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Autoimmune diseases linked to abnormal K^+ channel expression in double-negative $CD4^-CD8^-$ T cells*

Using the patch-clamp technique in combination with fluorescence microscopy we have found an abnormality in voltage-gated K^+ channel expression in T cells that represents the first molecular marker linking three disparate autoimmune diseases in mice. $CD4^-CD8^-$Thy-1.2$^+$ (double-negative or DN) lymphocytes from every known murine model for systemic lupus erythematosus, type-1 diabetes mellitus and experimental allergic encephalomyelitis exhibit abnormally high numbers of an unusual K^+ channel, termed type *l* compared to their phenotypic counterparts in normal mice. Other T cell subsets from these diseased mice retain their normal pattern of K^+ channel expression. The unique K^+ channel phenotype of DN Tcells arises in parallel with the onset of autoimmunity. Although mitogen-activated T cells and rapidly proliferating thymocytes exhibit large numbers of K^+ channels, these channels are of an electrophysiologically distinct type called *n*. Thus, abundant expression of type *l* K^+ channels appears to be a useful marker for DN Tcells associated with autoimmunity and may provide a valuable tool for delineating the role of DN T cells in the pathogenesis of autoimmune diseases.

1 Introduction

Mouse T cells exhibit three types of voltage-gated K^+ channels, termed *n*, *n'* and *l*, which are distinguished on the basis of voltage dependence, kinetics, pharmacology, and single channel conductance [1–4]. $CD4^+CD8^-$ (helper/inducer) T cells display 20–100 type *n* K^+ channels, whereas $CD4^-CD8^+$ (cytotoxic/suppressor) T cells express 20–200 types *l* or *n'* K^+ channels [1, 2]. Mitogen-activated T cells [5, 6] and rapidly proliferating thymocytes [1], possess about 20 times more K^+ channels than quiescent cells, which are exclusively type *n*. Thus, the pattern of K^+ channel expression reflects the T cell phenotype and the activation status of the cells. In this report we have taken advantage of the existence of murine models for SLE, type-1 diabetes mellitus and EAE to examine K^+ channel expression in T cells in autoimmune diseases. We have identified an alteration in K^+ channel expression which appears to be unique to double-negative $CD4^-CD8^-$Thy-1.2$^+$ (DN) T cells associated with autoimmune diseases.

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Abbreviations: **DN:** Double-negative $CD4^-CD8^-$Thy-1.2$^+$ (T cell) **lpr:** Lymphoproliferation **gld:** Generalized lymphoproliferative disease **NOD:** Non-obese diabetic **TEA:** Tetraethylammonium

2 Materials and methods

2.1 Mice

Mice were housed in the vivarium at U.C. Irvine, CA. Mice with autoimmune diseases were kindly provided by S. K. Datta (NZB × SWR)F_1, T. Santoro (BXSB-*Yaa*, MRL-+/+), D. McCurdy, D. Wofsy, F. Ebling and B. Hahn (NZB × NZW)F_1, M. Berman (BALB/c), B. Formby and G. Gutman (NOD), D. Kono (PLJ mice with acute EAE) and L. Steinman (SJL/PLJ mice with chronic EAE). The mice varied in age from 4–20 months: C57BL (6 and 19 months); BALB/c (1 and 7 months); BXSB-*Yaa* (2 mice aged 4 months); (NZB × NZW) (2 mice aged 6 months and one aged 9 months); (NZB × SWR) (three mice aged 7–8 months); diseased MRL-+/+ (two mice aged > 20 months); NOD mice (6 and 9 months); young MRL +/+ (6, 10, and 12 months); chronic EAE (two mice aged 16 months); acute EAE (two mice aged 4 months).

