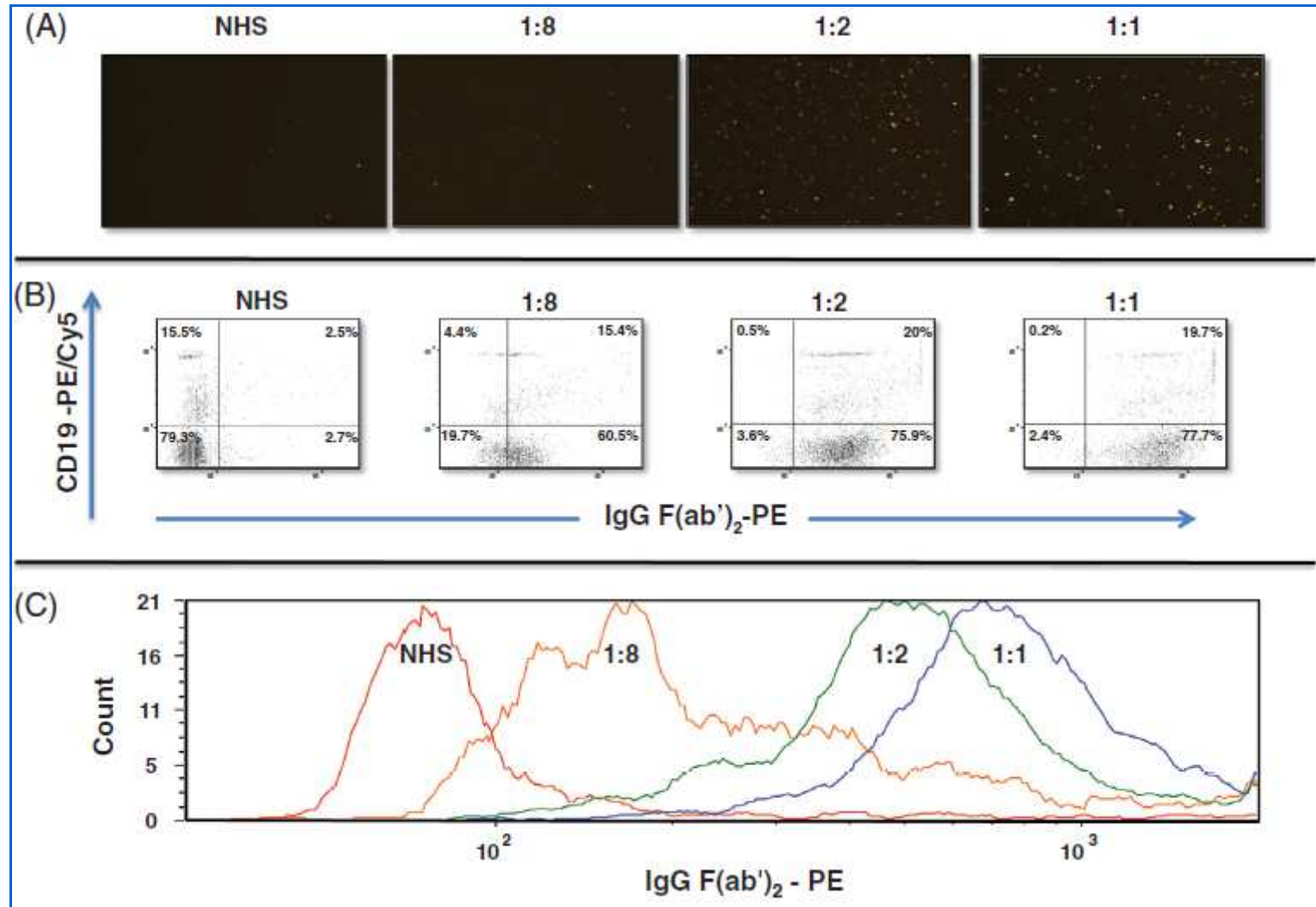
Uniform T cell (CD3+) and B cell (CD19+) populations (PE/Cy5).

T cell (A) and B cell (B) percent within the range of normal values.

