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Colorectal carcinoma rearranges cell surface protein topology and density in CD4$^+$ T cells

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Abstract

Previously, we described conserved protein clusters including MHC I and II glycoproteins, ICAM-1 adhesion molecules, and interleukin-2 and -15 receptors in lipid rafts of several human cell types. Differential protein–protein interactions can modulate function, thus influence cell fate. Therefore, we analyzed supramolecular clusters of CD4$^+$ T cells from draining lymph nodes and peripheral blood of colorectal carcinoma patients, and compared these to healthy controls. Superclusters of MHC I and II with IL-2/15 receptors were identified by confocal microscopy on all cell types. Flow-cytometric FRET revealed molecular associations of these proteins with each other and with ICAM-1 as well. In draining lymph nodes expression levels of all these proteins were lower, and interactions, particularly between IL-2/15 receptors and MHC molecules weakened or disappeared as compared to the control. Stimuli/local conditions can rearrange cell surface protein patterns on the same cell type in the same patient, having important implications on further function and cell fate.

Keywords: MHC I; MHC II; IL-2R; IL-15R; ICAM-1; Receptor clustering; FRET; Flow cytometry; Confocal microscopy; Membrane protein association; CD4$^+$ T cells; Colorectal carcinoma; Draining lymph node

The distribution and interactions of cell surface proteins play an important role in shaping transmembrane signaling. Interestingly, several cell types display similar association motifs. Homo- and heteroassociation of MHC I and II glycoproteins as well as their colocalization with ICAM-1 at both the nanometer (molecular) and submicrometer to micrometer (membrane microdomain) level were detected in the membrane of various activated and transformed cells, including T- and B-lymphoma [1–5] as well as uveal melanoma [6] and colon carcinoma [7,8] cell lines. Clustering/compartmentation of these molecules are often assisted by lipid rafts (membrane microdomains enriched in cholesterol and glycosphingolipids) [9], and are shown to facilitate antigen presentation [10–13]. In addition to molecules involved in antigen presentation, IL-2 [14–17] and IL-15 receptors [18] were also found to be localized in the same supramolecular clusters in lipid rafts of T lymphoma cell lines.

Abbreviations: MHC I/MHC II, class I/class II major histocompatibility complex protein; ICAM-1, intercellular adhesion molecule-1; IL-2/IL-15, interleukin-2/15; IL-2R/IL-15R, interleukin-2/interleukin-15 receptor; FRET, fluorescence resonance energy transfer; CLSM, confocal laser scanning microscopy; β_2m, beta-2 microglobulin; PBL, peripheral blood lymphocyte; DLN, draining lymph node.

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The vast majority of studies concerning the occurrence of these protein motifs have been performed on cell lines rather than on clinical samples. It is an intriguing question, whether these association motifs are present under different physiological/pathological conditions as well and whether they are influenced by the state of the cell. Here, we investigated the lateral organization and colocalization of MHC I, MHC II, IL-2Rα (CD25), IL-15Rα, and ICAM-1 in the plasma membrane of $CD4^+$ T cells derived from peripheral blood of healthy volunteers, or from the blood or draining lymph nodes (DLN) of patients with colorectal carcinoma.

Large-scale (~200 nm) colocalization of membrane proteins and their organization into membrane domains were detected by CLSM. Interactions at the molecular level were studied by FRET [19,20]. Our experiments revealed that MHC glycoproteins and ICAM-1 adhesion molecules formed similar patterns to those found previously in different human cell lines. Similar to T lymphoma lines, participation of IL-2Rα and IL-15Rα in these supramolecular complexes could also be observed on the $CD4^+$ subset of T lymphocytes. However, in the different T cell populations we could detect quantitative differences in the extent of these associations implicating their possible diagnostic value.

Materials and methods

Cells. Peripheral blood and DLNs from resected specimens of terminal ileum and cecum were drawn from several patients with colorectal carcinoma and no manifestation of inflammatory diseases. Peripheral blood was also taken from healthy volunteers. Peripheral blood mononuclear cells were separated by centrifugation over a Ficoll gradient (Sigma–Aldrich) according to standard procedures. Lymph nodes were minced with surgical scissors and filtered to separate cells from surrounding tissues. The fraction of $CD4^+$ T cells as determined by flow cytometry was 37% and 33% for PBLs of healthy volunteers and colorectal carcinoma patients, and 50% for DLNs. For CLSM experiments $CD4^+$ cells were separated by using MACS columns with CD4 depletion kit (Miltenyi Biotec).

