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Variazioni del gene di Perforina in pazienti con Diabete di Tipo1 (T1D)

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INTRODUZIONE

1- DIABETE DI TIPO 1 (T1D)

Il diabete di tipo 1 (T1D) rappresenta una delle più gravi forme cliniche di diabete mellito, in cui la distruzione delle cellule β del pancreas comporta generalmente l'incapacità di produrre insulina **[1-4]**. A tutt'oggi il T1D viene distinto in due forme: diabete mellito di tipo 1A (o immunomediato), che origina a seguito della distruzione delle cellule β e che può essere a insorgenza rapida (giovanile) o ad esordio lento (Latent Autoimmune Diabetes-LADA, in soggetti adulti) **[2-4]** e di tipo 1B (o idiopatico), meno frequente, le cui cause non sono ancora note e presente soprattutto in individui con discendenti asiatici o africani **[5]**.

L'eziologia della malattia resta ancora in gran parte sconosciuta, anche se è ampiamente accettato che l'origine del disturbo sia multifattoriale, particolarmente legata ad un'interazione tra predisposizione genetica da una parte e fattori ambientali dall'altra **[6–8]**. La suscettibilità genetica è stata ampiamente dimostrata per particolari combinazioni genetiche degli antigeni HLA: nello specifico sono stati identificati aplotipi predisponenti la malattia (DQA1*0301-DQB1*0302 e DQA1*0501-DQB1*0201) e protettivi (DQA1*0102-DQB1*0602) **[2]**. Altri geni associati sia a rare sindromi, come i geni AIRE e Foxp3, che a condizioni di autoimmunità, ad esempio PTPN22 e CTLA-4, sembrano svolgere un ruolo nella patogenesi del T1D **[9]**.

I fattori ambientali coinvolti nell'innesco della risposta autoimmune, che danno inizio alla distruzione delle cellule β, comprendono: virus (enterovirus, rosolia congenita, coxsackie virus) **[10, 11]**, tossine (nitrosamine) **[12]** e alimenti (proteine del latte, cereali, glutine) **[13–17]**.

L'eccessiva attivazione del sistema immunitario mediato dalle cellule T in soggetti predisposti, porta ad una risposta infiammatoria all'interno delle insule pancreatiche (insuliti) e ad una risposta umorale (mediata dai linfociti B) che conduce alla produzione di anticorpi diretti contro antigeni pancreatici. Sono stati individuati numerosi anticorpi rivolti verso le varie strutture delle insule pancreatiche, attualmente entrati anche nell'uso diagnostico: **ICA** (Islet Cell Autoantibodies) **[18]** rivolti ad antigeni bersaglio dell'insula estremamente eterogenei; sono presenti nella maggioranza dei casi di diabete 1A già in fase prediabetica, nel 70% dei casi all'insorgenza della malatti, nel 50% dopo sei mesi, nel 35% dopo due anni e nel 15% oltre i due anni. **GADA** (Glutamic Acid Decarboxylases Autoantibodie) **[19]** sono rivolti contro la Glutammato Decarbossilasi (GAD) da 65

kDa prevalentemente; sono presenti in circa il 60% dei casi di diabete di tipo 1A, sono caratterizzati da prolungata persistenza dopo l'esordio della malattia e sono quindi determinanti nella diagnosi del LADA. **IA-2** (IA-2A Autoantibodies) sono rivolti contro una tirosinfosfatasi che interviene nel trasporto dei segnali dal citoplasma alle vescicole secretorie delle cellule. Sono presenti in circa il 40% dei casi, caratterizzerebbero il rapido evolvere della malattia verso l'insulina-dipendenza e sono più frequenti nei giovani. **IAA** (Insulin Autoantibodies) [20] rivolti contro l'insulina nativa; sono un marcatore precoce di autoimmunità, nella prima infanzia sono i più frequentemente trovati (presenti in > 90% dei casi) e spesso in tale età la loro comparsa precede quella degli ICA, dei GADA e degli IA-2; sono caratterizzati da rapida scomparsa.

Un'evidenza che supporta la patogenesi autoimmune del T1D deriva dalla suscettibilità dei pazienti a sviluppare altre condizioni autoimmuni quali la tiroidite di Hashimoto, la malattia di Graves, la malattia di Addison, il morbo celiaco, e la miastenia grave [21–24].

L'associazione tra una risposta iperattiva del sistema immunitario e il T1D è dimostrato nei topi NOD (Non-Obese Diabetic), modello animale più comunemente studiato di diabete spontaneo, nei quali l'eliminazione dei linfociti T impedisce lo sviluppo di T1D; inoltre il trasferimento di cellule T da un donatore malato ad un ricevente sano si associa all'induzione della malattia [25].

Il mimetismo molecolare associato ad infezioni rappresenta una delle ipotesi per spiegare l'autoimmunita nel T1D, tuttavia è evidente che il precoce rimodellamento e /o omeostati che si assiste nella massa delle cellule β coinvolge il meccanismo apoptotico all'interno delle cellule stesse [26] che può innescare a sua volta l'autoimmunità.

Anche se la relazione tra apoptosi delle cellule β e autoimmunità rimane ancora da stabilire in modo completo, esistono evidenze per cui l'apoptosi mediata dalle cellule T rappresenta un meccanismo dominante nel T1D [25].

Sia i linfociti T-Helper ($CD4^+$) che i Citotossici ($CD8^+$) rappresentano importanti effettori finali nella distruzione delle cellule β [27–29], ma anche i macrofagi e le cellule dendritiche giocano un ruolo importante, non solo come cellule presentanti l'antigene, ma anche come fonte di radicali dell'ossigeno o di altri mediatori citotossici solubili [30].

Tuttavia, mentre sono note le cellule effettrici ed il loro ruolo nello sviluppo della malattia, ancora molto discusso rimane il capitolo riguardante le molecole coinvolte nella morte delle cellule β.

Perforina, Fas e il suo ligando (FasL), TNFα, IL-1, INFγ, NO sono tutte molecole che sembrano coinvolte nell'apoptosi cellulare, anche se non è ancora nota quale sia tra queste la molecola che svolge il ruolo cruciale nella fase finale di sviluppo della malattia [31].