2.2 Clinical features

(NZB × SWR)F_1 and old MRL-+/+ mice had significant proteinuria (> 300 mg protein/100 ml); BXSB-*Yaa* mice had palpable cervical lymphadenopathy; (NZB × NZW)F_1 mice had terminal disease characterized by alopecia, rapid breathing, a hunched-up position and inability to move around the cage; NOD mice were polyuric and polydypsic, and had the clinical features seen in (NZB × NZW)F_1 mice; chronic EAE mice were killed at 16 months of age, 12 months after developing a chronic relapsing disease resulting in hind limb paralysis following injection with 400 mg myelin basic protein; acute EAE mice were killed when they developed hind limb paralysis, 2 weeks after injection of myelin basic protein; young MRL-+/+ had no gross evidence of clinical disease. The mice were killed by cervical dislocation, and single-cell suspensions prepared from the spleen and LN. T cells were enriched by passage through a nylon wool column.

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2.3 Staining

Cells were first stained with rat anti-mouse CD4 and rat anti-mouse CD8 antibodies, then with PE-conjugated goat anti-rat IgG, and finally with fluoresceinated rat anti-mouse Thy-1.2. DN T cells ($CD4^-CD8^-Thy\text{-}1.2^+$) were identified as green cells, other T cell subsets were yellow, and B cells and monocytes were unstained. To investigate K^+ channel expression in helper and cytotoxic T cells from diseased mice, we stained cells with PE-conjugated anti-CD4 and FITC-conjugated anti-CD8. Helper cells ($CD4^+CD8^-$) appeared orange and cytotoxic cells ($CD4^-CD8^+$) were green. Antibodies were purchased from Becton Dickinson, Mountain View, CA (rat anti-mouse CD4, rat anti-mouse CD8, fluoresceinated rat anti-Thy-1.2, PE-conjugated anti-CD4, and FITC-anti-CD8) and Caltag, San Francisco, CA (affinity-purified, mouse absorbed PE-conjugated goat anti-rat IgG). These staining protocols do not affect channel expression [1, 2]. In most experiments, chambers were coated with polylysine (0.5 mg/ml) to improve cell adherence to the dish. This procedure did not alter channel expression when compared with cells plated into uncoated chambers.

2.4 Electrophysiology

2.4.1 General remarks

After phenotypic identification by epifluoresence microscopy, single T cells were patch-clamped at room temperature (22° to 26 °C). Details of the giga-ohm voltage-clamp technique used here are described elsewhere [1–7]. The cells under investigation were bathed in Ringer solution (in mM): 160 NaCl, 4.5 KCl, 2 $MgCl_2$, 1 $CaCl_2$, and 5 Na-Hepes (pH 7.4). The patch pipette contained 134 KF, 11 K_2-EGTA, 1.1 $CaCl_2$, 2 $MgCl_2$ and 10 K-Hepes (pH 7.2). In Ringer solutions containing tetraethylammonium (TEA^+) chloride, NaCl was replaced by appropriate TEA^+ concentrations keeping the osmolarity constant. The bath solution could be changed during recordings by bath perfusion.

2.4.2 Identification of K^+ channel type

We identified K^+ channels on the basis of their inactivation properties, channel closing kinetics and sensitivity to block by TEA^+ as described previously [1–5]. Typically, type *n* K^+ channels are use dependent, close slowly upon repolarization with a time constant of about 30 ms at − 60 mV and are blocked by TEA^+ (K_D = 8 mM). Type *l* K^+ channels are not use dependent, close more rapidly on repolarization with a time constant of 2 ms at − 60 mV, and are much more sensitive to block by TEA^+ (K_D = 0.1 mM). Type *n'* K^+ channels are not use dependent, close slowly as do type *n* K^+ channels upon repolarization, but are less sensitive to block by TEA^+ ($K_D \sim 100$ mM).

2.4.3 Determination of maximal K^+ conductance (g_K) and number of K^+ channels per cell

The g_K was calculated from the largest K^+ current recorded in each cell. We used a reversal potential of − 80 mV to calculate g_K [2]. The number of K^+ channels per cell was calculated by dividing g_K by the single-channel conductances of the appropriate channel type; the single-channel conductances are 18, 27, and 17 pS for *n*, *l* and *n'*, respectively [1, 2].