Monoclonal antibodies (mAbs) and labeling. The heavy and light (β2m) chains of MHC I were labeled by W6/32 and L368 mAbs (hybridomas provided by F. Brodsky, UCSF). IL-2Rα, IL-15Rα, and CD4 were targeted by anti-Tac, 7A4-24, and OKT-4. MEM-111 specific to ICAM-1 (CD54) and MEM-75 specific for the transferrin receptor were kindly provided by V. Hořejsí (Institute of Molecular Genetics, Czech Academy of Sciences). Antibodies or their Fab fragments were conjugated with succinimidyl esters of Alexa 488 (Invitrogen), Cy3 or Cy5 dyes (Amersham Pharmacia) ([5]). Cells (~10^6 in 100 μl) were incubated with 10 μg mAbs in PBS for 40 min on ice to avoid receptor aggregation/internalization. After washing cells were fixed with 1% formaldehyde/PBS for 30 min on ice.

Analysis of colocalization by CLSM. Colocalization of membrane species at the few-hundred nanometer scale was studied using an Olympus FluoView 1000 confocal microscope. Cells were doubly or triply labeled with markers specific for distinct molecular species and carrying spectrally different fluorophores that can be detected separately. Alexa Fluor 488 was excited at 488 nm, Cy3 at 543 nm and Cy5 at 633 nm. Fluorescence was detected through 505–550, 560–615 and 655–755 nm band-pass filters. Images of 512 × 512-pixel, 1.5-μm thick optical sections were obtained from the bottom (near the coverslip) or the top layer (near the slide) of the cells with a 60× UPLSAPO oil immersion objective (NA 1.3). Images were low-pass filtered to reduce noise. Colocalization of fluorescent markers in the pixels of the multicolor images was numerically evaluated by calculating the Pearson's correlation coefficients (C) between the intensity distributions from the different channels [16].

Flow-cytometric FRET. FRET measurements were carried out on a Becton–Dickinson FACStar Plus flow cytometer with a Spectra Physics Ar ion laser operating in "all lines" mode. Cell debris was excluded from analysis by gating on the forward and side scatter signals. $CD4^+$ T cells were selected based on the I_1 signal (excitation: 488 nm, detection: 527–543 nm) from Alexa 488-conjugated OKT4 mAbs. FRET efficiency was determined as the degree of donor (Cy3) quenching in the presence of the acceptor dye (Cy5). Cells were labeled either with Cy3-conjugated mAbs alone or with both Cy3- and Cy5-labeled mAbs. The fluorescence of Cy3 (I_2) was excited at 514 nm and detected between 550 and 620 nm. The spectral spillover of Alexa 488 was corrected for by using the factor S, determined from cells labeled with Alexa 488-OKT4 alone:

$$S = \frac{I_2}{I_1} \tag{1}$$

Where, I_1 and I_2 are background-corrected intensities in the Alexa 488 and Cy3 channels. Quenching efficiency was determined as follows:

$$E = 1 - \frac{I_{2,\mathrm{da}} - SI_{1,\mathrm{da}}}{I_{2,\mathrm{d}} - SI_{1,\mathrm{d}}} \tag{2}$$

Where, $I_{2,\mathrm{d}}$ and $I_{2,\mathrm{da}}$ are the background-corrected mean intensities of the donor plus the spillover from the Alexa 488 fluorescence in the absence or presence of acceptor. $I_{1,\mathrm{d}}$ and $I_{1,\mathrm{da}}$ are the background-corrected mean intensities detected in the Alexa 488 channel for the same sample.

Determination of expression levels. The number of binding sites on the cell surface was determined from flow-cytometric fluorescence intensity histograms of cells labeled with dye-conjugated Fab fragments. The means of histograms corrected for the spectral overspill (i.e. $I_2 - SI_1$) were converted to numbers of binding sites by calibration with fluorescent microbeads (Rainbow Fluorescent Particles, BD Biosciences, Pharmingen).

