Defect of TCR–mediated signal pathway of thymocytes in Autoimmune Nonobese Diabetic mouse thymus*

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Abstract **Objective** To study the mechanism responsible for T cell alterations in NOD mice. methods we examined whether a defect exists in the thymus of NOD mice at the level of TCR-mediated signaling after activation by anti-TCR and ConA. **Reaults** thymocytes from NOD mice respond weakly to anti-TCR -induced proliferation, compared with thymocytes from BALB/c mice. This defect correlates with the age,and both NOD CD4+CD8- and CD4-CD8+ mature thymic T cells respond pooly to anti-TCR.The defect can be partially revearsed by the addition of rIL-2 to NOD thymocytes. In contrast to their low proliferative response to anti-TCR, NOD thymocytes respond normally to the combinations of PMA plus the Ca2+ ionophore ionomycin and PMA plus anti-TCR but weakly to anti-TCR plus ionomycin. **Conclusion** the age-related NOD thymocyte unresponsiveness to anti-TCR results from a defect in the signaling pathway of T activation that occurs upstream of protein kinase C activation.

Key Words Defect; TCR signal pathway; NOD mice

Abbreviations: NOD: nonobese diabetic; IDDM: insulin-dependent diabetes mellitus; DAG: diacylglycerol; PKC: protein kinase C; PLC: phospholipase C; IP3: inositol trisphosphate; PE: phycoerythrin ; NCS: newborn calf serum.

T cell tolerance to self-antigen is considered to reflect a combination of central and peripheral tolerance. Central tolerance leads to deletion of immaure T cells in the thymus and is largely responsible for elimination autoreactive T cells. The nonobese diabetic (NOD) strain of mice generally die from type 1 diabetes, reflecting the T cell-mediated destruction of pancreatic β cells, but also develop generalized autoimmune disease affecting multiple organs.

Adoptive cell transfer experiments indicate that both CD4+ T cells are essential for the development of disease[1]. How these T cells escape from self-tolerance and induce IDDM is not well understood. In the BioBreeding rat model of IDDM, a change in thymic T cell development occues, and this involves an increase in the frequency of immature subsets in thymus, accompanied by a reduction in the proportion of mature thymic T cells [2].These changes in thymic T cell maturation are prerequisites for the development of IDDM in BioBreeding rats and are thought to be induced by a defect in bone marrow-derived thymic APC.

To examine the mechanism responsible for observation of altered thymic T cell distribution and function in NOD mice[3], we examined the ability of NOD thymic costimulatory signal to activated thymocytes and in vitro proliferative capacity of NOD thymocytes after activation by different stimuli.

MATERIALS AND METHODS

Expreimental animals Male and female NOD mice between 4 and 12 weeks of age were checked for glycosuria with reagent strips. Age- and sex-matched BALB/c were used as controls obstained from the Animal Breeding Laboratory, Institute of Genetics, Chinese Academy of Sciences.

Monoclonal antibody ConA, PMA, the calcium ionophore purchased from Sigma Chemical Co. The anti-TCR mAb (H57.597), produced in our laboratory as ascites; nti-CD28mAb, PE-labeled anti-CD4 (192.19), FITC-labeled anti-CD8 , were purchased from PharMingen(San Diego, CA).

Cell activation Mice were killed by cervical dislocation, thymic The cells were washed and suspended in cold 2% -NCS-RPMI1640 medium.Thymocytes (2×10^5/well) were cultured for 72h at 37℃ in 96-well round-bottomed plates, in a final volume of 200μl of 2%-NCS-RPMI1640 supplemented with different stimuli of PMA (1ng/ml),ionomycin (100nM), anti-TCR

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mAb(50ug mg/L 37℃ 2 h)plus anti-CD28 mAb(20μ g/ml , Pharmacia) , and ConA (2.5μ g/ml). Cell proliferation was assessed by addition of 1μ Ci of ^{3}H thymidine 18h before termination of culture, and incorporation of 3H thymidine was determined by liquid scintillation counting, by using an LKB rackβ -counter (LKB Instruments,Inc., Galthersburg, MD).