3 Results

3.1 Murine SLE is associated with altered K^+ channel in $CD4^-CD8^-Thy\text{-}1.2^+$ T cells, irrespective of the genetic background of the mouse

K^+ channel expression was investigated in mAb-defined T cell subsets from genetically distinct murine models for SLE with varied clinical features and immunological abnormalities [8–25]. Fig. 1a shows K^+ outward currents in splenic and LN DN T cells from normal mice and from mice with SLE. In these experiments, the membrane potential of the cells was held at − 80 mV, and then depolarizing pulses were applied to + 40 mV. This protocol opens all the voltage-gated K^+ channels in the cell. The normal mouse (BALB/c) DN T cell shows small voltage-gated K^+ currents which are half-blocked by 10 mM TEA^+, indicating that it expresses low numbers of type *n* K^+ channels [1–5]. The DN T cell from the mouse with SLE $(NZB \times NZW)F_1$ displays large K^+ currents which are half-blocked by 0.1 mM TEA^+, indicating that this cell possesses many type *l* K^+ channels [1–5].

Fig. 1b demonstrates the maximum K^+ conductance, g_K, of splenic DN T cells from normal mice and mice with SLE. DN T cells of three normal strains of mice (C3H, C57BL, BALB/c) express small numbers (10–20 K^+ channels/cell) of types *n*, *l*, or *n'* K^+ channels. In marked contrast, 68 of 76 phenotypically similar cells from 7 murine models for SLE [$(NZB \times NZW)F_1$ $(NZB \times SWR)F_1$, BXSB-Yaa, MRL-+/+, MRL-*lpr*, C3H-*lpr*, C3H-*gld*] expressed exclusively type *l* K^+ channels, averaging 180/cell. These DN T cells are most likely T cells since we previously demonstrated that cells displaying an abundance of type *l* K^+ channels in *lpr/gld* mice expressed the TcR α/β ($F23.1^+$) [2]. Occasionally we observed cells with small numbers of type *n* K^+ channels; these may be normal DN T cells. Helper ($CD4^+CD8^-$) and cytotoxic ($CD4^-CD8^+$) T cells from mice with SLE displayed a small number of K^+ channels like phenotypic counterparts from normal mice (data not shown). Thus, SLE is associated with the augmented expression of type *l* K^+ channels in DN T cells, regardless of the genetic background of the mouse or the clinical and immunological features that may be unique to a particular strain.

3.2 Altered K^+ channel expression in DN T cells parallels the development of disease

Fig. 2 demonstrates that DN T cells from young MRL-+/+ mice, prior to the onset of autoimmunity, displayed about 10 K^+ channels/cell which were types *n*, *l*, or *n'*, a pattern similar to that of T cells from normal mice. Older MRL-+/+ mice with lupus nephritis expressed ~ 200 type *l* K^+ channels/cell. Similar results were obtained in DN T cells from young and old *lpr* mice (Fig. 2 and [2]). These data