Results

Expression levels of MHC I, MHC II (HLA-DR), ICAM-1, IL-2Rα and IL-15Rα were determined by flow

Table 1
Expression levels of membrane proteins on $CD4^+$ T cells

Epitope	No. binding sites (×10^3)[a]		
	Control blood	Colorectal	
		Blood	DLN
β_2m	268.4 ± 36.4 (14)	356.9 ± 76.5 (4)	140.3 ± 20.8 (18)*
MHC I heavy chain	216.8 ± 28.1 (14)	257.5 ± 30.6 (6)	126.1 ± 14.6 (18)*
MHC II (HLA-DR)	42.9 ± 3.2 (24)	36.7 ± 7.1 (10)	31.1 ± 4.0 (16)*
IL-2Rα	54.2 ± 5.3 (12)	57.6 ± 8.4 (6)	35.0 ± 4.4 (9)*
IL-15Rα	16.5 ± 0.9	13.1 ± 4.6	6.4 ± 2.3*
ICAM-1	10.7 ± 1.2 (12)	12.1 ± 1.2 (6)	6.1 ± 0.5 (5)*

[a] Data represent means ± SEM values. Numbers in parentheses represent number of independent experiments.
* Values significantly different from the control ($p < 0.05$).

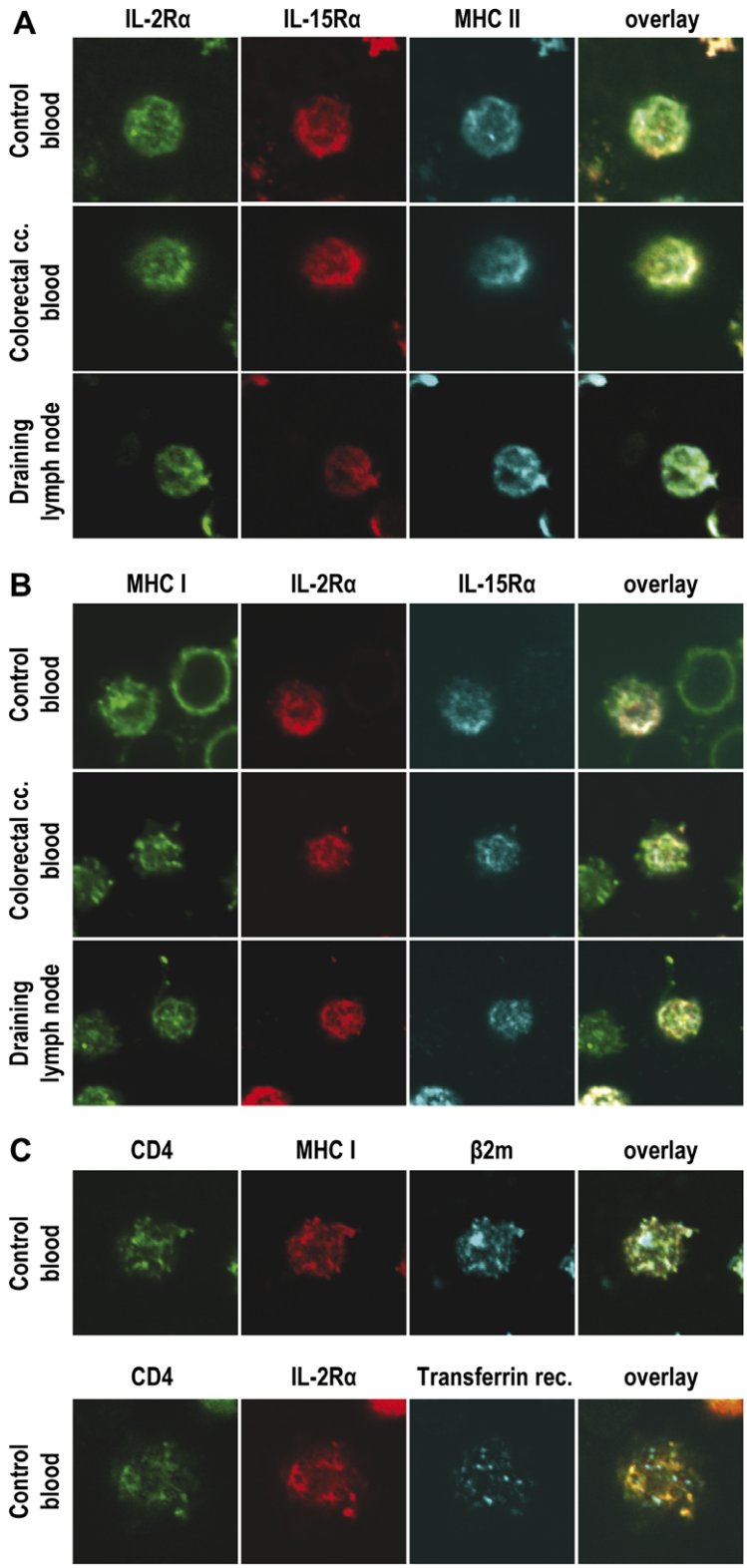

Fig. 1. Colocalization of membrane proteins detected by CLSM. (A) IL-2Rα, IL-15Rα, and MHC II were labeled by Alexa 488-anti-Tac, Cy3-7A4 24 and Cy5-L243 mAbs. Samples were drawn from healthy controls (top), or the blood (middle) or DLNs (bottom row) of colorectal carcinoma patients. Areas where membrane species colocalize are indicated with mixed colors in the overlay. (B) MHC I heavy chain, IL-2Rα, and IL-15Rα were labeled by Alexa 488-W6/32, Cy3-anti-Tac, and Cy5-7A4 24 mAbs. (C) Positive and negative controls for colocalization experiments. (Top) CD4, MHC I heavy chain, and β2m were labeled by Alexa 488-anti-CD4, Cy3-W6/32, and Cy5-L368 mAbs. (Bottom) CD4, IL-2Rα, and transferrin receptors were labeled by Alexa 488-OKT4, Alexa-546-anti-Tac, and Cy5-MEM75. Correlation coefficients calculated from pixel intensities of the different channels are shown in Table 2. The size of each frame is 17.4 μm.

cytometry (see Table 1). We could not detect significant differences between peripheral $CD4^+$ T cells of healthy volunteers or those of colorectal carcinoma patients. On the other hand, cells isolated from DLNs of colorectal carcinoma patients exhibited 40–50% lower expression for MHC I, ICAM-1, and IL-15Rα, whereas the amount of MHC II and IL-2Rα was 28–35% lower than in peripheral cells.