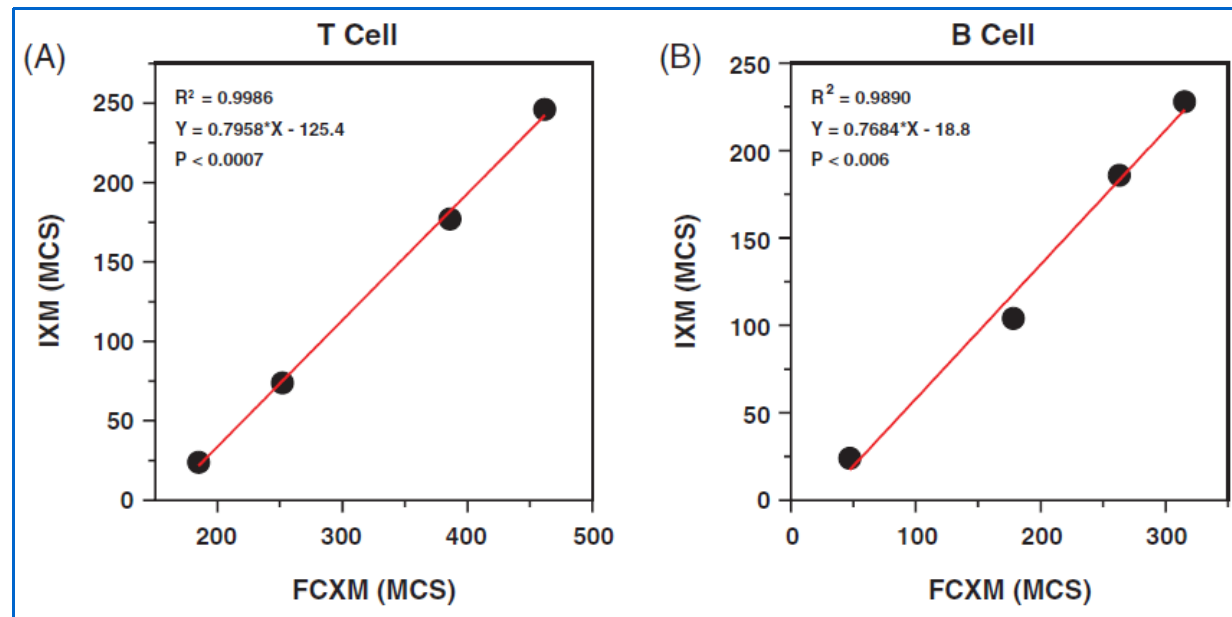
The overlay analysis of the CD3 and CD19 antigen expression in multiple cells demonstrate that the system is capable of identifying uniform T cell (C) and B cell populations, respectively.



T cell image cytometry crossmatch (IXM) serum titration analysis. (A) Intensity of the events captured on digital image by PE modules at different dilutions. (B) Comparable to flow cytometry analysis, the intensity of the events is presented in scatter plots. (C) The cell population shift represented in an overlay graph for easy comparison with the negative control (NHS).



B cell image cytometry crossmatch (IXM) serum titration analysis. (A) Intensity of the events captured on digital image by PE modules at different dilutions. **(B)** Comparable to flow cytometry analysis, the intensity of the events is presented in scatter plots. **(C)** The cell population shift represented in an overlay graph for easy comparison with the negative control (NHS).



	T cell (n = 39)		B cell (n = 39)
	IXM		IXM
	Positive	Negative	Positive
FCXM			
Positive	16	1	21
Negative	0	22	2
Sensitivity (95% CI)	94.1% (71.3%-99.9%)		100% (83.1%-100%)
Specificity (95% CI)	100% (84.6%-100%)		88.9% (65.5%-100%)
PPV (95% CI)	100% (79.4%-100%)		91.3% (72.1%-100%)
NPV (95% CI)	95.7% (78.1%-99.9%)		100% (79.4%-100%)
P-value	<0.001		<0.001

Correlation between IXM and FCXM. Median channel shift (MCS) from both systems were analyzed by linear regression, showing a significant correlation.

Performance characteristics of IXM compared to FCXM

This feasibility study demonstrates that the FCXM test could be easily adapted to the Cell Vision CBA image cytometer without compromising specificity and sensitivity.

The low instrumentation cost, minimal maintenance, and simple operation allow for implementation or transition from the FCXM to the IXM method.

Innovazioni Interpretative ⁽¹⁾

Controindicazione vs Rischio di AMR/Perdita del Trapianto

Am J of Transplant 2003; 3: 1488-1500

Pre-Transplant Assessment of Donor-Reactive, HLA-Specific Antibodies in Renal Transplantation: Contraindication vs. Risk

H. M. Gebel, R. A. Bray and P. Nickerson

From its first clinical description, the simple complement dependent assay was recognized as neither sufficiently specific nor sensitive to identify all relevant antibodies.

