Upregulated inducible co-stimulator (ICOS) and ICOS-ligand in inclusion body myositis muscle: significance for CD8+ T cell cytotoxicity

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Summary
Interactions between inducible co-stimulatory molecule (ICOS) and ICOS-ligand (ICOS-L) are crucial for T-cell co-stimulation, effector cell differentiation and memory CD8+ T-cell activation. Because in the muscle of patients with sporadic inclusion body myositis (sIBM) clonally expanded CD8+ T cells invade major histocompatibility complex (MHC) class I-expressing muscle fibres, we investigated ICOS-ICOS-L interactions and correlated their expression with perforin, a marker for cytotoxic effector function by autoinvasive CD8+ T cells. The mRNA from 20 muscle biopsies of sIBM, 20 non-inflammatory or dystrophic controls, two dermatomyositis (DM) and two polymyositis (PM) patients was reverse transcribed and reamplified by semi-quantitative and quantitative reverse transcription±polymerase chain reaction (RT±PCR), using primers for ICOS, ICOS-L and perforin. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH)-normalized ratio of ICOS, ICOS-L and perforin expression was compared with the degree of endomysial inflammation. Protein expression of ICOS, ICOS-L and perforin was confirmed by immunohistochemistry. We demonstrate that ICOS-L mRNA was upregulated in sIBM (arbitrary units, median 6 SEM: 48.6 ± 14.9) compared with controls (6.2 ± 17.8, P < 0.05) and significantly correlated with the expression of ICOS (53.9 ± 16.6 versus 6.7 ± 8.9 in controls, P < 0.001). By triple labelling immunohistochemistry, the CD8+ T cells in sIBM and PM were found to invade ICOS-L- and MHC class I-co-expressing muscle fibres. Among the autoinvasive CD8+ T cells, however, only a subset of ~5–10% were ICOS positive, and thereby perceptive for ICOS-ICOS-L signalling at the immunological synapse. In contrast, in Duchenne muscular dystrophy and DM, although ICOS and ICOS-L mRNA expression was also increased, the majority of ICOS-L- and ICOS-positive cells were in the perimysial regions and connective tissue. The mRNA for perforin was increased in sIBM (28.1 ± 8.7) compared with controls (4.3 ± 11.2, P = 0.18), and significantly correlated with mRNA of ICOS, ICOS-L and the degree of endomysial inflammation as assessed in coded haematoxylin/eosin tissue sections. By triple immunohistochemical staining and cell counting, perforin granules were found in 71% of the autoinvasive CD8+ T cells that were also ICOS positive. Our data indicate that in sIBM there is upregulation of ICOS-ICOS-L co-stimulatory signalling in association with enhanced perforin expression by the autoinvasive CD8+ T cells. The findings support previous suggestions that in IBM, the muscle fibres have the capacity for antigen presentation, thereby activating a specific subset among the autoinvasive CD8+ T cells to exert a cytotoxic effect. The observations strengthen the immunopathogenesis of sIBM, and offer the basis for future therapeutic interventions targeting ICOS-ICOS-L co-stimulatory interactions.

Keywords: inclusion body myositis; co-stimulation; autoimmunity; muscle inflammation; perforin

Abbreviations: APC = antigen-presenting cell; DM = dermatomyositis; DMD = Duchenne muscular dystrophy; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; ICOS(-L) = inducible co-stimulatory molecule(-ligand); MHC = major histocompatibility complex; PM = polymyositis; RT±PCR = reverse transcription±polymerase chain reaction; (s)IBM = (sporadic) inclusion body myositis