Large-scale colocalization of IL-2 and -15 receptors, CD4 and MHC glycoproteins was studied by multicolor CLSM (Fig. 1A and B). The pairwise colocalization of molecular species was numerically characterized by the Pearson's correlation coefficients (Table 2). The images as well as the high correlation coefficients indicate a substantial overlap between the distributions of MHC I and II with both IL-2Rα and IL-15Rα. As positive control the light and heavy chains of MHC I were used (Fig. 1C), yielding correlation coefficients greater than 0.8. Similar high correlation was observed between IL-2Rα and IL-15Rα. The correlation coefficients between IL-2Rα and MHC I, IL-15Rα, and MHC I are higher in cells from DLNs than in PBLs. As negative control, the distinct localization of transferrin receptors (known to be localized in coated pits [21]) and IL-2Rα (found earlier in lipid rafts, [16,18]) is shown (Fig. 1C).

FRET measurements were performed to check whether the molecules in the microscopically observed large-scale clusters also display molecular (2–10 nm) proximity (Table 3). Representative histograms demonstrating donor quenching are presented in Fig. 2. As a rule-of-thumb, FRET efficiencies >3–5% can be considered to be significantly different from zero referring to molecular association. As positive control, the FRET efficiency between the light and heavy chains of MHC I was determined (Table 3A and B, and Fig. 2A and B) resulting values between 23% and 39% for all the cell types. Since MHC I molecules form homoclusters,

Table 2
Colocalization of cell surface molecules on $CD4^+$ T cells characterized by the Pearson's correlation coefficients calculated from CLSM images ($N \geqslant 10$)

Epitopes		$C \pm$ SEM		
		Control blood	Colorectal	
			Blood	DLN
CD4	MHC I h.c.	0.76 ± 0.02	0.84 ± 0.05	0.82 ± 0.04
IL-2Rα	IL-15Rα	0.73 ± 0.06	0.78 ± 0.05	0.85 ± 0.03
IL-15Rα	MHC II	0.71 ± 0.05	0.70 ± 0.04	0.73 ± .04
IL-2Rα	MHC II	0.73 ± 0.04	0.75 ± 0.04	0.78 ± 0.03
IL-15Rα	MHC I h.c.	0.58 ± 0.07	0.53 ± 0.06	0.76 ± 0.05
IL-2Rα	MHC I h.c.	0.48 ± 0.07	0.52 ± 0.08	0.68 ± 0.05
β_2m (positive control)	MHC I h.c.	0.84 ± 0.02	0.81 ± 0.06	0.81 ± 0.01
IL-2Rα (negative control)	Transferrin receptor	0.38 ± 0.05	n.d.	n.d.