Over time, more sensitive and specific lymphocyte crossmatch assays were developed that effectively decreased the incidence of early antibody-mediated rejection.

We believe that a positive FCXM should neither be considered an indisputable contraindication to transplantation nor be completely disregarded.

Rather, the FCXM should be used to provide an assessment of risk for rejection and/or graft loss not predicted by a negative serologic crossmatch.

Impact of HLA antibodies detected only by a flow cytometric crossmatch

Primary transplant	Pt (n)	CXM method	PRA method	Defn T+ FXCM ¹	% T+ FXCM	Early graft loss (<3 mo) FP vs. FN	Acute rejection FP vs. FN	Survival 1 yr FP vs. FN
Iwaki (38)	113	CDC	CDC	2 SD	16%	22% vs. 4%		
Cook (39)	196	CDC		10/256	18%	22% vs. 7%		
Kerman (43) ²	89	CDC	CDC	20/256	13%			83% vs.
Mahoney (42)	67	AHG	AHG	40/1024	18%	33% vs. 7%		67% vs.
Ogura (45)	841	CDC	CDC	50/1024	18%	20% vs. 7%		75% vs.
Lefor (48)								75% vs.
Pelletier (50) ²								86% vs.
Kimball (51)								44% vs.
Kerman (53) ² (Cadaveric)								81% vs.
Kerman (53) ² (Haplo LRD)								89% vs.
El Fettouh (54)								3 year
Karpinski (56)								80% vs.
Early graft loss (<3 months)								
					FCXM +		FCXM -	
Primary transplant					20%		5%	
Regrafts					60%		15%	
Regrafts	Pt (n)	CXM method	PRA method	Defn T+ FXCM ¹	% T+ FXCM	Early graft loss (<3 mo) FP vs. FN	Acute rejection FP vs. FN	Survival 1 yr FP vs. FN
Iwaki (38)	23	CDC	CDC	2 SD	26%	67% vs. 12%		
Cook (39)	35	CDC		10/256	18%	56% vs. 21%		
Mahoney (42)	23	AHG	AHG	40/1024	43%	60% vs. 0%		40% vs.
Kerman (43)	47	CDC	CDC	20/256	62%		76% vs. 44%	48% vs.
Mahoney (47)	103	AHG	AHG	40/1024	34%	34% vs. 13%		66% vs.
Bryan (52)								
AHG-ve	174	AHG	AHG			32%		70%
Flow-ve	106			2 SD		8%		88%

Risk assignment based on crossmatch interpretation

Crossmatch Donor-reactive, HLA-specific, IgG antibody	Risk categories		
	High	Intermediate	Neg
Current positive direct CDC	X		
Current positive AHG-CDC	X		
Current positive flow crossmatch only	X		
Historic positive direct CDC		X ¹	
Historic positive AHG-CDC		X ¹	
Historic positive flow crossmatch only		X	
Current and historic negative direct CDC			X ^{1,2}
Current and historic negative AHG-CDC			X
Current and historic negative flow crossmatch			X

¹High risk if current flow crossmatch is positive.
²Intermediate risk if remote flow crossmatch is positive.
³Negligible risk if accurate sensitization history can be ensured.
 CDC = complement-dependent cytotoxicity.

- ✓ **High risk**: Clinical programs may consider this category a contraindication to transplantation.
- ✓ **Intermediate risk**: Clinical program may consider this category a contraindication to transplantation; transplanted, patients may require augmented immunosuppression and post-transplant monitoring.
- ✓ **Negligible risk**: Clinical programs may proceed to transplant with no change in their normal practice.

Human leukocyte antigen typing and crossmatch: A comprehensive review

Summary of the pre-transplant risk assessment of immunological challenge.