Diversi lavori riportano l'utilizzo di Fas (recettore di morte che induce apoptosi nei linfociti attivati) da parte dei CTL nell'apoptosi delle cellule β [32–34], un ruolo confermato anche attraverso la nostra identificazione di una sostanziale popolazione di pazienti T1D che presentano una difetta funzionalità della proteina [35]. Il ruolo di Fas nella risposta immune è confermato nei pazienti affetti da Sindrome Autoimmune Linfoproliferativa (ALPS), rara malattia autoimmune caratterizzata manifestazioni autoimmuni eterogenee e accumulo di linfociti negli organi linfatici secondari, caratterizzati da difetti ereditari della funzionalità di Fas [36–39].

Un'altra molecola ampiamente associata alla distruzione delle cellule β è perforina. I granuli dei CTL e delle cellule natural killer (NK) contengono perforina e granzimi, che vengono rilasciati sulle cellule bersaglio dopo il riconoscimento da parte delle cellule citotossiche. Perforina polimerizza sulla membrana delle cellule bersaglio formando dei pori che permettono l'ingresso di granzimi, i quali innescano l'apoptosi nella cellula bersaglio attraverso l'attivazione delle caspasi [40]. La citotossicità mediata da perforina svolge un ruolo cruciale anche nell'eliminazione delle cellule infettate da virus e nello spegnimento della risposta immunitaria [41–43].

Il ruolo di perforina nel T1D è confermato anche nei topi NOD, infatti, l'eliminazione di perforina riduce sensibilmente l'incidenza del diabete nei topi NOD e inoltre blocca l'insorgenza del diabete indotto da virus nei topi transgenici RIP-LCMV [44, 45], suggerendo quindi un ruolo importante della proteina sia nei modelli spontanei di diabete che in quelli artificiali.

2-PERFORINA

I linfociti citotossici (CL), che comprendono i linfociti T citotossici (CTL) e le cellule natural killer (NK), sono in grado di riconoscere cellule infettate da virus o cellule trasformate e di distruggerle attraverso meccanismi perforina-dipendenti e/o utilizzando recettori di morte. Entrambi questi meccanismi rappresentano sistemi fondamentali per l'immuno sorveglianza e l'immuno regolazione.
I granuli presenti all'interno dei CL contengono perforina ed altre proteine pro-apoptotiche che vengono segregate insieme al fine di uccidere le cellule bersaglio.
Perforina, codificata da un gene presente in singola copia ed altamente conservato tra le specie (dai pesci ai primati), svolge un ruolo cruciale per il funzionamento degli altri costituenti dei granuli citotossici (ad es. granzimi), coinvolti anch'essi nell'induzione della morte delle cellule bersaglio, tramite meccanismi caspasi-dipendenti e/o caspasi-indipendenti **[46].**
L'esocitosi dei granuli citotossici avviene a seguito della formazioni di sinapsi immunologiche tra i CL e le cellule bersaglio; la fusione dei granuli con la membrana plasmatica si risolve con il rilascio di perforina e serino-proteasi pro-apoptotiche (granzimi) insieme ad altre molecole quali granulosina e chemochine.
Perforina è una proteina di circa 67 kDa, in grado di formare pori sulla membrana delle cellule bersaglio; la sua espressione è regolata durante il differenziamento linfocitario da segnali di recettori di attivazione (recettori delle cellule T, NKG2D) e da citochine (IL-2, IL-15, IL-21).
Il gene umano di perforina, localizzato sul cromosoma 10q22 **[47]**, è costituito da tre esoni, due dei quali (esone 2 e 3) codificanti la proteina di 555 aminoacidi.
La proteina presenta una sequenza segnale di 21 aa, seguita da un dominio centrale con forte omologia con la proteina C9 del complemento che si ritiene formare un'α elica antipatica in grado di inserirsi nel doppio strato lipidico della cellula bersaglio.
Approssimativamente, gli ultimi 200 aa della sequenza di perforina vanno a costituire due dominii che sono stati identificati in precedenza in altre famiglie di proteine: un dominio EGF-like (Epidermal Growth Factor) e una regione di circa 130 aa omologa al dominio C2 della protein-chinasi C **[48, 49]**.
Il dominio C2 all'estremità carbossil-terminale di perforina mostra una stretta omologia di sequenza con il dominio C2 di altre proteine Ca^{2+}-dipendenti coinvolte nel traffico vescicolare o nella trasduzione del segnale **[50–52].** Recenti studi hanno dimostrato il ruolo cruciale di questo dominio nella prima fase dell'attività membranolitica di perforina, la fase di legame alla membrana Ca^{2+}-dipendente **[53]**.

Diversamente dalle altre proteine contenenti un dominio C2, perforina sembra esercitare la sua attività nel compartimento extracellulare, dove la concentrazione di Ca^{2+} libero è alta (> 1 mM). Ne consegue che la proteina necessita almeno di una concentrazione 100uM di Ca^{2+} per legare efficientemente la membrana della cellula bersaglio. La bassa affinità di perforina per il Ca^{2+} potrebbe d'altronde essere necessaria per proteggere i CL dall'autolisi durante la sintesi di perforina e il suo traffico all'interno della cellula. Inoltre, l'accumulo di perforina nei granuli, all'interno dei quali il pH viene mantenuto basso (< 5), si risolve nella protonazione dei residui di aspartato all'interno del dominio C2 necessari per legare il Ca^{2+}, non permettendo quindi il legame alla membrana e impedendo l'attivazione di perforina prima della sua esocitosi.

La proteina contiene due siti di N-glicosilazione. Viene sintetizzata come precursore inattivo: una volta processato nel carbossiterminale, rilascia circa 20 aminoacidi e si trasforma nella sua forma attiva [49] (**Figura 1**). In seguito alla sintesi nel reticolo endoplasmatico rugoso, le molecole di perforina si spostano attraverso i compartimenti del Golgi dove continua la modificazione post-traduzionale e vengono infine impaccate nei granuli litici dei CTL e delle cellule NK.