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Introduction
Emerging evidence suggests that muscle fibres are capable of acting as antigen-presenting cells (APCs) in vitro (Goebels et al., 1992) and in vivo (Wiendl et al., 2003). In addition to the presentation of the antigen via major histocompatibility complex (MHC) class I or II molecules on myofibres (Karpati et al., 1988; Emslie-Smith et al., 1989; Goebels et al., 1992; Murata and Dalakas, 1999; Wiendl et al., 2000), the expression of co-stimulatory molecules is indispensable for stimulation and clonal expansion of (autoreactive) T cells. A well-characterized co-stimulatory pathway is the interaction of CD28 on T cells with B7-1 (CD80)/B7-2 (CD86) on APCs. Muscle fibres are incapable of expressing these ‘classical’ co-stimulatory molecules, but they can express BB-1 as another member of the B7 co-stimulatory molecules (Behrens et al., 1998; Murata and Dalakas, 1999). As more members of the B7-family have now been discovered (reviewed in Carreno and Collins, 2002; Sharpe and Freeman, 2002), the inducible co-stimulator- ligand (ICOS-L; B7RP-1, B7h, B7-H2) (Swallow et al., 1999; Yoshinaga et al., 1999) has emerged as a key molecule. ICOS-L is expressed on monocytes, dendritic cells and also in a variety of non-lymphoid tissues. It interacts with its receptor ICOS (Hutloff et al., 1999) on T cells and can promote Th1 and Th2 responses (Carreno and Collins, 2002; Sharpe and Freeman, 2002). Interestingly, myofibres can also express ICOS-L under pathological conditions, as recently described (Wiendl et al., 2003). This indicates that this molecule plays a crucial role in antigen presentation by myofibres. Further, ICOS signalling has been shown to be important for the CD8+ T cell effector function, especially for the recall response rather than the initial priming (Wallin et al., 2001). Because the most important effector molecule of CD8+-mediated cytotoxicity in polymyositis (PM) muscle is perforin (Goebels et al., 1996), quantification of perforin expression can be used as a read-out system for those CD8+ T cells that exert effector functions after ICOS-ICOS-L co-stimulation.

We describe here the expression of ICOS-L co-stimulatory molecules in sporadic inclusion body myositis (sIBM) muscle and their correlation with the perforin-expressing, cytotoxic, ICOS+ T cells that invade MHC class I-expressing muscle fibres. The results are fundamental in strengthening the immunopathology of sIBM and solidifying the concept that an antigen-directed T cell-mediated cytotoxicity by auto-invasive T cells plays a key role in the mechanisms of the disease.

Methods
Patients
We investigated the muscle biopsies of 20 sIBM patients who fulfilled the typical clinical, electrophysiological and histopatho-logical criteria of the disease (Dalakas, 1991). The patients were admitted to the National Institutes of Health (NIH) Clinical Center and studied under Institutional Review Board (IRB)-approved clinical protocols. All subjects gave informed consent.

Control muscle specimens were obtained from three patients with Duchenne muscular dystrophy (DMD), five patients with non-inflammatory myopathy (due to mutations in the β-myosin chain gene), three with amyotrophic lateral sclerosis, one with a desmin myopathy and eight with morphologically normal muscle. In addition, we analysed muscle biopsy samples from two patients with dermatomyositis (DM) and two patients with PM.