Donor crossmatch result	Crossmatch method	Current or historical	Antibody screening results	Interpretation of immunological risk
Positive T and B lymphocyte	CDC (DTT)	C	IgG HLA class I DSA	High risk ¹ Hyperacute rejection (veto to transplantation)
Positive B lymphocyte	CDC (DTT)	C	IgG HLA class II DSA	High risk ¹
Positive B lymphocyte	CDC (DTT)	C	Weak IgG HLA class I DSA	Intermediate risk ²
Positive T and B lymphocyte	FCXM (CDC neg)	C	IgG HLA class I DSA	Intermediate risk ²
Positive B lymphocyte	FCXM (CDC neg)	C	IgG HLA class II DSA	Intermediate risk ²
Positive T and B lymphocyte	CDC (DTT)	H	IgG HLA class I DSA	High risk ³
Positive B cell	CDC (DTT)	H	IgG HLA class II DSA	High risk ³
Positive B lymphocyte	CDC (DTT)	H	Weak IgG HLA class I DSA	Intermediate risk ²
Positive T and B lymphocyte	FCXM (CDC neg)	H	IgG HLA class I DSA	Intermediate risk ²
Positive B lymphocyte	FCXM (CDC neg)	H	IgG HLA class II DSA	Intermediate risk ²
Positive T and B lymphocyte	CDC (neg DTT)	C or H	IgM HLA class I DSA	Standard risk
Positive B lymphocyte	CDC (neg DTT)	C or H	IgM HLA class II DSA	Standard risk
Positive T and B lymphocyte	CDC (neg DTT)	C or H	IgM non-HLA (often autoreactive)	Standard risk
Positive B lymphocyte	CDC (neg DTT)	C or H	IgM non-HLA (often autoreactive)	Standard risk
Negative T and B lymphocyte	FCXM	C or H	IgG HLA class I or II DSA (detected by Luminex SAB alone)	Standard risk
Positive T and/or B lymphocyte	CDC and/or FCXM	C or H	Negative (Luminex Ab detection and/or SAB)	Standard risk (IgM/IgG non-HLA, often showing <i>in vitro</i> autoreactivity)
Positive T; Negative B lymphocyte	CDC and/or FCXM	C or H	Positive (Luminex SAB-not donor-specific) or negative	Standard risk (results suggest antibody is not HLA-specific)
Negative T and B lymphocyte	FCXM	C or H	Positive (Luminex SAB) not donor HLA-specific	Standard risk
Negative T and B lymphocyte	CDC and/or FCXM	C or H	Negative (Luminex Ab detection and/or SAB)	Standard risk

The Clinical Importance of Flow Cytometry Crossmatch in the Context of CDC Crossmatch Results

R.J. Graff, P.M. Buchanan, N. Dzebisashvili, M.A. Schnitzler, J. Tuttle-Newhall, H. Xiao, E. Schadde, A. Gheorghian, and K.L. Lentine

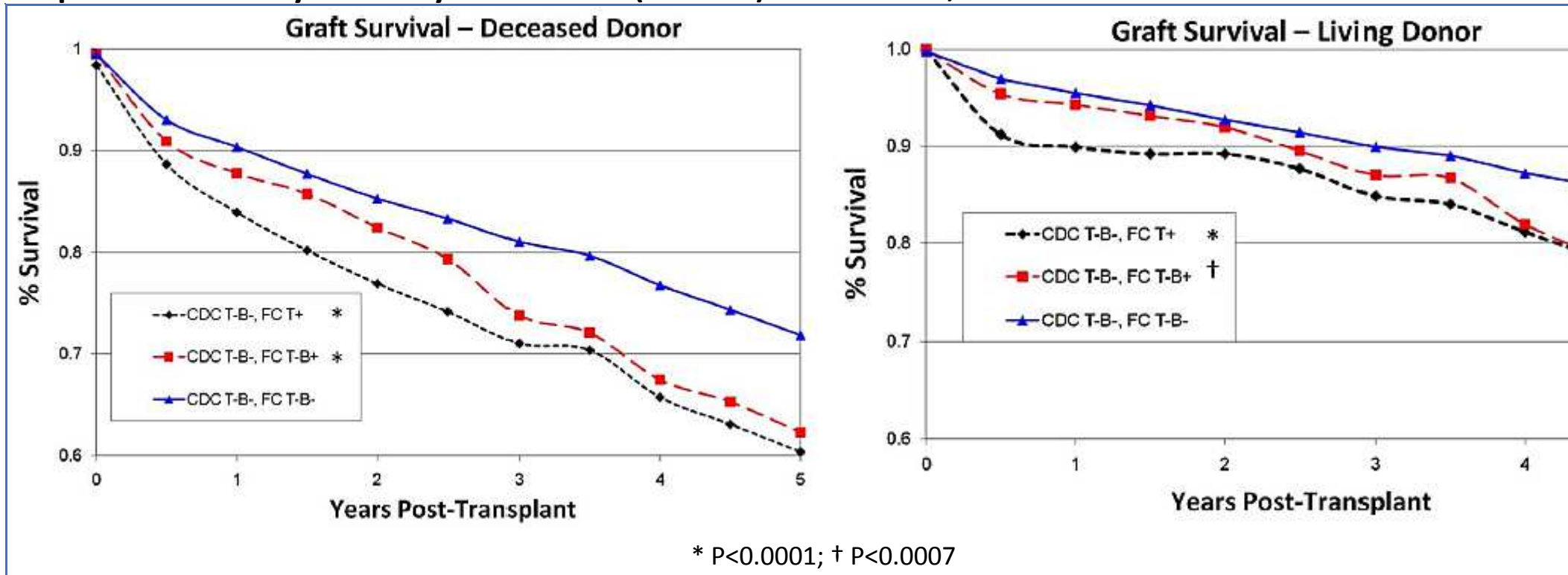
The complement-dependent microcytotoxicity crossmatch (CDCXM) is a standard method for evaluating the presence of preformed antibodies before transplantation.