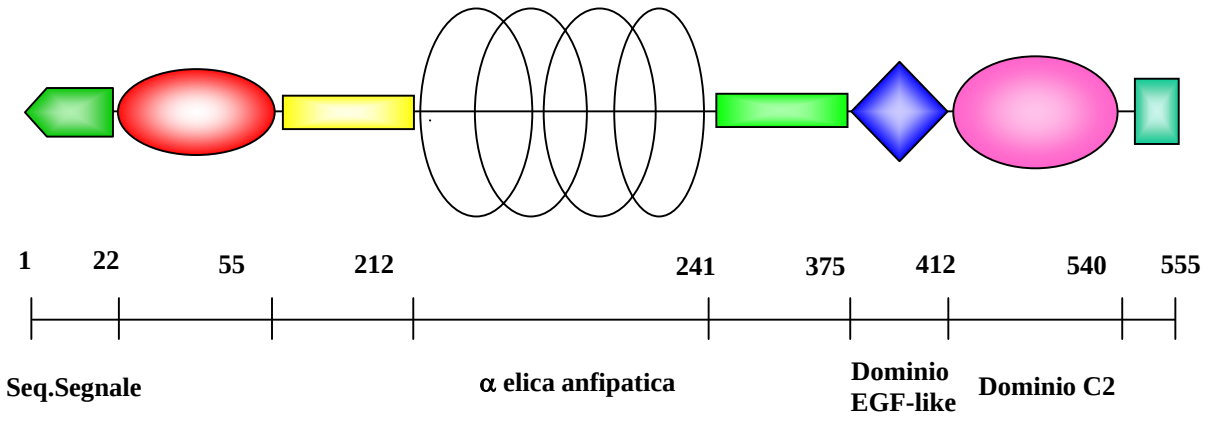

Figura 1. Domini strutturali di perforina.

2.1 Deficit di perforina nell'uomo: la Linfoistiocitosi Emofagocitica Familiare (FHL)

La Linfoistocitosi Emofagocitica (HLH) è una malattia caratterizzata da una condizione iperinfiammatoria determinata da un'incontrollata inefficace risposta immune. I sintomi diagnostici comprendono: febbre persistente, splenomegalia, citopenia, ipertrigliceridemia e/o fibrinopenia ed emofagocitosi a livello del sistema reticolo-endoteliale [54].

Generalmente la HLH viene classificata in forma primaria (o familiare, FHL) e secondaria [55, 56].

La diagnosi di FHL, condizione autosomica recessiva, è legata alla familiarità della malattia e, anche se non sempre uno studio familiare è possibile, la recente identificazione di difetti genetici nei pazienti FHL ha reso la diagnosi più accurata.

Le mutazioni identificate cadono nel gene di Perforina (*PRF1*) nei pazienti FHL2 [57–64], nel gene di Munc13-4 (*UNCI13D*) nei pazienti FHL3 [64–66] e nel gene di Syntaxin11 (*STX11*) nei pazienti FHL4 [64, 67, 68].

Nel complesso, mutazioni a carico di Munc13-4, Syntaxin11 e Perforina sono responsabili del 30-70% dei casi di FHL, suggerendo che altri difetti genici che possono predisporre all'FHL devono ancora essere identificati.

Perforina è coinvolta nella citotossicità mediata dai granuli presenti nelle cellule NK e nei CTL e, dopo l'identificazione di mutazioni nel suo gene, è stato possibile stimare che la FHL2 può svilupparsi anche dopo l'infazia, fino ai primi cinquant'anni di vita [69], mentre l'insorgenza della HLH è tipicamente giovanile [70].

Nei pazienti affetti da HLH l'attività citotossica dei CTL e delle cellule NK è severamente ridotta, se non assente e le mutazioni in perforina coincidono con una marcata riduzione della funzionalità e stabilità della proteina [57, 60, 62]. I linfociti citotossici dei pazienti HLH sono incapaci di eliminare le cellule bersaglio e reprimere le cellule presentanti l'antigene. Questo determina un'attivazione ed un'espansione incontrollata dei linfociti T CD4+ e CD8+ ed una eccessiva produzione di citochine infiammatorie [71]

Tra le molte variazioni note in perforina, la sostituzione nucleotidica +272C/T, che determina a livello proteico il cambiamento dell'Alanina in posizione 91 con una Valina (A91V), rappresenta la variazione più comune identificata in perforina, con una frequenza allelica che varia tra 3% e 17% in diverse popolazioni di controli [63, 72, 73].

Molti studi hanno suggerito un nesso tra la A91V e forme atipiche (tardive nello sviluppo) di FHL2 [63, 69, 72, 74]. E' stato inoltre proposto un ruolo della variazione nella predisposizione di diversi tipi

di tumore, inclusi linfomi a cellule T e B [75] e nella Leucemia Acuta Linfoblastica (ALL) dei bambini [76]. Infine, recentemente, la variazione è stata associata anche alla DALD (Dianzani Lymphoproliferative Disease) [77]. Nella maggior parte dei casi (ad eccezione dei pazientiFHL), la A91V si manifesta in eterozigots, anche se sono stati individuati alcuni casi di omozigosità sia nella ALPS che nella DALD. A livello proteico, la variazione A91V sembra alterare la conformazione della proteina riducendone l'attivazione proteolitica ed aumentando la sua degradazione [78, 79].

Un altro polimorfismo sospetto di perforina, che determina il cambiamento amino acidico N252S, è stato individuato in pazienti FHL che hanno sviluppato la malattia in età molto precoce [57, 62].

La variazione è stata recentemente associata alla Sindrome Autoimmune Linfoproliferativa (ALPS), dove è stato dimostrato aumentare il rischio di sviluppare la malattia di circa 63 volte [77].

SCOPO DEL LAVORO

Recentemente nel nostro laboratorio è stato dimostrato che la sostituzione A755G del gene di perforina, che determina a livello aminoacidico il cambiamento dell'asparagina in posizione 252 con una serina (N252S), rappresenta un fattore di rischio per lo sviluppo della Sindrome Autoimmune Linfoproliferativa (ALPS), rara malattia autoimmune ereditaria causata da difettivà funzionalità di Fas. E' stato anche dimostrato che la variazione C272T nello stesso gene, che conduce alla sostituzione aminoacidica A91V è associata allo sviluppo della DALD (variante incompleta dell'ALPS).

Essendo noto il convolgimento di perforina, proteina ad attività citolitica presente nei granuli dei linfociti citotossici (CTL) e delle cellule natural killer (NK), nel meccanismo di distruzione delle cellule β del pancreas che si assiste nel Diabete di Tipo 1 (T1D), ho valutato, nel mio secondo anno di dottorato, se le due variazioni di perforina associate all'ALPS/DALD, potessero rappresentare un fattore di rischio anche per lo sviluppo di una malattia autoimmune più comune come il T1D. Il lavoro di seguito riportato completa i primi dati ottenuti, dimostrando il coinvolgimento della variazione N252S di perforina nella suscettibilità alla malattia.