Flow cytometry Thymocytes of isolated each individual subgroups were prepared for fluorescent Ab staining. For two color analysis, staining was performed directly with anti-CD8-FITC and either with anti-CD4-PE. Electronic compensation was performed based on the isotype and positive controls of the cells stained with each respective fluorescent mAb. The expression of cell surface molecules was analyzed by flow cytometry equipped with two lasers(FACS calibur, BD company). In all cases, gains were adjusted so that dead cells were excluded from living cells. Data acquisition and analysis were performed using Cell Quest Software (Becton Dickinson).

Statistical analyses The results were analyzed according to the Student's t-test.

RESULTS

NOD thymocytes proliferate poorly in response to anti–TCRmAb plus CD28mAb or ConA.

To determine whether previous observation of altered thymic T cell repertoire[2] and function[3] in prediabetic NOD mice are mediated by a defect in signal transduction by thymic T cells. we examined the capacity of NOD thymocytes to proliferate in response to either anti-TCRmAb or ConA, which both activate T cells through the TCR/CD3 complex. The responses to anti-TCRmAb plus CD28mAb and ConA of thymocytes from either young prediabetic NOD mice or age- and sex-matched BALB/c control mice were measured. Assays were optimized to detect maximal thymocyte proliferation. After 72h in culture, the response of NOD thymocytes obstained from both female and male mice was significantly lower ($p<0.005$) than that of control BALB/c T cells. There was an approximate 16-fold difference in the anti-TCRmAb plus CD28mAb-induced proliferative response for NOD and BALB/c thymocytes. At the concentration of ConA (2.5μ g/ml), the NOD response was about 20 to 30% of that of control BALB/c(Table 1).

NOD thymic T cell unresponsiveness to anti – TCR is age dependent.

To determine whether the development of the relative unresponsiveness of NOD thymic T cells to anti-TCRmAb is age dependent, we examined the capacity of thymic T cells from NOD mice at different ages to respond to anti-TCRmAb and compared it with that of BALB/c mice. Only small differences in the proliferative response to anti-TCRmAb were observed between thymocytes from 4-6 week-old NOD and BALB/c mice. However, quite a significant reduction in the proliferative response of NOD thymocytes was observed at 10 weeks of age. In contrast, the anti-TCRmAb-induced proliferstive responses of thymocytes from BALB/c at 10 weeks of age were relatively unchanged (Figure 1). These data demonstrate that reduced proliferation in response to anti-TCRmAb is an age-related response characteristic of NOD but not control thymocytes.

In order to determine whether the thymic defect in NOD mice is expressed by different thymic T cell subsets, anti-TCRmAb-activated thymocytes from 4- and 10-week-old NOD mice were double-stained with FITC-CD8 plus PE-CD4. Both $CD4^{+}CD8^{-}$ and $CD4^{-}CD8^{+}$ T cell subsets from 4-week-old NOD mice increased about 8.9-and 10.9-fold in number, respectively, and comprised about 72% (39.2% $CD4^{+}$ $CD8^{-}$ plus 32.6%$CD4^{-}$ $CD8^{+}$) of the total blast cells . However, in 10-week-old NOD mice, the frequency of these T cell subsets among the thymocyte blasts totaled about only 31%(16.7%$CD4^{+}$ $CD8^{-}$ plus 14.0%$CD4^{-}$ $CD8^{+}$ T cells), with the respective fold increases being only 2.1 and 1.2. The frequencies of the immature $CD4^{-}$ $CD8^{-}$ plus $CD4^{+}$ $CD8^{+}$ T cell blasts in 4- and 10-week-old NOD mice were 28.2% and 69.3%, respectively. Insignificant proliferative responses were obtained with the $CD4^{-}$ $CD8^{-}$ and $CD4^{+}$ $CD8^{+}$ immature T cell subsets. Thus, the age-dependent reduction in the frequency of blasts was evident among both the $CD4^{+}$ $CD8^{-}$ and $CD4^{-}$ $CD8^{+}$ T