Extraction of mRNA and reverse transcription–polymerase chain reaction (RT–PCR)
RT–PCR was performed as previously described (Raju et al., 2003). Total RNA was extracted from muscle biopsies using the RNeasy Kit from Quiagen (Valencia, CA) following the supplier’s instructions. In brief, we homogenized the tissue with a 0.5 mm needle and syringe, followed by dissolving it in 350 μl of lysis buffer. The RNA was eluted in 30 μl of water and stored at −80°C. The cDNA synthesis was performed with SuperScript II Reverse Transcriptase from Invitrogen (Carlsbad, CA), following the supplier’s instructions. The resulting cDNA was stored at −20°C. For amplification, we used 0.5 μl of cDNA in a 20 μl reaction with 0.5 U of Taq DNA polymerase, 0.5 μU of Taq antibody, 1.5 mM MgCl2 and 0.2 mM dNTP in PCR buffer (all from Invitrogen) with 0.5 μM primer mix. Primers were obtained from XX IDT DNA Technologies (Coralville, IA): GAPDH (glyceraldehyde 3-phosphate dehydrogenase) forward 5′-GGTAAGGTGGGAGTCAACG and reverse 5′-CAAAATGTTCATGGATGACC (Raju et al., 2003); ICOS forward 5′-GTGGCTCACTGGGAGTGGAAAT and reverse 5′-GTCAACCTGGTTCTGCAAT, coding for a 321 bp product (accession number: AJ277832); ICOS-L (B7H2) forward 5′-GTTGAAAGGGCTGGATCAACG and reverse 5′-TGGTTCTGCAAT, coding for a 412 bp product (accession number: M28393). The PCR was performed in a Peltier Thermal Cycler PTC-200 from MJ research (Watertown, MA) and optimized in pilot experiments. The cycle protocol consisted of 4 min at 94°C, denaturation for 30 s at 94°C, annealing for 30 s at 55°C for GAPDH or ICOS, or at 63°C for ICOS-L or perforin, and extension for 1 min at 72°C. Cycle numbers were 24 for GAPDH, 38 for ICOS and ICOS-L, and 40 for perforin. Pilot experiments revealed that the amplification of all three targets remained within the exponential phase. The amplified products (15 μl) were analysed by electrophoresis in a 2% agarose gel (SeaKem LE from BME, Rockland, ME) with 0.2 μg/ml ethidium bromide (Gibco, now Invitrogen) for 50–60 min at 100 V in a Horizon 11-14 gel apparatus (Invitrogen, now InVitrogen). Fluorescent bands were visualized in an Alpha Imager (Bio-Rad, Hercules, CA) and the optical density of each ICOS, ICOS-L or perforin band was compared with the respective GAPDH band after background subtraction using the Alpha Imager software (Bio-Rad). All experiments were repeated at least once, and in the case of a weak mRNA expression, even two or
three times. In 12 selected muscle biopsies from four control patients and eight sIBM patients, we analysed the mRNA expression of ICOS, ICOS-L and perforin in relation to GAPDH by quantitative (real-time) RT–PCR. The reactions were run in duplicates on an Opticon 2 DNA engine (MJ research), using 6-carboxy-fluorescein (FAM)-labelled probes and specific primers (Applied Biosystems, Foster City, CA), following the standard cycle protocol and instructions provided by the supplier.

**Immunohistochemistry**

Frozen sections (5 μm) of muscle biopsy specimens were fixed in acetone for 10 min at −20°C and air dried for 10 min. Unspecific binding was reduced by 30 min incubation with 5% bovine serum albumin (BSA) and 3% goat or chicken serum in Tris-buffered saline (TBS; 0.05 M pH 7.4 Tris, 0.15 M saline). All primary and secondary reagents were diluted in 1% BSA in TBS. The following primary anti-human antibodies were used at various concentrations: polyclonal goat anti ICOS at 5 μg/ml (R&D, Minneapolis, MN); monoclonal (clone 2D3) mouse anti-ICOS-L at 20 μg/ml (Labvision, Fremont, CA); monoclonal mouse (clone 34930) anti-CD4 at 1.7 μg/ml (R&D); polyclonal rabbit anti-CD8 at 1 μg/ml (Spring Bioscience, Fremont, CA); fluorescein isothiocyanate (FITC)-conjugated monoclonal mouse (clone KP1) anti-CD68 at 3.3 μg/ml (DAKO, Glostrup, Denmark); monoclonal rat (clone YTH 862.2) anti-HLA-A,B,C at 6.7 μg/ml (Serotec, Oxford, UK); and monoclonal mouse (clone 2B2.76) anti-perforin (US Biological, Swampscott, MA) at 20 μg/ml. Apart from incubation for 20 min with anti-CD8, sections were incubated with the primary antibodies for 1 h at room temperature and immunoreactivity was detected using Alexa-488-, Alexa-594- or Alexa-350-conjugated secondary goat or chicken antibodies against mouse, rat, goat or rabbit IgG (all from Molecular Probes, Eugene, OR). To avoid cross-reactions in double and triple labellings, we used a protocol similar to the one previously described (Schmidt et al., 2003). In the triple labelling experiments for co-localization of ICOS, CD4 and CD8, another blocking incubation with 6% goat serum for 30 min was added, owing to the fact that not all the secondary antibodies were of goat origin. For double and triple labellings, all secondary antibodies were pre-adsorbed by incubating for 15 min at 37°C with sera from species different from the host of the target antibody used in the same staining. Negative controls were performed by omission of one of each primary antibody in every staining. All sections were embedded in 30% glycerol in TBS and stored at 4°C in a dark chamber. Immunofluorescence microscopy and digital photography were performed with an inverted microscope (Axiovert 200 M, Zeiss, Göttingen, Germany) using appropriate filters for green (488 nm), red (594 nm) and blue (350 nm) fluorescence and the Axiovision 3.0 software (Zeiss). In coded haematoxylin/eosin sections from all IBM and control patients, we analysed the semi-quantitative degree of endomysial inflammation, ranging from 0 to 3, by evaluating the entire area of two cross-sections from one biopsy at 100× magnification. Two analyses were performed, one by each investigator, J.S. and G.R.