RISULTATI E DISCUSSIONE

BRIEF REPORT

Variations of the Perforin Gene in Patients With Type 1 Diabetes

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OBJECTIVE—Perforin plays a key role in cell-mediated cytotoxicity. Mutations of its gene, *PRF1*, cause familial hemophagocytic lymphohistiocytosis but have also been associated with lymphomas and the autoimmune/lymphoproliferative syndrome. The aim of this work was to investigate the role of *PRF1* variations in type 1 diabetes.

RESEARCH DESIGN AND METHODS—We typed for the N252S and A91V variations in an initial population of 252 type 1 diabetic patients and 816 control subjects and a second population of 365 patients and 964 control subjects. Moreover, we sequenced the coding sequence and intron-exons boundaries in 200 patients and 200 control subjects.

RESULTS—In both cohorts, allelic frequency of N252S was significantly higher in patients than in control subjects (combined cohorts: 1.5 vs. 0.4%, odds ratio 6.68 [95% CI 1.89–7.48]). Sequencing of the entire coding region detected one novel mutation in one patient, causing a P477A amino acid change not detected in 199 patients and 300 control subjects. Typing for HLA-DQA1 and DQB1 alleles showed that type 1 diabetes–predisposing DQα/DQβ heterodimers were less frequent in patients carrying N252S or P477A than in those carrying wild-type *PRF1*. We previously found that natural killer (NK) activity is not decreased in most N252S heterozygotes, but we detected one whose NK activity was normal at the age of 12 but strikingly low in early childhood. Here, we discovered that NK function was low in three heterozygotes in early childhood, one homozygous adult, and in the subject carrying P477A.

CONCLUSIONS—These data suggest that N252S and possibly other *PRF1* variations are susceptibility factors for type 1 diabetes development. *Diabetes* 57:1078–1083, 2008

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ALPS, autoimmune lymphoproliferative syndrome; HLH, hemophagocytic lymphohistiocytosis; NK, natural killer; PBMC, peripheral blood mononuclear cells.

In type 1 diabetes, autoimmune damage is mainly due to β-cell destruction by autoreactive cytotoxic T-cells by an inflammatory response organized by autoreactive TH1 cells (1,2). Ethnic variations in its incidence point to involvement of genetic and environmental factors (3,4). Susceptibility genes may include genes coding for molecules involved in immune response control and immune effector functions. Further genes may be those involved in switching off the immune response and leading to homeostatic control of the size of the peripheral lymphocyte pool and reducing the risk of autoimmunity due to cross-reactions between nonself and self antigens (3–5).

Involvement of genes participating in this switching off has been initially suggested for CTLA-4, a receptor expressed by activated T-cells that delivers negative signals upon ligation by B7.1 and B7.2. The role of these negative signals is well documented by CTLA-4–deficient mice developing severe lymphoproliferation and lymphoid infiltration of multiple organs (6). A link with type 1 diabetes has been suggested by its association with *CTLA-4* gene polymorphisms associated with decreased receptor function (7–9).

A second link with defective switching off of the immune response came from our observation that a substantial proportion of type 1 diabetic patients display defective function of Fas, a death receptor triggering apoptosis of activated lymphocytes (10). The role of Fas in the immune response is shown by the finding that inherited defects of Fas function cause the autoimmune lymphoproliferative syndrome (ALPS), a rare autoimmune disease characterized by heterogeneous autoimmune manifestations, and lymphocyte accumulation in the spleen and lymph nodes (11–14).

A third mechanism involved in downmodulation of the immune response is perforin-mediated cytotoxicity (15). Cytolytic granules of $CD8^+$ cytotoxic T-lymphocytes and natural killer (NK) cells contain perforin and granzymes and are released on the target cell upon its recognition by cytotoxic cells.

Perforin polymerizes on the target cell membrane where it forms pores that allow the entry of granzymes, which trigger apoptosis of the target cell by cleaving caspases (15). This cytotoxicity is crucial to kill virus-infected cells and clear viral infections but may also be involved in downmodulation of the immune response by fratricide of effector lymphocytes and antigen-presenting cells (16–18). Biallelic loss-of-function mutations of the perforin gene (*PRF1*) have been associated with ~30% of cases of

familial hemophagocytic lymphohistiocytosis (HLH), a rare life-threatening immune deficiency that occurs in infants and young adults (19–23). Furthermore, 25% of patients display mutations of the *MUNC 13-4* gene involved in perforin storage in the lytic granules and exocytosis (24). Intriguingly, some of these variations, themselves insufficient to cause HLH, seem to act as predisposing factors for the development of ALPS (25). This possibility was first suggested by the observation of an ALPS patient with a *Fas* gene mutation inherited from the father and with a *PRF1* mutation inherited from the mother. Since both parents and the patient's brother, who carried the *Fas* mutation only, were healthy, it appeared that both mutations contributed to the development of ALPS (25). This was confirmed in a larger group of ALPS patients, where two HLH-associated amino acid substitutions of *PRF1* were detected, i.e., N252S and A91V (26). The frequency of N252S was increased in typical forms of ALPS and increased the risk of its development by about 62-fold, whereas that of A91V was increased in an incomplete variant of ALPS and increased this risk by about threefold (26).

The aim of this work was to assess involvement of *PRF1* in type 1 diabetes by evaluating the frequency of N252S and A91V in two cohorts of patients and control subjects. Results showed that frequency of N252S, but not A91V, was increased in both cohorts of type 1 diabetic patients. Perforin variations may thus be involved in type 1 diabetes development in some patients.

RESEARCH DESIGN AND METHODS

We analyzed two independent cohorts of type 1 diabetic patients and randomly selected, ethnically matched, healthy control subjects. Patients and control subjects were enrolled from the Diabetes Centers of the Maggiore Hospital of Novara (Novara, Italy), the S. Giovanni Battista Hospital of Turin and the Regina Margherita Children Hospital of Turin (Turin, Italy), the Giannina Gaslini Children's Hospital, University of Genoa (Genoa, Italy), and IRCCS Policlinico S. Matteo (Pavia, Italy). Patients were consecutive Italian patients followed by these Diabetes centers (Novara, n = 98; Turin, n = 389; Genoa, n = 158; and Pavia, n = 76); control subjects were consecutive Italian donors obtained from the transfusion services of the respective hospitals.