The flow cytometry crossmatch (FCXM) is more sensitive, but there is controversy regarding translation of its increased sensitivity to clinically relevant graft outcomes.

We analyzed Organ Procurement and Transplant Network registry data for living (and deceased donor kidney transplants (6538 and 6488 respectively) performed in 1995 to 2009 after both CDCXM and FCXM testing.

Graft survival according to crossmatch results was compared by survival analysis.

Kaplan-Meier graft survival by flow cytometry crossmatch (FCXM) result in patients with negative complement dependent microcytotoxicity crossmatch (CDCXM). * $P < 0.0001$; † $P < 0.0007$



In conclusion, this analysis of national data with 5-year follow-up supports the important prognostic implications of positive FCXM even when CDCXM is negative.

Thus, FCXM⁺ results should not routinely be dismissed as “overly sensitive” when CDCXM is negative.

Clinical Significance of Pretransplant Donor-Specific Antibodies in the Setting of Negative Cell-Based Flow Cytometry Crossmatching in Kidney Transplant Recipients

O.O. Adebiyi, J. Gralla, P. Klem, B. Freed, S. Davis, A.C. Wiseman and J.E. Cooper

....Six hundred and sixty kidney and kidney–pancreas recipients with a negative pretransplant FCXM from September 2007 to August 2012 without desensitization therapy were analyzed with a median follow-up of 4.2 years.

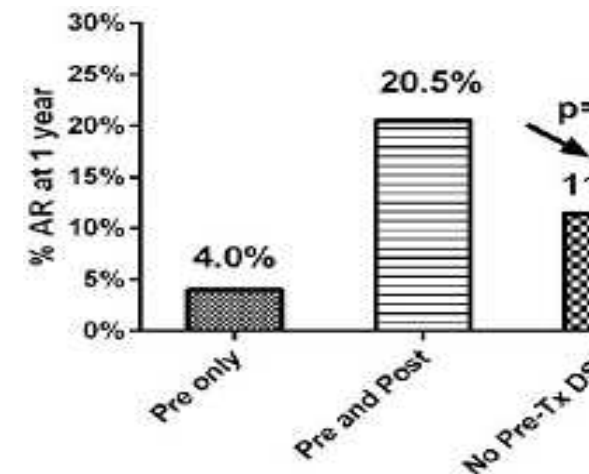
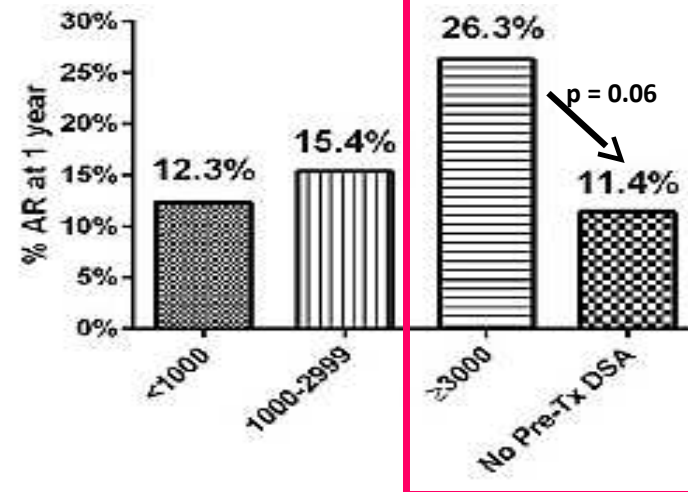
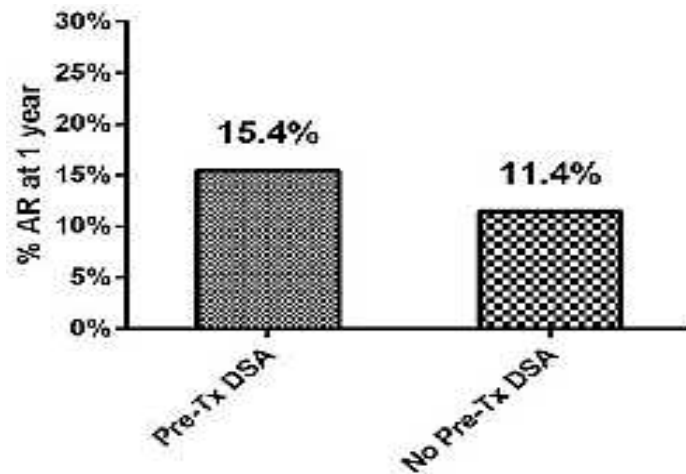
....All patients underwent cell-based FCXM and SAB analysis on current and historic sera prior to transplantation. One hundred and sixty-two patients (24.5%) had DSA detected prior to transplant.