Table 3
FRET efficiencies measured between Fab-labeled cell surface molecules on $CD4^+$ T cells

Epitopes		$E \pm$ SEM (%)[a]		
Donor	Acceptor	Control blood	Colorectal	
			Blood	DLN
(A) β_2m	β_2m	18.0 ± 2.2 (11)	21.8 ± 0.6 (6)*	15.0 ± 2.7 (9)
	MHC I h.c.	25.9 ± 2.6 (13)	31.0 ± 3.0 (6)	26.1 ± 3.6 (8)
(B) MHC I h.c.	β_2m	34.9 ± 3.0 (13)	38.5 ± 5.3 (6)	23.0 ± 3.7 (9)*
	MHC I h.c.	14.9 ± 2.3 (10)	10.9 ± 2.0 (5)	8.0 ± 1.3 (9)*
(C) MHC II	β_2m	25.8 ± 1.9 (20)	23.9 ± 3.2 (8)	10.5 ± 2.1 (16)*
	MHC I h.c.	28.9 ± 2.4 (21)	20.6 ± 3.7 (8)*	10.0 ± 1.8 (15)*
	MHC II	10.5 ± 3.1 (7)	5.5 ± 1.2 (4)*	8.0 ± 2.7 (7)
(D) IL-2Rα	β_2m	27.0 ± 2.7 (12)	28.5 ± 2.2 (6)	10.1 ± 2.8 (8)*
	MHC I h.c.	36.2 ± 3.0 (11)	24.5 ± 6.5 (6)*	9.3 ± 2.4 (8)*
	MHC II	8.3 ± 2.5 (6)	2.9 ± 2.8 (6)*	0.9 ± 2.2 (6)*
	IL-2Rα	7.7 ± 4.2 (8)	9.3 ± 1.4 (6)	0.5 ± 2.2 (9)*
(E) IL-15Rα	β_2m	19.0 ± 1.7 (2)	38.9 ± 2.5 (2)*	16.4 ± 13.3 (2)
	MHCI h.c.	30.4 ± 2.0 (2)	25.4 ± 2.2 (2)*	n.m.
(F) ICAM-1	β_2m	26.3 ± 2.1 (12)	29.6 ± 2.5 (6)	12.6 ± 2.1 (8)*
	MHC I h.c.	23.3 ± 2.6 (12)	31.4 ± 4.9 (6)*	9.5 ± 1.4 (7)*

[a] Means ± SEM values, in parentheses the number of independent experiments.
* Significantly different from the control ($p < 0.05$).

these values represent the net of intra- and intermolecular FRET processes. MHC I and II exhibited significant homo-clustering on all three cell types (Table 3A–C). The high FRET efficiencies measured from donor-labeled MHC II, IL-2Rα, and ICAM-1 imply the heteroassociation of these proteins with the acceptor-labeled MHC I, irrespective of the origin of the cell (Table 1C–E).

Beyond the aforementioned similarities, significant differences were observed in the extent of hetero-associations. *E* values measured on DLN cells were lower than those from the control samples for all observed donor-acceptor pairs (MHC II + MHC I, IL-2Rα + MHC I, IL-2Rα + MHC II, ICAM-1 + MHC I, IL-15Rα + MHC I). $CD4^+$ peripheral T cells of patients with colorectal carcinoma exhibited lower *E* values for the MHC II + MHC I and the IL-2Rα + MHC I FRET pairs as compared to the control. However, the values were still higher than for the DLN cells. On the other hand, the hetero-association of ICAM-1 and MHC I increased for these cells.

With the exception of MHC II, the extent of homoassociations was the lowest for DLN-derived cells. IL-2Rα showed homoassociation in peripheral blood-derived cells, whereas practically no FRET was detected on DLN cells, referring to the monomeric state of the receptor chain. For MHC II, the lowest value could be detected for peripheral blood cells of colorectal carcinoma patients.

Discussion

Previously, we and others have described co-clustering of MHC glycoproteins, ICAM-1 and IL-2/15R on various T lymphoma cell lines [4,6,14,16–18]. Our earlier studies showed the association of these molecular superclusters with lipid rafts, which are critically involved in the establishment of the spatio-temporal organization of immunologically relevant molecules [16–18]. In this study, we demonstrated that co-clustering is present not only in transformed cells, but in the activated $CD4^+$ PBLs and DLN-derived T cells expressing IL-2/15R as well.