The first cohort consisted of 882 patients and 816 control subjects and the second of 368 patients and 964 control subjects. Patients and control subjects were unrelated, Caucasian, and Italian. Overlaps between different sites were ruled out.

All subjects gave informed consent according to the Declaration of Helsinki (International Committee of Medical Journal Editors, 1996). The research was approved by the ethical committee of the Maggiore Hospital of Novara.

Amplification of *PRF1* and mutation detection. Genomic DNA was isolated from peripheral blood mononuclear cells (PBMCs) using standard methods. In the first cohort, exons 2 and 3 of the *PRF1* coding region were amplified in standard PCR conditions. The primers used for amplification have previously been described (19). PCR products were purified with the EXO/SAP kit (GE Healthcare, Piscataway, NJ). Sequencing was performed with the ABI PRISM® BigDye™ Terminator kit (Applied Biosystems, Foster City, CA) on an automatic sequencer (Applied Biosystems 3100 Genetic Analyzer) according to the manufacturer's instructions with the amplification primers plus two internal primers (forward 5′-CAGGTCAACATAGGCATCCACG-3′; reverse 5′-GAACAGCAGGTCGTTAATGGAG-3′) for exon 3. In the second cohort, genotyping of +272 C/T and +768 A/G single nucleotide polymorphisms was performed with the TaqMan 5′ allelic discrimination assay (Applied Biosystems).

Allelic specific primers and fluorogenic probes were used for discrimination (for +272 C/T see ref. 27; for +768 A/G Cod.4331876, Applied Biosystems). Genotyping of each sample was automatically attributed by the SDS, version 1.3, software for allelic discrimination. All mutations were then confirmed by sequencing.

Cytotoxicity assays. NK activity of PBMC was assessed by a standard 4-h ^{51}Cr release assay with K562 cells as the target. Results are expressed as specific lysis percentage, calculated as follows: (sample ^{51}Cr release − spontaneous release)/(maximal release − spontaneous release) × 100.

Flow cytometry. Analysis of lymphocyte subset in PBMCs was performed by direct immunofluorescence and flow cytometry. Perforin expression was evaluated in fixed and permeabilized cells (Cytofix-Cytoperm, BD PharMingen, San Diego, CA) using a phycoerythrin-conjugated anti-perforin antibody (BD PharMingen) and flow cytometry.

HLA-DQA1 AND DQB1 genomic typing. HLA-DQA1 and DQB1 genes were typed at a high resolution level using the reverse PCR-SSO technique, as previously described (28), in 28 patients carrying N252S or P477A, 51 random patients carrying wild-type *PRF1*, and 677 healthy children recruited from the cord blood bank of the Pavia center. The control subjects were ethnically matched with the patients and checked for absence of diabetes in their families; their ages ranged from 6 months to 4 years (mean age 2.6 years).

Statistical analysis. Statistical analysis was performed with GraphPad Instat (GraphPad Software, San Diego, CA). Allelic frequencies were compared with the χ^2 test or Fisher's exact test, as indicated. The Mann-Whitney test was used for NK activity. All *P* values are two tailed, and the significance cutoff was $P < 0.05$.

RESULTS

Search for the N252S and A91V variations of perforin. In *PRF1*, the C/T substitution in position 272 (rs35947132) of the cDNA (numerations are referred to the GenBank cDNA clone M28393, ATG = +1) and an A/G substitution in position 755 (rs28933375) cause an A91V and a N252S amino acid variation at the protein level, respectively, and have been associated with both HLH and ALPS. By sequencing the genomic DNA, we initially assessed the frequency of these substitutions in 352 type 1 diabetic patients and 816 control subjects. The N252S variation was found in nine type 1 diabetic patients and two control subjects (all heterozygotes). Its allelic frequency was significantly higher in type 1 diabetic (1.3%) than in control (0.1%) subjects ($P = 0.0006$) and conferred an odds ratio (OR) of 10.55 (95% CI 2.12–70.82) for type 1 diabetes development. The A91V variation was carried by 19 type 1 diabetic patients (18 heterozygotes and one homozygous) and 72 control subjects (69 heterozygotes and three homozygotes), and its allelic frequency was not significantly different in the two groups (type 1 diabetic vs. control subjects: 2.8 vs. 4.0%) (Table 1). The frequency of both variations in the control subjects was similar to that reported in other studies, and their genotypic distributions did not deviate significantly from Hardy-Weinberg equilibrium in any group.

These data indicate that N252S, but not A91V, may be a predisposing factor for type 1 diabetes development. To confirm this observation, we evaluated both variations in a second cohort of type 1 diabetic patients (*n* = 365) and control subjects (*n* = 964) using the TaqMan 5′ allelic discrimination assay. N252S was found in 13 type 1 diabetic patients and 13 control subjects (all heterozygotes). Its allelic frequency was higher in type 1 diabetic patients (1.8%) than in the control subjects (0.7%) ($P = 0.0179$) and conferred an OR of 2.67 (95% CI 1.16–6.15) for type 1 diabetes development. A91V was carried by 36 type 1 diabetic patients (35 heterozygotes and one homozygotes) and 118 control subjects (115 heterozygotes and three homozygotes), and its allelic frequency was not significantly different in the two groups (type 1 diabetic vs. control subjects 5 vs. 6.3%, respectively) (Table 1). The genotypic distributions of these variations did not deviate significantly from Hardy-Weinberg equilibrium in either group. No difference was found between the N252S carriers and the other patients with regard to sex distribution, age at diagnosis of type 1 diabetes, or presence of a second concomitant autoimmune disease (Table 2).