Multivariable analysis of rejection in the first posttransplant year

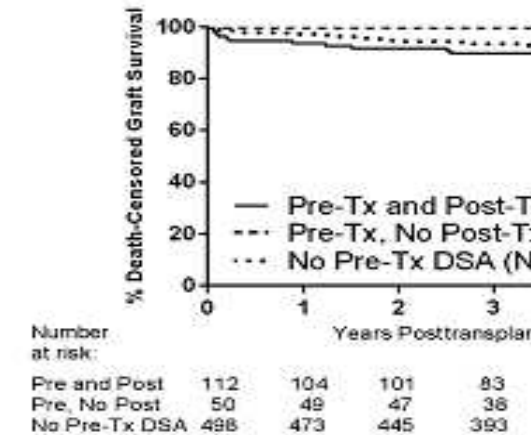
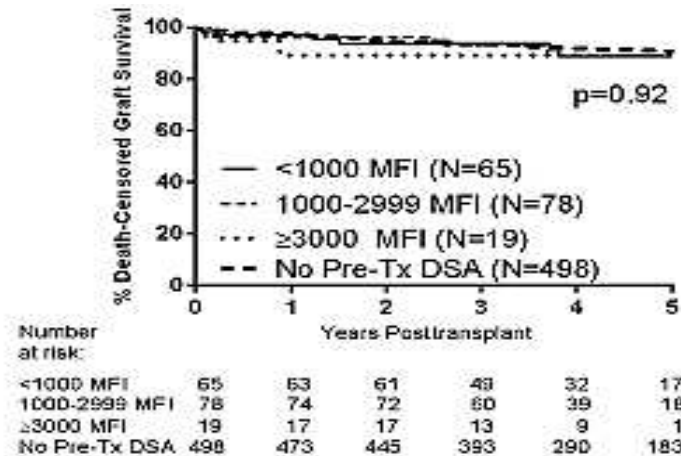
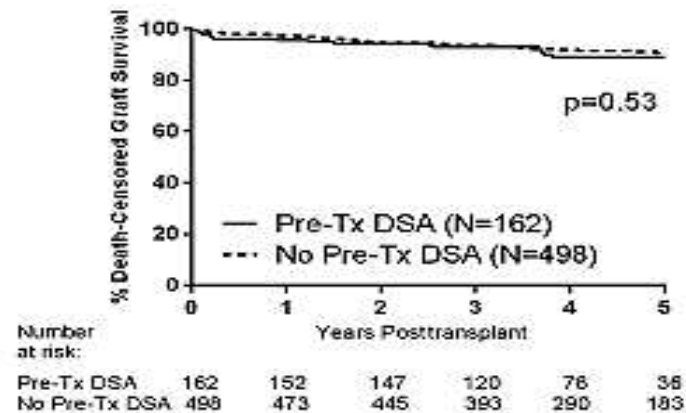
Variable	Relative risk (95% CI)
Pretransplant DSA versus no pretransplant DSA	1.15 (0.73–1.83)
Maximum pretransplant DSA strength <3000 versus no pretransplant DSA	1.04 (0.63–1.71)
Maximum pretransplant DSA strength ≥3000 versus no pretransplant DSA	2.26 (1.01–5.05)
Pretransplant DSA class I versus no pretransplant DSA	0.75 (0.37–1.53)
Pretransplant DSA class II or mixed versus no pretransplant DSA	1.56 (0.93–2.63)
Pretransplant DSA >6 months before crossmatch versus no pretransplant DSA	1.16 (0.48–2.79)
Pretransplant DSA <6 months before crossmatch versus no pretransplant DSA	1.06 (0.59–1.89)
Pretransplant DSA at crossmatch versus no pretransplant DSA	1.45 (0.70–3.01)
Persistent DSA posttransplant versus no pretransplant DSA	1.45 (0.90–2.33)
Pretransplant DSA only versus no pretransplant DSA	0.37 (0.09–1.51)