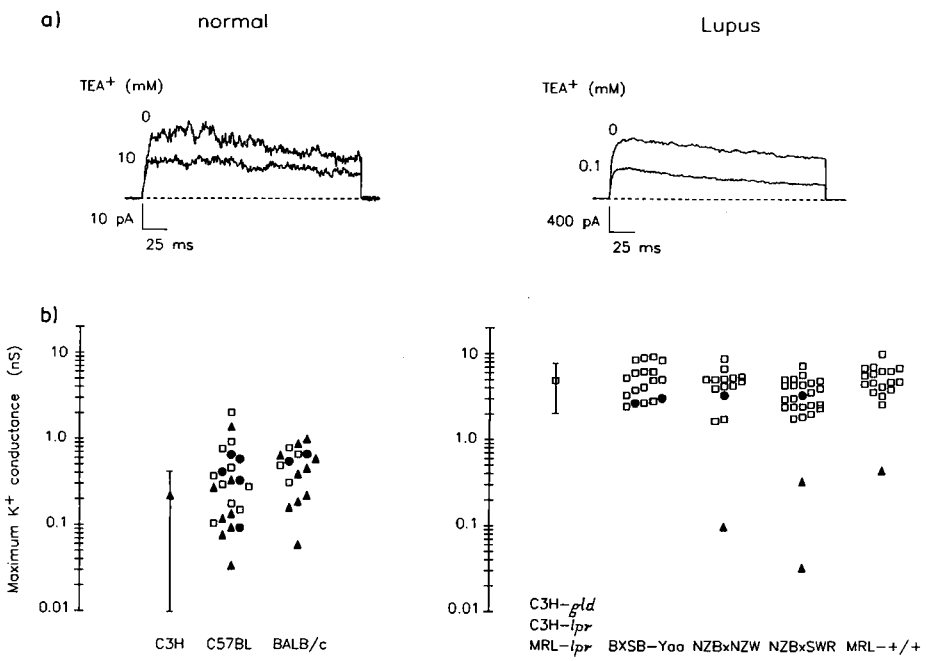

Figure 1. K+ channel expression in splenic and LN-derived DN Tcells in normal mice and mice with SLE. (a) Representative whole-cell K+ currents in DN Tcells in normal mice (*left*, BALB/c) and mice with SLE [*right*, (NZB × NZW)F_1]. Note the different current scales. K+ currents were elicited by 200 ms depolarizing pulses to 40 mV (holding potential − 80 mV). Two current traces superimposed before and during application of a Ringer solution containing 10 mM (left) or 0.1 mM (right) TEA^+, respectively. (b) Maximum K+ conductances (g_K). ▲, □, ●, represent *n, l, n'* K+ channel types. g_K was determined from the maximum peak K+ current elicited at + 40 mV, assuming a reversal potential for K+ of − 80 mV. Each point represents g_K in one single cell. Data from C3H ($n = 12$), C3H-*lpr* ($n = 22$), C3H-*gld* ($n = 26$), and MRL-*lpr* ($n = 31$) are taken from [2, 3], respectively, and are included in the graph for comparison. These data are shown as mean ± SD. Dividing g_K by the single channel conductance [1] gives an estimate of the numbers of channels/cell. Average g_K (mean ± SEM) of DN T cells: normal mice, 405 ± 53 pS, $n = 51$; SLE mice, 4550 ± 254 pS, $n = 76$; *lpr/gld* mice, 4935 ± 325 pS. $n = 79$.

suggest that elevation of type *l* K+ channel expression in DN Tcells parallels the development of SLE. Interestingly, DN Tcells from diseased SLE mice induce B cells to secrete cationic anti-DNA autoantibodies *in vitro* [8–10], whereas phenotypically similar cells from lupus-prone mice, prior to the onset of disease, do not exhibit this property [8, 9]. The development of lupus nephritis appears to be dependent on the deposition of the cationic IgG anti-double-stranded autoantibodies on the negatively charged glomerular base-