TABLE 1
Frequency distribution of the N252S and A91V variations of *PRF1* in two independent cohorts of type 1 diabetic patients and healthy control subjects

	Population 1		Population 2		Total population	
	Patients*	Control subjects	Patients*	Control subjects	Patients*	Control subjects
A91V						
Alleles†						
A	684 (97.2)	1,557 (95.4)	693 (95)	1,907 (93.7)	1,377 (96)	3,264 (94.5)
V	20 (2.8)	75 (4.6)	37 (5)	121 (6.3)	57 (4)	196 (5.5)
Statistic‡	N.S.		N.S.		N.S.	
Genotypes§						
AA	333 (94.6)	744 (91.2)	329 (90.1)	846 (87.7)	662 (92.3)	1,590 (89.3)
AV	18 (5.1)	69 (8.5)	35 (9.6)	115 (11.9)	53 (7.4)	184 (10.3)
VV	1 (0.3)	3 (0.3)	1 (0.3)	3 (0.3)	2 (0.3)	6 (0.4)
N252S						
Alleles†						
N	695 (98.7)	1,630 (99.9)	717 (98.2)	1,915 (99.3)	1,412 (98.5)	3,545 (99.6)
S	9 (1.3)	2 (0.1)	13 (1.8)	13 (0.7)	22 (1.5)	15 (0.4)
Statistic‡	10.55 (2.13–70.82)		2.67 (1.16–6.15)		3.68 (1.83–7.48)	
Genotypes§						
NN	343 (97.4)	814 (99.8)	352 (96.4)	951 (98.7)	695 (96.9)	1,765 (99.2)
NS	9 (2.6)	2 (0.2)	13 (3.6)	13 (1.3)	22 (3.1)	15 (0.8)
SS	0	0	0	0	0	0

Data are n (%) or OR (95% CI). *Type 1 diabetic patients. Data shown are number of †chromosomes or §subjects. Genotypic distribution did not deviate significantly from Hardy-Weinberg equilibrium in any group (data not shown). ‡χ^2 test calculated on allelic frequencies; *P* values are two tailed: population 1, $P = 0.0036$; population 2, $P = 0.0175$; population 3, $P = 0.0007$.

Analysis of the *PRF1* gene. To assess whether type 1 diabetic patients carry other variations of *PRF1*, the entire coding sequence plus intron-exon boundaries were sequenced in 200 type 1 diabetic patients and 300 control subjects. Besides A91V and N252S, four other known nucleotide variations were detected but were not further evaluated because they did not change the amino acid or influence the splicing sites. Two, C822T (rs885821) and T900C (rs885822), had been previously reported as common polymorphisms not associated with HLH. Their frequency was similar in the patients and the control subjects (C822T, 15 vs. 12%; T900C, 43 vs. 40%). The other two (G435A and A462G) are known to be in perfect linkage disequilibrium with N252S and were in fact only detected in all subjects carrying this variation (25). Moreover, we detected a novel variation in one type 1 diabetic patient, a C/G substitution in position 1,429 (C1429G) causing a P477A amino acid change at the protein level. This variation was not found in any other subject.

Analysis of NK function and HLA-DQ typing. We had previously found, like other workers, that NK activity is not decreased in subjects carrying N252S. However, we also described an ALPS patient heterozygous for N252S whose NK activity was normal at age 12 years but had been almost undetectable at age 3 years and extremely low at the age 5 years (26). We suggested that, in this patient, N252S was associated with factors decreasing NK function in early childhood, followed by normalization on the part of unknown compensatory mechanisms. Because in this study we detected three N252S heterozygous subjects the early childhood, we assessed whether their N252S was associated with defective NK function by evaluating NK cell count, NK activity, and perforin expression in their PBMCs (Table 3). Two (patients 1 and 2) had type 1 diabetes, whereas the third was the healthy sister of patient 1.

Moreover, we performed the same analyses in patient 1's healthy father, who was homozygous for N252S, in his wild-type mother, and in the type 1 diabetic patient heterozygous for the novel P477A mutation (patient 3). Results showed that NK activity was defective in patient 1, his sister, and father, whereas it was low but in the normal range in the mother not carrying the mutation; all these subjects displayed normal perforin expression. In patient 2, NK activity was borderline and perforin expression was decreased. In patient 3 (P477A), both NK activity and perforin expression were defective. NK cell counts were normal in all subjects. Specificity of the NK function defect was assessed by evaluating NK function in 10 children in early childhood (age <5 years) and lacking *PRF1* variations; 5 were healthy, whereas 5 had type 1 diabetes. Results showed that NK activity of these children was similar to that displayed by our random control subjects (reported in Table 3) and significantly higher ($P < 0.01$)

TABLE 2
Demographic and clinical parameters of type 1 diabetic patients with and without N252S

					Second autoimmunity		
PRF1 N252S	n*	Male/female†	Age (years)‡	Age at diabetes diagnosis (years)	Thyroid	Celiac disease	Multiple sclerosis
N	606	374/821	27 (17–36)	13.5 (8–21)	7	7	1
S	22	15/7	26 (20–36)	13 (6–21)	2	0	0

Data are n or median (interquartile range). *Subjects. †Male/female ratio of control subjects was 1,068/712. ‡Median age of control subjects was 32 years (27–36).

TABLE 3
NK activity, perforin expression, and proportion of NK cells in PBMC of type 1 diabetic patients carrying the N252S and P477A perforin variations

PRF1 variation	Subject	Age (years)	NK activity (effector-to-target ratio)* 100:1	30:1	10:1	Perforin expression† Percent	MFI-R	Peripheral blood NK cells (%) $CD3^-$ $CD16^+$	$CD3^-$ $CD56^+$
N252S‡	Patient 1	2	7§	4§	2	13	11	6	5
N252S‡	Patient 1's sister	5	14§	7§	2	15	9	6	4
N252S‖	Patient 1's father	30	8§	4§	2	23	7	7	9
	Patient 1's mother	28	32	10	2	32	8	21	20
N252S‡	Patient 2	4	25	10	3	17	3.5§	4	5
P477A‡	Patient 3	3	6§	0§	2	9	2§	4§	5
	Control subjects¶	—	40 (15–66)	26 (8–50)	14 (2–31)	23 (17–26)	9 (5–20)	11 (5–31)	17 (4–27)

Data are *n* or median (5th–95th percentile) unless otherwise indicated. *NK activity is expressed as specific cell lysis percent, and it is the mean of triplicate assays, whose SD was always <10% of the mean. Spontaneous cell lysis was always <10% of maximal cell lysis. †Perforin expression is shown as proportion of positive cells (%) and mean fluorescence intensity ratio (MFI-R). ‡Heterozygous. §Represents <5th percentile of control subjects. ‖Homozygous. ¶*n* = 13 controls.

than that displayed by patients 1, 2, and 3 and by patient 1's sister (Fig. 1).