Using fluorescent microscopy as detailed above, we assessed the percentage of ICOS+ perforin+ and ICOS- perforin+ T cells that co-expressed CD8 in triple labellings of sIBM muscle sections. In six representative sIBM patients with a moderate to high expression of the respective target mRNAs, we evaluated 106–177 CD8+ T cells in 10 visual fields per patient, assessing only moderate to severe T-cell infiltration to avoid miscounts in areas with a very high cell density.

**Statistics**

For statistical analysis of the non-Gaussian distributed mRNA expression results, the non-parametric Mann–Whitney U test and the non-parametric Spearman r correlation were used. An unpaired t test was used for the analysis of the normally distributed results of the semi-quantitative histology grades. All statistics were calculated using GraphPad Prism V4.0 (San Diego, CA).

**Results**

**Upregulated mRNA expression of ICOS-L and correlation with ICOS in IBM**

The mRNA expression of ICOS and ICOS-L was much stronger in the 20 IBM muscle specimens compared with non-inflammatory myopathies or the non-myopathic control muscle biopsies, as shown for representative subjects (Fig. 1). Normalized to GAPDH expression, ICOS-L was significantly upregulated in IBM (all data in arbitrary units, given as median ± SEM: 48.6 ± 14.9, 6.2 ± 17.8, P < 0.05, Fig. 2A). As counter-receptor on activated and memory T cells, ICOS was also upregulated in the same fashion in IBM (53.9 ± 16.6, P < 0.05, Fig. 2B). Moreover, there was a significant correlation of the ICOS-L with the ICOS mRNA expression in all 40 patients (Spearman r = 0.37, P < 0.05, not shown). Since ICOS and ICOS-L do not upregulate one another, it is crucial to assess their co-localization by double labelling immunohistochemistry as reported below.

Within the control group, the DMD patients accounted for three out of four individuals with the highest ICOS levels, and for two out of three subjects with the strongest expression of ICOS-L. This is explained by the inflammatory infiltrates present in the muscle from the DMD patients. However, the tissue distribution of ICOS and ICOS-L was fundamentally different in DMD compared with IBM (see below).

The degree of upregulation of ICOS and ICOS-L-expression in IBM was similar to that of the two other inflammatory myopathies, PM and DM (data not shown), which is in accord with previous findings (Wiendl et al., 2003).

In addition to the semi-quantitative RT–PCR, quantitative (real-time) RT–PCR was performed in 12 representative muscle biopsies from four control and eight sIBM patients, revealing a similar upregulation of the mRNA expression of ICOS and ICOS-L.

**ICOS-L signal is localized on myofibres in IBM**

By immunohistochemistry, it was confirmed that the myofibres in IBM expressed ICOS-L, whereas muscle fibres from patients without muscle pathology showed almost no signal (Fig. 3A–C). The ICOS-L signal was found on the surface of almost all fibres in IBM, including healthy myofibres away from the inflammation. The most intense staining, however, was noted in fibres surrounded by CD8+ T-cell infiltrates. In contrast, in DMD, only a few fibres showed a weak ICOS-L expression, despite the presence of some
endomysial inflammation (not shown). Further, the majority of ICOS-L signal in DMD was in the perimysial fibrotic regions, not around the muscle fibres, which is consistent with the ability of fibroblasts to express ICOS-L (Swallow et al., 1999).