CI, confidence interval; DSA, donor-specific antibodies; PRA, calculated panel-reactive antibody. Bold values = statistically significant at $p < 0.05$.

One year incidence of Acute Rejection



Five-year death-censored graft survival



....In conclusion, pretransplant DSA in the setting of a negative FCXM confers minimal immunologic risk in the intermediate term, does not necessitate desensitization therapy and should not represent a barrier to renal transplant.

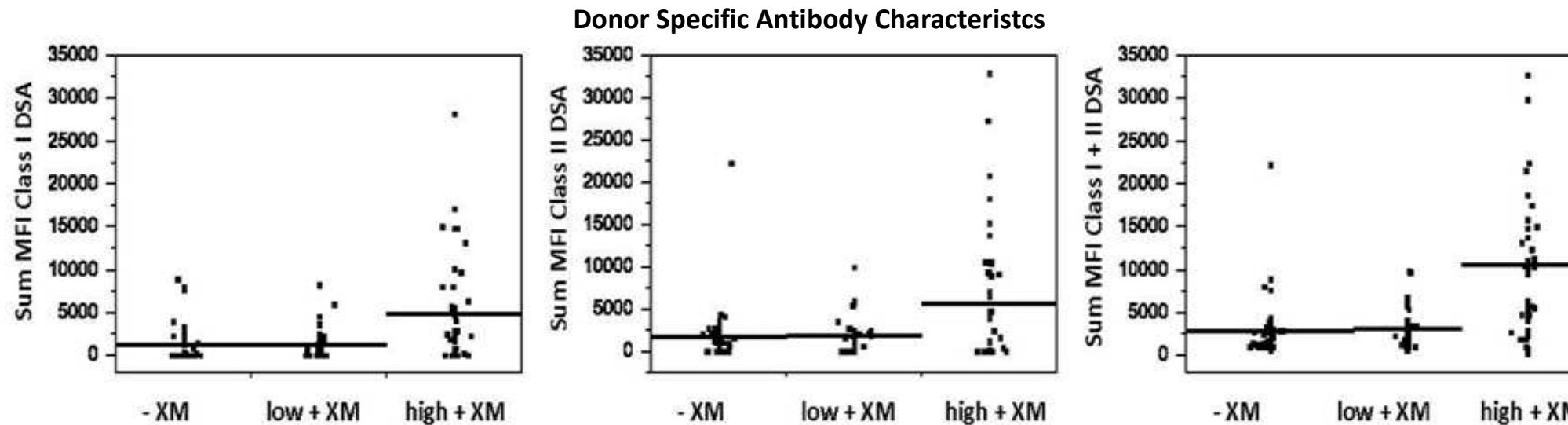
Kidney Transplant with Low Levels of DSA or Low Positive B-Flow Crossmatch