Finally, we evaluated the frequencies of DQαβ diabetogenic heterodimers in type 1 diabetic patients carrying or not carrying the N252S or P477A variations and in 677 healthy control subjects. In this analysis, susceptible heterodimers were those comprising a DQα chain with an arginine at position 52 and a DQβ chain with a nonaspartic acid at position 57; a subject can have one, two, or four susceptible heterodimers, the higher the number, the higher the risk for type 1 diabetes (29). Table 4 shows that the distribution of subjects carrying zero, one, two, or four susceptible heterodimers was significantly different in the two patient groups (overall $P = 0.028$). The proportion of carriers of four predisposing heterodimers was significantly lower in the N252S/P477A group than in the wild-type group (34.8 vs. 65.4%, $P = 0.015$), but it was higher in both patient groups than in the control subjects (1.0%, $P < 0.0001$ vs. both patient groups).

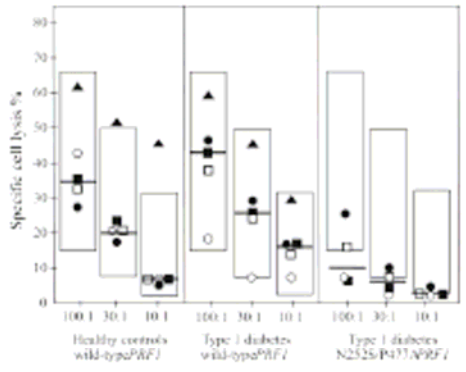

FIG. 1. NK activity in PBMC of type 1 diabetic patients carrying the N252S and P477A perforin variations and in age-matched subjects carrying wild-type *PRF1* with or without type 1 diabetes. All subjects were aged <5 years. NK activity was assessed at the 100:1, 30:1, and 10:1 effector-to-target ratios; each subject is marked with the same symbol in the different effector-to-target ratios. Gray boxes indicate the 5th–95th percentile interval of NK activity assessed in normal random control subjects (those of Table 3). Horizontal lines indicate the medians. Statistical analysis was performed with the Mann-Whitney test (type 1 diabetic patients: N252S/P477A vs. wild-type, $P = 0.0008$; N252S/P477A vs. control subjects, $P = 0.0009$).

DISCUSSION

This paper stems from our finding that variations of *PRF1* may be a predisposing factor for development of ALPS, a rare inherited autoimmune disease (26). It shows that *PRF1* variations may also predispose to development of type 1 diabetes.

Of the two *PRF1* variations associated with ALPS, i.e., N252S and A91V, only N252S was associated with type 1 diabetes, since its frequency was significantly higher in two independent groups of type 1 diabetic patients than in the respective control subjects. The OR calculated in the combined cohorts (717 patients and 1,780 control subjects) was 3.68 (95% CI 1.83–7.48; $P = 0.00007$). *PRF1* is located on chromosome 10q22, far from the known type 1 diabetes susceptibility loci located on this chromosome, i.e., IDDM10 (10p11-q11) and IDDM17 (10q25). However, other genes involved in cell death (*PPIF*) or cell-mediated cytotoxicity (*PRG1*) are located nearby *PRF1*, and we cannot rule out linkage disequilibrium between the *PRF1*

TABLE 4
Distribution of subjects with different numbers of type 1 diabetes–predisposing HLA-DQαβ heterodimers in type 1 diabetic patients carrying or not carrying the N252S or P477A variations and in control subjects

Predisposing HLA-DQαβ heterodimers	N252S/P477A *PRF1*	Wild-type *PRF1*	Control subjects*
n	23	81	677
0	5 (21.7)	7 (8.6)	356 (52.6%)
1	1 (4.3)	6 (7.4)	185 (27.3%)
2	9 (39.1)	15 (18.5)	125 (18.5%)
4	8 (34.8)	53 (65.4)	11 (1.0%)
Statistics†			
Overall *P*	0.028‡		<0.0001§
4-carriers	0.015‡		<0.0001§

Susceptible heterodimers are HLA-DQα52Arg and DQβ57nonAsp. *Healthy babies recruited from the cord blood bank of the transfusion center. †Fisher's exact test calculated on overall frequencies or comparing the frequency of subjects carrying 4 predisposing heterodimers (4-carriers). ‡N252S/P477A mutated patients vs. nonmutated (wild-type) patients. §Control subjects vs. diabetic patients (the same significance was obtained with each patient group).

variations and those that may play a role in the effector phase of β-cell destruction.

Several works have shown that A91V decreases perforin function by altering its conformation, decreasing its cleavage to the active form, and increasing its degradation (30–33). By contrast, the functional significance of N252S has been debated because it occurs within the membrane-attack complex, a region critically involved in the pore-forming activity of perforin, but several works have associated it with normal NK function and perforin expression (19,31–33). However, these studies were performed on cells from N252S heterozygotes or artificial systems where mutated forms of perforin were transfected in reporter cell lines to assess their expression. We have recently described an ALPS patient heterozygous for N252S who displayed a striking deficiency of NK activity when he was aged 3–5 years followed by normalization when he was aged 12 years (26). Therefore, we suggested that N252S heterozygosis per se or other factors associated with it may decrease NK function in early childhood.

Our present findings substantiate this possibility, since it describes three new children heterozygous for N252S with low NK function in their early childhood. A follow-up will show whether compensatory mechanisms eventually normalize their NK function. A second point is that we also detected low NK function in an adult homozygous for N252S. In the presence of homozygosity, therefore, these mechanisms may not be sufficient. However, N252S homozygosity is not sufficient to induce development of type 1 diabetes, ALPS, or HLH. It is noteworthy that variations altering NK function in early childhood may be particularly significant for type 1 diabetes since it is the outcome of a transient autoimmune aggression that destroys β-cells, generally in childhood.

Intriguingly, one type 1 diabetic patient displayed a novel *PFR1* mutation causing the P477A substitution. This mutation was only found in this subject and has never been detected in HLH patients. Its location within the carboxy-terminal C2 domain of perforin suggests that it may have functional significance, since this domain plays a key role in Ca^{2+}-dependent binding of perforin to membranes, the first step of perforin-mediated lytic activity (34). In line with this possibility, our analysis of PBMC from this patient detected defective NK activity and normal perforin expression. In addition to the recurrent N252S mutation, therefore, other sporadic perforin mutations might favor type 1 diabetes development, and the global predisposing effect of perforin variation in type 1 diabetes development may be higher than that calculated for N252S.