In PM, the staining pattern was similar to IBM, with ICOS-L-positive myofibres predominantly in areas surrounded by CD8+ T-cell infiltrates. In DM, the only ICOS-L-positive myofibres were in the perifascicular regions.

Fibroblasts, endothelial cells (see Fig. 3G) and inflammatory cells between the fascicles also showed a strong ICOS-L signal. As revealed by double labelling immunohistochemistry, most of the ICOS-L-positive lymphoid cells were macrophages, found endomysially in IBM and PM, but perimysially in DM (not shown), which is consistent with the presence of ICOS-L on APCs. A few CD8+ T cells also expressed ICOS-L (not shown), as observed by others (Ling et al., 2000; Wiendl et al., 2003).

**Fig. 1** The mRNA expression of ICOS and ICOS-L in relation to GAPDH, amplified from the muscle tissue of two representative sIBM (right) and two non-myopathic control patients (left). The three lanes for each sample demonstrate the ICOS (left lane), ICOS-L (middle lane) and GAPDH (right lane) expression. ICOS-L and ICOS are clearly overexpressed in both sIBM patients compared with the non-dystrophic control subjects. The presence of ICOS mRNA in the control patient 1 is explained by a relatively high proportion of blood vessels and connective tissue in the rather small biopsy sample, as revealed by haematoxylin/eosin staining.

**A subset of autoinvasive CD8+ T cells expresses ICOS**

Dual fluorescent labelling immunohistochemistry for ICOS and CD8 revealed that 1–2 out of ~20 autoinvasive CD8+ T cells (~5–10%) in IBM muscle were double positive (Fig. 4A). This indicates that only a subset of CD8+ T cells, even those in proximity to myofibres, had been activated previously to express ICOS. A similar percentage of CD4+ T cells, partly found in close contact with CD8+ T cells, also showed ICOS double immunoreactivity (Fig. 4B).

In PM, the endomysial ICOS-positive CD8+ and CD4+ T cells were seen at a similar frequency and ratio as in IBM. In contrast, in DM and DMD muscle, ICOS-expressing CD4+ T cells invade MHC class I- and ICOS-L-overexpressing myofibres

Because for sufficient stimulation and clonal expansion of T cells, APCs need to express MHC molecules in conjunction with co-stimulatory signals, we examined ICOS-L upregulation in MHC class I-expressing muscle fibres, as previously shown for the BB-1 molecule (Behrens et al., 1998; Murata and Dalakas, 1999). Most MHC class I-expressing myofibres (Fig. 3D) were also ICOS-L positive (Fig. 3F), as confirmed by co-localization (Fig. 3E). Both MHC class I and ICOS-L were most prominent in the muscle fibres surrounded by CD8+ T-cell infiltrates (Fig. 3A and D–F). Using a fluorescent triple labelling, we observed CD8+ T cells invading myofibres that were double positive for MHC class I and ICOS-L (Fig. 3G–I), supporting further the functional implications of co-stimulation via the ICOS-ICOS-L pathway. A similar pattern of ICOS-L and MHC class I double-positive myofibres was also frequent in PM, especially in the fibres invaded by CD8+ T cells. In DM or DMD, only a few myofibres were ICOS-L- and MHC class I-double positive but, most importantly, no invasion by CD8+ T cells was observed in those fibres (not shown).
and CD8+ T cells were infrequent and present only in the perimysium and around blood vessels, but not among the autoinvasive T cells.