According to our CLSM experiments the large scale co-distribution patterns of the studied membrane proteins were similar in the cells irrespective of their origin. On the other hand, the molecular patterns were significantly different at the nanometer scale. Because FRET not only depends on distance but also on the acceptor density, the proper interpretation of FRET efficiencies requires consideration of the expression levels as well. The mere fact that generally larger differences could be observed in the FRET efficiency values than those in the expression levels (e.g. between IL-2Rα and MHC I) suggests that in addition to changes in acceptor density, there should be changes in the intermolecular separations as well. We revealed significant differences in the expression levels as well as the pairwise interactions on PBLs and DLN-derived cells from colorectal carcinoma patients as compared to PBLs. These results show that pathological conditions change not only expression levels, but also protein–protein interactions on the surface of $CD4^+$ T cells. Thus cell surface protein patterns may have a diagnostic value.

Although, the role of these association motifs is unclear, it is probable that the interactions between the molecules within the supercluster can modulate the function of each participant, thereby influence cell fate. Earlier it was demonstrated that the interaction of MHC I molecules with the insulin receptor (IR) bears functional significance. Increasing the MHC I-to-IR ratio also enhanced phosphorylation of IR substrate-1 and the activation of phosphoinositide 3-kinase. MHC I molecules themselves were phosphorylated on tyrosine and associated with phosphoinositide 3-kinase when cells were stimulated with insulin [22]. The existence of a similar regulatory cross-talk cannot be excluded in the relation of MHC I to IL-2R either. Further studies are needed to clarify the functional consequence of

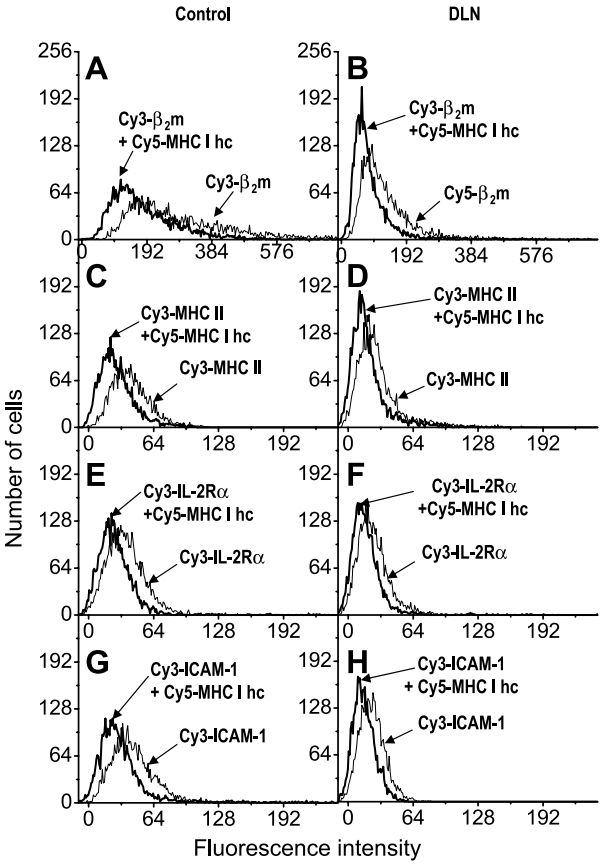

Fig. 2. Measurement of protein–protein interactions by flow-cytometric FRET. Representative distributions of the fluorescence intensity of donor-conjugated (Cy3) Fabs measured on $CD4^+$-lymphocytes in the absence (thin line) or presence (thick line) of acceptor-conjugated (Cy5) Fabs. (A, C, E, and G) Control cells; (B, D, F, and H) DLN cells of colorectal carcinoma patients. Both the mean donor intensities and their shifts due to energy transfer are significantly larger for control cells than for DLN cells implying a higher degree of clustering on controls. $CD4^+$ subpopulations were gated out using the I_1 signal from the Alexa 488-OKT-4 Fabs. Specificity of Fabs: L368 (β2m), W6/32 (MHC I heavy chain), L243 (HLA-DR), anti-Tac (IL-2Rα), and MEM-111 (ICAM-1). Mean FRET efficiencies calculated from the histograms are summarized in Table 3.

the differences found between the protein patterns of DLN-derived versus peripheral blood-derived T cells.

$CD4^{+}CD25^{hi}$ T cells (also called regulatory T cells or Tregs) contribute to the natural suppression of the anti-tumor immune response [23,24]. Their occurrence in DLNs in colorectal carcinoma lends special importance to our results. These cells are potential targets of anti-tumor antibody therapies, thus knowledge of the cell surface distribution and interactions of their membrane proteins (of which CD25 is a potential target) are crucially important for the rational design of such therapies.

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