Underappreciated Option for Highly-Sensitized Transplant Candidates

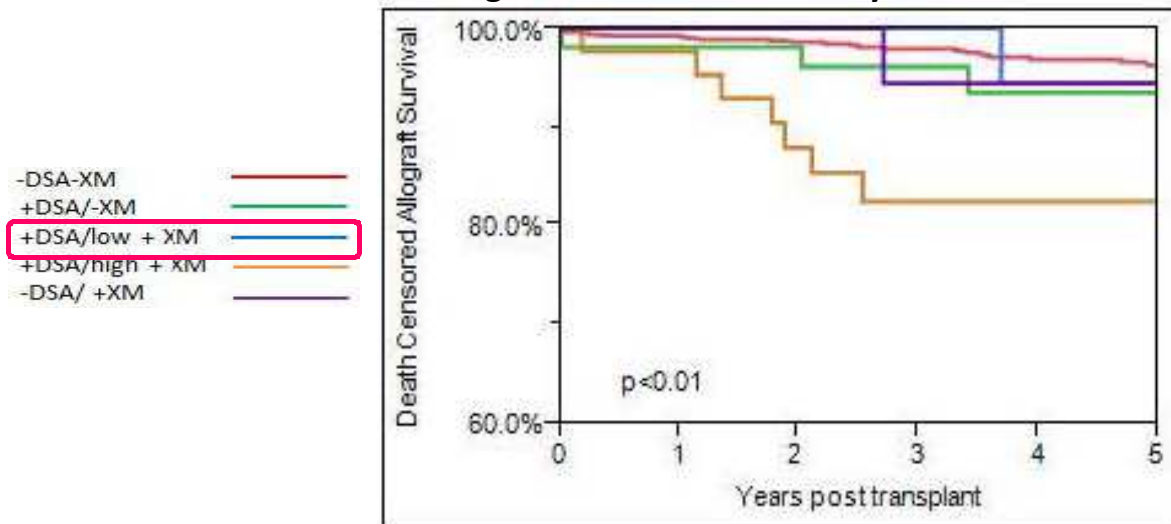
C. Schinstock, M. Gandhi, W. Cheungpasitporn, D. Mitema, M. Prieto, P. Dean, L. Cornell, F. Cosio, and M. Stegall

.... We retrospectively compared the outcomes of 954 patients transplanted with varied levels of baseline DSA detected by single antigen beads and B flow cytometric crossmatch (XM).

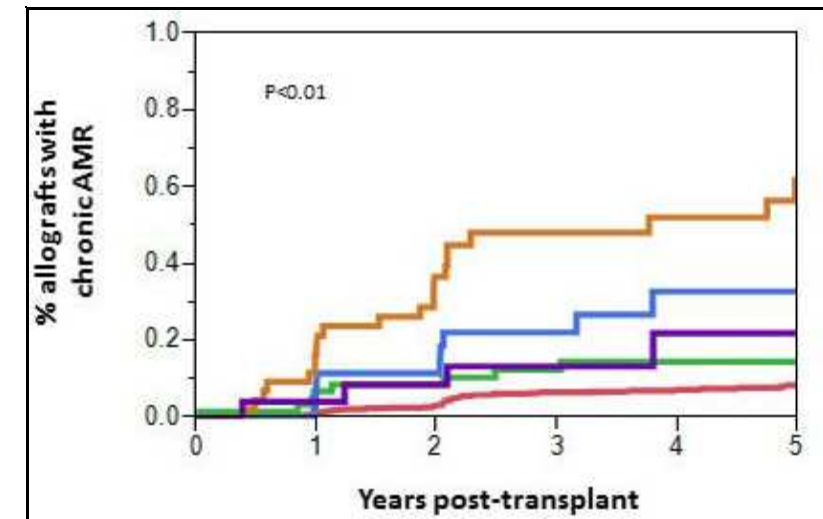
.... Patients were grouped as follows: -DSA/-XM, +DSA/-XM, +DSA/low+XM, +DSA/high+XM, and -DSA/+XM followed for a mean of 4.1 ± 1.9 years (similar among groups, $p=0.49$).



Allograft Survival stratified by DSA and B-FCXM



Incidence of Chronic AMR



Conclusion

.... Kidney transplantation with low level DSA with or without a low positive XM is reasonable option for highly sensitized patients and may be advantageous compared to waiting for a negative XM deceased donor.

....The risk for CAMR is low in patients with no DSA even if the XM is positive.....

Conclusioni

- ✓ Nel trapianto, la citometria a flusso rappresenta il metodo più sensibile per dimostrare il legame degli anticorpi anti-HLA alle corrispondenti molecole HLA «non self» espresse sulle cellule dell'organo trapiantato.
- ✓ Nel corso degli anni, l'introduzione di molteplici innovazioni metodologiche, ha portato ad un significativo incremento della sensibilità e riproducibilità del FCXM.
- ✓ Fornendo informazioni di notevole rilevanza clinica rispetto alla classica tecnica di citotossicità complemento-mediata, l'utilizzo prospettico dell'FCXM migliora l'allocatione dell'organo e quindi la sopravvivenza del trapianto per i riceventi (presenza di anti-HLA DSA).
- ✓ L'FC-XM, assieme ad una attenta valutazione dei DSA preformati meccanici, rappresentano la metodologia di elezione per una accurata selezione «immunologicamente» più idoneo a ricevere l'organo da un potenziale donatore.



Grazie