**Upregulated effector perforin correlates with ICOS-ICOS-L and accumulates near the immunological synapse of autoinvasive CD8+ T cells**

Since perforin, the major effector used by CD8+ T cells, is present in inflammatory myopathies (Goebels et al., 1996), a functional ICOS-ICOS-L cross-talk should be meaningful if the cytotoxic, autoinvasive CD8+ T cells are present in ICOS-ICOS-L-positive regions. Indeed, by immunohistochemical double labelling, perforin granules were found in autoinvasive CD8+ T cells in IBM and PM (Fig. 4C–E), but not in DMD muscles. More importantly, triple labelling immunohistochemistry revealed that the ICOS-positive autoinvasive CD8+ T cells expressed perforin at their contact sites with the myofibres (immunological synapse, Fig. 4F). Triple immunolabelling in six representative sIBM patients was quantified by fluorescent microscopy of 10 visual fields with moderate to severe inflammation, revealing that among the CD8+ T cells, 11.0 ± 2.9% (mean ± SD) were double positive for ICOS, and 26.1 ± 4.7% were double positive for perforin. Moreover, 71% of the ICOS+ CD8+ T cells were triple positive, co-expressing the effector molecule perforin (data not shown).

Consistent with the immunohistochemical observations, the perforin mRNA expression was upregulated in IBM (median ± SEM, arbitrary units: 28.1 ± 8.7) compared with controls (4.3 ± 11.2, P = 0.18, Fig. 5). Two out of four control group patients with the highest perforin expression were specimens from DMD patients, concurring with the elevated ICOS and ICOS-L mRNA expression observed in DMD, as mentioned above. The perforin expression by quantitative (real-time) RT–PCR in 12 representative patients (detailed

**Fig. 3** Fluorescent single, double or triple labelling immunohistochemistry in muscle biopsy specimens of sIBM versus control muscles, using antibodies for ICOS-L, MHC class I and CD8, visualized with secondary fluorochrome reagents. (A–C) ICOS-L (red) is strongly expressed in sIBM on the surface of the muscle fibres, even on those not invaded by T cells (overview in A, single myofibre in B), but it is absent in a morphologically normal muscle (C). (D–F) Double labelling of one section for MHC class I (green, D) and ICOS-L (red, F) shows strong co-localization of MHC class I and ICOS-L (yellow, E). (G–I) One section with two muscle fibres and one blood vessel (asterisk, G), triple labelled for ICOS-L (red, G), MHC class I (green, I; yellow, merged with ICOS-L, H) and CD8 (blue, overlay with MHC class I, I). Note the CD8+ T cells (arrows, I) invading myofibres that co-express ICOS-L and MHC class I (yellow, H). Scale bars = 30 µm in A and C; 20 µm in B and G–I; 50 µm in D–F.
above) revealed results comparable with our semi-quantitative assessment.

In addition to the more important co-localization by double labelling immunohistochemistry, we observed a significant correlation of elevated perforin mRNA with the ICOS mRNA level ($r = 0.34$, $P < 0.05$), and with ICOS-L mRNA expression ($r = 0.43$, $P < 0.01$) (data not shown).

In PM patients, perforin expression was strong and comparable with that in IBM. In contrast, in DM, a lower perforin expression was detectable (data not shown).

**ICOS, ICOS-L and perforin mRNA expression correlates with the degree of endomysial inflammation**

The functional implications of the ICOS-ICOS-L cross-talk were investigated further by comparing the degree of cellular inflammation in haematoxylin/eosin-stained sections with the mRNA expression of these molecules. The degree of inflammation was higher in the IBM muscles (mean ± SD: 2.2 ± 0.8) compared with controls (0.9 ± 0.8, $P < 0.0001$) (data not shown). The degree of cellular inﬁltrates in the 40 patients correlated signiﬁcantly with the individual amount of mRNA expression for ICOS ($r = 0.56$, $P < 0.001$), ICOS-L ($r = 0.38$, $P < 0.05$) and perforin ($r = 0.43$, $P < 0.01$) (Fig. 6A–C). This suggests that ICOS-ICOS-L signalling mirrors the degree of inﬂammation. Although this does not indicate causality, it supports the putative functional role of these co-stimulatory molecules in the pathogenesis of sIBM. In analogy to the upregulated mRNA of ICOS and ICOS-L in some of the controls, two of the four patients with the strongest cellular inﬁltrates were DMD patients.