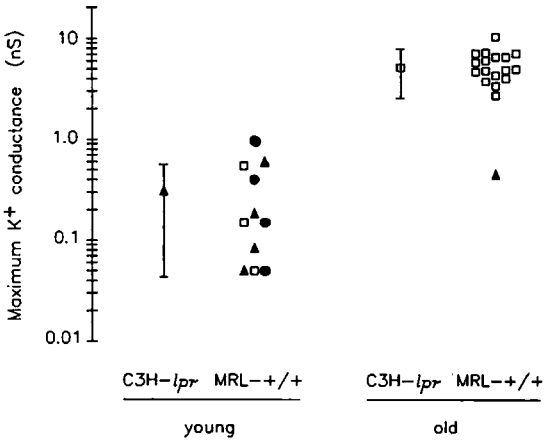

Figure 2. K+ channel expression in DN T cells in young and old SLE mice. g_K of DN T cells from three young MRL-+/+ mice, before the onset of disease and two old MRL-+/+ mice with lupus nephritis. MRL-+/+ mice: each data point represents g_K in one single cell; *lpr* mice: mean ± SD of published data included for comparison [5, 6]. ▲, □, ●, represent *n, l, n'* K+ channel types. Average g_K (mean ± SEM) of DN T cells: young MRL-+/+ mice, 295 ± 91 pS, $n = 11$; old MRL-+/+ mice, 5306 ± 518 pS, $n = 18$; young *lpr* mice, 324 ± 67 pS, $n = 17$; old *lpr* mice, 5223 ± 565 pS, $n = 22$.

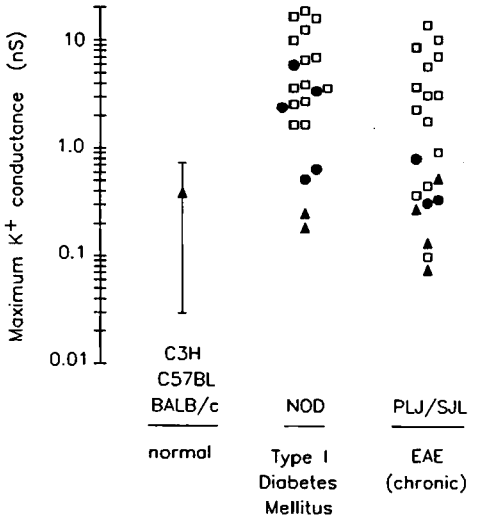

Figure 3. K+ channel expression in DN Tcells in normal mice, and in mice with type-1 diabetes mellitus (NOD) and chronic EAE. NOD and EAE: each data point represents g_K in one single cell; normal mice: mean ± SD of cells shown in Fig. 1. ▲, □, ●, represent *n, l, n'* K+ channel types. Average g_K (mean ± SEM) of DN T cells: NOD mice, 5795 ± 1253 pS, $n = 21$; chronic EAE ($g_K > 1000$ pS), 6083 ± 1277 pS, $n = 10$; chronic EAE ($g_K < 1000$ pS), 396 ± 84 pS, $n = 11$.

ment membrane [21–25]. An intriguing possibility is that the expression of numerous type *l* K^+ channels by DN T cells reflects their aberrant helper activity.

3.3 DN T cells from diseased non-obese diabetic (NOD) mice possess numerous type *l* K^+ channels

The only other known model for spontaneously developing autoimmune disease is the non-obese diabetic mouse that progressively becomes diabetic with age, apparently due to T cell-mediated destruction of pancreatic islet cells [26, 27]. The clinical features of diabetes in these mice resemble human type-1 diabetes mellitus [26, 27]. To determine whether altered ion channel expression in DN T cells links SLE and other autoimmune diseases, we examined splenic DN T cells from NOD mice which had clinical evidence of diabetes. Interestingly, the majority of DN T cells from diabetic NOD mice exhibit large numbers of type *l* K^+ channels, averaging ~ 200 channels/cell (Fig. 3). A few cells (4/21) displayed small numbers of K^+ channels and may represent normal cells. Hence, elevated numbers of type *l* K^+ channels in DN T cells are a characteristic of both type-1 diabetes mellitus and SLE.