Nevertheless, perforin variations are a rare predisposing factor: N252S was carried by 1.5% patients and P477A by 0.5%. N252S-mediated predisposition seems to require concurrence of HLA-predisposing alleles, since about 80% of patients carrying N252S also carried HLA-DQ heterodimers involved in type 1 diabetes susceptibility. However, susceptibility dependent on HLA-DQ seems to be lower in patients carrying the *PRF1* variations than in those carrying wild-type *PRF1*, which suggests that *PRF1* contributes to the onset of type 1 diabetes additionally and independently from HLA. *PRF1* variations may thus be a predisposing factor for type 1 diabetes. Perforin-mediated cytotoxicity is the main effector system in clearance of virus-infected cells but may also be involved in downregulation of the immune response due to its involvement in fratricide of effector lymphocytes and antigen-presenting cells (15–18,35–39). Defects of both of these functions may predispose to autoimmunity by prolonging the immune response and increasing the risk of cross-reactions between viral and self antigens by molecular mimicry.

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CORSI FREQUENTATI:

I° anno:

Corso di lingua inglese tenuto dal Prof. Irving Bell.

SEMINARI INTERNI AL DIPARTIMENTO DI SCIENZE MEDICHE

Anno Accademico 2005/06

18 Novembre 2005 Dr.Diego Cotella "Cardiac Potassium channel regulation by accessori subunits"

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13 Marzo 2006 Dr.Antonia Follenzi "Il trapianto di cellule endoteliali (les) nel fegato di topo ha implicazioni per la terapia cellulare e genica dell'emofilia"

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6 Aprile 2006 Dott. Francesco Forconi Aspetti immunogenetici e terapeutici della "hairy cell leukemia"

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CONGRESSI FREQUENTATI:

I° anno: XXVIII Congresso Nazionale della Società italiana di Patologia, Università degli studi di Pavia, 19-22 Settembre, 2006.

II° anno: XIV Telethon Convention, Palazzo dei Congressi, Salsomaggiore Terme, 12-14 Marzo, 2007.

III° anno: EUROPEAN BIOLPINE CONVENTION. Inflammation and autoimmunity:bridging pubblic and private research. Collereto Giacosa (TO)**,** 6-7 Novembre, 2007.

COMUNICAZIONE A CONGRESSI:

I° anno:

"Variazioni nel gene di perforina in pazienti con sclerosi multipla" Orilieri E., Cappellano G., Comi C., Chiocchetti A., Cerutti E., Castelli L., Monaco F., Dianzani U. (XXVIII Congresso Nazionale della Società italiana di Patologia, Università degli studi di Pavia, 19-22 Settembre, 2006). (PRESENTAZIONE POSTER).

"Variazioni nel gene di perforina nei pazienti con autoimmunità/linfoproliferazione e difettiva funzionalità di Fas" Cerutti E., Cappellano G., Chiocchetti A., Clementi R., Ferretti M., Orilieri E, Dianzani I., Ferrarini M., Bregni M., Danesino C, Bozzi V., Putti M.C., Cometa A., Locatelli F., Maccario R., Ramenghi U., Dianzani U. (XXVIII Congresso Nazionale della Società italiana di Patologia, Università degli studi di Pavia, 19-22 Settembre, 2006). (POSTER).

"Variations of the perforin gene in patients with autoimmunity/lymphoproliferation and defective Fas function" Cappellano G., Chiocchetti A., Cerutti E., Ferretti M., Orilieri E., Dianzani I., Ramenghi U., Dianzani U. (16thEuropean Congress of Immunology; PARIS, 6-9-September, 2006). (POSTER).

II° anno:

"Search for genetic alterations of the Fas system in the autoimmune/lymphoproliferative syndrome (ALPS)". Dianzani U., Chiocchetti A., Cappellano

G., Orilieri E., Cerutti E., Ferretti M., Clementi R., Notarangelo L., Ramenghi U. (XIV Telethon Convention, Palazzo dei Congressi, Salsomaggiore Terme, 12-14 Marzo, 2007). (PRESENTAZIONE POSTER).

III° anno:

EUROPEAN BIOLPINE CONVENTION. INFLAMMATION AND AUTOIMMUNITY:BRIDGING PUBBLIC AND PRIVATE RESEARCH. COLLERETTO GIOCOSA (TO) 6-7 NOVEMBRE 2007.

"Variations of the perforin gene in patients with multiple sclerosis" Giuseppe Cappellano, Cristoforo Comi, Elisabetta Orilieri, Annalisa Chiocchetti, Sara Bocca, Elena Boggio, Ilaria Seren Bernardone, Sandra Dalfonso, Mara Giordano, Franco Perla, Maurizio Leone, Francesco Monaco, and Umberto Dianzani. (PRESENTAZIONE POSTER)

"Variations of the perforin gene in patients with type 1 diabetes" Elisabetta Orilieri, Giuseppe Cappellano, Massimo Ferretti, Elisa Cerutti, Francesco Cadario, Franco Cerutti, Graziella Bruno, Annalisa Chiocchetti, and Umberto Dianzani. (PRESENTAZIONE POSTER).

VI SIICA NATIONAL CONFERENCE, UNIVERSITÀ LA SAPIENZA, ROMA, 1114 GIUGNO 2008.

"Variations of the perforin gene in patients with autoimmune diseases". Elisabetta Orilieri, Giuseppe Cappellano, Annalisa Chiocchetti and Umberto Dianzani (PRESENTAZIONE POSTER).

"The 423qpolymorphism of the xiap gene influences macrophage function and is associated with idiopatic periodic fever". A. Chiocchetti , M. Gattorno, M. Ferretti, E.Orilieri, G.Cappellano, A. Martini, U. Dianzani (PRESENTAZIONE POSTER).

PUBBLICAZIONI:

Chiocchetti A, Miglio G, Mesturini R, Varsaldi F, Mocellin M, Orilieri E, Dianzani C, Fantozzi R, Dianzani U, Lombardi G. **Group I mGlu receptor stimulation inhibits activation-induced cell death of human T lymphocytes**. *Br J Pharmacol.* 148(6): 760-8, 2006.

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