**Discussion**

We demonstrate that in IBM, the myoﬁbres expressing MHC class I molecules speciﬁcally upregulate ICOS-L. Further, we show that among the autoinvasive CD8$^+$ T cells, there is a preferential subset that expresses the counter-receptor ICOS and the cytotoxic effector perforin and makes cell–cell contact with the myoﬁbres. The results extend previous observations that myoﬁbres express the functional co-stimulatory molecules used by professional APCs and provide evidence that in sIBM, the muscle ﬁbres form an immunological synapse with the sensitized, autoinvasive T cells.

Expression of peptide–MHC complex and co-stimulatory molecules is a prerequisite for the APC to activate the autoinvasive T cells. It has been shown that among the co-stimulatory molecules, BB-1 is upregulated on MHC-expressing myoﬁbres in PM and sIBM muscles (Behrens et al., 1998; Murata and Dalakas, 1999). The ‘classical’ B7-CD28/cytotoxic T-lymphocyte antigen 4 (CTLA-4) co-stimulatory pathway, however, is predominantly involved in the initial T cell priming. In contrast, the new members of the B7 family, most importantly ICOS-L (B7RP-1, B7h, B7-H2) expressed on APCs (Yoshinaga et al., 1999; Swallow et al., 1999) and its counter-receptor ICOS on T cells (Hutloff et al.,...)
are fundamental for re-activation and stimulation of the effector functions in memory T cells, especially in tissues devoid of the ‘classical’ B7 molecules (reviewed in Carreno and Collins, 2002; Sharpe and Freeman, 2002). The functional importance of ICOS-ICOS-L co-stimulation in autoimmunity has been shown in experimental models of allergic airway disease (Dong et al., 2001; Gonzalo et al., 2001), transplantation (Harada et al., 2003) and multiple sclerosis (Dong et al., 2001; Rottman et al., 2001; Sporici et al., 2001), where blocking of ICOS signalling during the effector phase, but not during the initial priming, ameliorates the course of the disease. It has been demonstrated recently that in inflammatory myopathies, the muscle fibres not only express ICOS-L, but this molecule is also functional, arguing for an important role for ICOS-ICOS-L interactions in these disorders (Wiendl et al., 2003). Our present observations, based on a large number of biopsy specimens from patients and controls, utilizing semi-quantitative and quantitative RT–PCR in conjunction with immunohistochemistry, further support the functional significance of ICOS-ICOS-L co-stimulatory signalling in IBM. Because myofibres co-expressing ICOS-L and MHC class I were found to make cell–cell contact with ICOS-positive and perforin-expressing CD8+ T cells, these molecules seem to be essential in forming the immunological synapse between muscle fibres and CD8+ T cells.

A novel finding in this study was the observation that ICOS was present only on a subpopulation of the autoinvasive CD8+ T cells, indicating that only a small percentage of T cells is relevant for the T cell-mediated cytotoxicity. This is consistent with the expression of the chemokine receptor CXCR3 by only a small subpopulation of autoinvasive CD8+ T cells (Raju et al., 2003). Despite reports of T-cell heterogeneity in some patients with sIBM (Bender et al., 1998; Fyhr et al., 1998; van der Meulen et al., 2002), the majority of studies observed a restricted T-cell receptor repertoire in IBM muscle among a small, but important subset of clonally expanded CD8+ T cells (Lindberg et al., 1994; Bender et al., 1998; Amemiya et al., 2000; Muntzing et al., 2003). These observations are also in line with recent findings in a mouse model of allergic airway disease, where only a small subpopulation of ~10% of antigen-experienced ICOS+ CD4+ T cells accounted for the vast majority of inflammatory effector cells (Lohning et al., 2003). It appears, therefore, that the striking endomysial inflammation noted in IBM consists of a large number of bystander, non-antigen-specific T cells, whereas only 5–10% of T cells among the autoinvasive CD8+ T cells make up a powerful, ICOS-positive subset that plays a key role for T cell effector functions. The importance of ICOS-ICOS-L signalling in reactivating only the cytotoxic and autoaggressive T cells relevant to the lesion is supported further by the observation that ICOS signalling has augmented the effector CD8+ T-cell responses in a tumour rejection model (Wallin et al., 2001). The convincing role of ICOS-ICOS-L interactions in enhancing CD8+ T-cell cytotoxicity, however, may not be equally applicable to all disorders in humans and animals, further calling for functional studies. For example, the cytotoxic effect by T lymphocytes in an infectious mouse model did not seem to depend solely on ICOS signalling (Kopf et al., 2000), and ICOS-L failed in vitro to augment the lytic activity in a human glioblastoma model (Schreiner et al., 2003). Apart from direct effects on cytotoxic activity, ICOS-ICOS-L interactions may also exert their effects via priming and expansion of CD8+ T cells or by modulation of the cytokine milieu (Kopf et al., 2000; Schreiner et al., 2003).