3.4 Chronic EAE is associated with abundant type *l* K^+ expression in DN T cells

We have extended our observation on genetically determined models for autoimmune disease to an acquired autoimmune disorder. Inoculation of SJL/PLJ mice with myelin basic protein induces an immune response, leading to demyelination and acute EAE [28, 29]. Some mice progress into a chronic relapsing form of encephalomyelitis (chronic EAE) which resembles multiple sclerosis in humans [28, 29]. DN T cells from mice with acute EAE had normal K^+ channel expression (data not shown). In contrast, 10 of the 21 splenic cells examined from mice with chronic EAE had an average of ~ 200 type *l* K^+ channels/cell, similar to DN T cells from mice with SLE and type-1 diabetes mellitus (Fig. 3). The remaining cells had few K^+ channels and may represent normal cells.

4 Discussion

K^+ channel expression was investigated in mAb-defined T cell subsets from mice with autoimmune diseases and control mice using the patch-clamp technique. We demonstrate that DN T cells from every murine model for SLE, type-1 diabetes, and chronic EAE display an abundance of type *l* K^+ channels (Fig. 2, [2]). Phenotypically similar cells from normal strains of mice (Figs. 2 and 3), or from mice immunized with heat-killed *E. coli* or injected with CFA (data not shown), do not express this high level of type *l* K^+ channels. The pattern of K^+ channel expression in DN T cells associated with autoimmunity is also dramatically different from that in normal mitogen-activated T cells [5, 6] and rapidly proliferating thymocytes [1, 6], which exhibit large numbers of type *n* K^+ channels. Furthermore, the $CD4^+CD8^-$ (helper/inducer) and $CD4^-CD8^+$ (cytotoxic/suppressor) cells from diseased mice display the same type and roughly the same number of K^+ channels as their counterparts in normal mice. Thus, elevated expression of type *l* K^+ channels appears to be a unique feature of DN T cells cells associated with symptoms of autoimmune diseases.

Several lines of evidence indicate that DN T cells contribute to the pathogenesis of autoimmunity. Increased numbers of DN T cells are found both in humans and mice with SLE [8–10]. These DN T cells induce B cells from lupus-prone mice to secrete pathogenic cationic anti-DNA autoantibodies *in vitro* [8–10]. Deposition of the cationic IgG anti-double-stranded autoantibodies of the negatively charged glomerular basement membrane results in inflammation and lupus nephritis [21–25]. Severe lymphoproliferation of TcR α/β expressing DN T cells in mice with the *lpr* or *gld* mutations is associated with the accelerated onset of SLE [11–18]. These proliferating DN T cells secrete B cell differentiation factors [30, 31], which may enhance autoantibody production. Increased numbers of TcR γ/δ-bearing DN T cells have also been recently reported in the inflamed joints of patients with rheumatoid arthritis [32, 33]. The expansion of DN T cells in autoimmune diseases seems to coincide with the appearance of $CD4^+CD8^-$ autoreative T cells [8–10], suggesting a link between these two T cell populations. The demonstration that treatment with anti-CD4 antibodies concomitantly reduces the DN T cells population along with the $CD4^+CD8^-$ T cells in the lymphoid organs of MRL-*lpr* mice [34] supports this conclusion. Anti-CD4 therapy also ameliorates the pathology of murine SLE [34, 35] type-1 diabetes [27], EAE [28, 29], collagen arthritis [36] and myasthenia gravis [37], suggesting that these autoimmune diseases share a dependence on autoreactive $CD4^+$ cells and possibly DN T cells [28, 34–43].

In conclusion, augmented type *l* K^+ channel expression appears to be a valuable marker for DN T cells associated with murine SLE, type-1 diabetes mellitus and chronic EAE. These results focus attention on the possible role of DN T cells in the pathogenesis of autoimmune diseases and emphasize the potential value of combining electrophysiological approaches with immunological and molecular techniques in the study of autoimmunity. However, it is not clear from our results whether the abundance of type *l* K^+ channels in DN T cells reflects a causal relationship with autoimmunity or whether it is secondary to the disease process. Investigation of the effects of type *l* K^+ channel-specific drugs (*e.g.* TEA^+) on the development of autoimmunity could clarify this issue.

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