Fig. 6 In 20 sIBM and 20 control subjects, the semi-quantitative grade of cellular inflammation in haematoxylin/eosin staining was correlated to the GAPDH-normalized mRNA expression of ICOS, similar to Figs 2 and 5. The cellular infiltration (y-axis) correlates significantly with the mRNA expression (y-axis) of ICOS (A, P < 0.001), ICOS-L (B, P < 0.05) and perforin (C, P < 0.01).
The presence of ICOS signals on a small subset of endomyosial CD4+ helper T cells observed in our study may enhance the cytotoxicity of the specific CD8+ effector T cells via cytokine secretion.

The upregulated perforin mRNA levels observed in IBM muscles compared with DM and DMD controls suggests that the perforin-expressing CD8+ T cells are also the effector cells in IBM, in a pattern similar to that reported for PM (Goebels et al., 1996). As mentioned earlier, the ICOS+ CD8+ T cells observed in our study are presumably memory cells, which are known to contain smaller amounts of cytotoxic granules compared with the effector CD8+ T cells (Kaech et al., 2002; Barber et al., 2003). However, these cells can be readily re-activated to exert a killing effect as fast and effectivley as the effector CD8+ T cells, as recently shown in an infectious disease model (Barber et al., 2003; Byers et al., 2003), arguing for a crucial role for memory CD8+ T cells in autoimmune disorders. Our observation that perforin granules, present in the majority of endomyosial ICOS+ CD8+ T cells, were directed towards the surface of the myofibres supports the view that in a chronic, indolent disease such as IBM, the cytotoxic function is mediated mostly by such activated memory T cells. The finding is specific for IBM and PM, but not DM, where the inflammation is not antigen driven.

The demonstration that in sIBM the effector molecule perforin accumulates near the site of the immunological synapse between ICOS+ CD8+ T cells and ICOS-L-expressing myofibres suggests that the ICOS-ICOS-L interactions are critical in initiating or perpetuating T cell-mediated cytotoxicity. The finding is significant, not only in a better understanding of the immunopathomechanisms of sIBM, but also in designing treatment strategies aiming at the co-stimulatory ICOS-ICOS-L pathway. Perhaps the difficulty in treating IBM and other chronic autoimmune disorders with immunosuppressive drugs is that the majority of the autoinvasive T cells have a bystander rather than an antigen-specific role. The mild reduction of endomyosial inflammation in patients with sIBM after unsuccessful, non-specific, immunotherapy (Dalakas et al., 2001) may reflect a proportional reduction of these ‘reactive’ cells instead of eliminating the disease-specific T cells. Along these lines, the recent observation that the powerful anti-T-lymphocyte globulin that caused significant T-cell reduction (presumably including the relevant cytotoxic T cells) was effective in IBM (Lindberg et al., 2003) supports our contention and opens the avenue for more, target-specific, anti-T cell therapeutic interventions.

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References


Swallow MM, Wallin JJ, Sha WC, B7h, a novel costimulatory homolog of B7.1 and B7.2, is induced by TNFalpha. Immunity 1999; 11: 423–32.