Table 1 anti-TCR,ConA-induced proliferative response of thymic T cell from 10-week-old NOD and BALB/c mice*

3H Thymidine incorporation(cpm)	NOD	BALB/c
Anti-TCR plus CD28mAb	8640±2516	93742±21360
ConA	2110±540	12864±1320

*The results shown represent the mean values±SEM of triplicate cultures obtained from four independent experiments.

cell subsets. In contrast to the results obtained from NOD mice, no differences in the frequencies of the various T cell subsets among the blast cells were detected in 4- and 10-week-old BALB/c mice before and after thymocyte activation by anti-TCRmAb. Proliferative responses were augmented about 4-15-fold for BALB/c $CD4^+$ $CD8^-$ and $CD4^-$ $CD8^+$ T cells, respectively.

NOD thymocytes respond to PMA plus ionomycin or anti-TCRmAb plus PMA but fail to respond to anti-TCRmAb plus ionomycin.

The phorbol ester PMA and the Ca^{2+} inophore ionomycin trigger the stimulation of T cells by bypassing early signaling events, i.e., by activation of PKC and elevation of Ca^{2+} ion concentration, respectively.Thus, these agents can be used to define whether the age-related defect in NOD thymocyte responsiveness to anti-TCRmAb occurs before or after PKC activation. PMA plus ionomycin elicited a comparable proliferative response in thymocytes from both 10-week-old NOD and BALB/c mice (11.126 ± 1.856 and 17.000 ± 2.114cpm). Similar responses were induced by PMA plus ConA in NOD and BALB/c thymocytes. In constrast, the response of NOD thymocytes to anti-TCRmAb plus ionomycin remained low, in comparison with that of BALB/c thymocytes. NOD and BALB/c thymocytes did not proliferate in response to either PMA or ionomycin alone(data not shown). These observations demonstrate that PKC activity is inducible in thymocytes in 10-week-old NOD mice(Figure 2).

Addition of rIL-2 to NOD thymocytes only partially restores their response to anti-TCRmAb.

To observe that NOD thymic T cells cann't be stimulated by anti-TCRmAb for a high proliferative response in the presence of a constimulatory signal provided by anti-CD28mAb raised the possibility that the concentration of IL-2 synthesized and secreted by NOD thymocytes upon interaction with NOD thymic APC was too low to stimulate the proliferation of NOD thymic T cells. We tested this possibility by comparing the proliferative responses of NOD with BALB/c thymocytes cultured for 72h in the presence of anti-TCRmAb and varying concentrations of rIL-2. Results indicate that exogenous rIL-2 only partially restores the response of NOD thymocytes to anti-TCRmAb. At all rIL-2 cocentrations used, the absolute proliferative responses of NOD thymocytes were about one half as great as those of BALB/c thymocytes. Over the range of rIL-2 concentration tested, about a 30-fold higher dose of rIL-2 was required to yield an NOD thymocyte response equivation to that of BALB/c thymocytes(Figure 3).

To analyze the number of proliferating blasts among the mature thymic T cell subsets, Thymocytes from 4-10-week -old NOD mice were cultured in the presence of anti-TCRmAb and 800U/ml rIL-2 for 72h and then were analyzed for expression of surface CD4 and CD8. The addition of rIL-2 to anti-TCRmAb activated thymocytes in 10-week-old NOD mice that increased the percentages of both $CD4^+$ $CD8^-$ and $CD4^-$ $CD8^+$T cell blasts from 16.7% to 30.0%and from 14.0 to 34.5%,respectively. However, a significant difference in the frequency of $CD4^+$ $CD8^+$ T cells in 10 week-old NOD mice vs 4-week-old mice was still observed. Therefore, rIL-2 can partially restore thymic T cell unresponsiveness in NOD mice by stimulating the proliferation of both $CD4^+$ $CD8^-$ and $CD4^-$ $CD8^+$ thymic T cells(Figure 4).

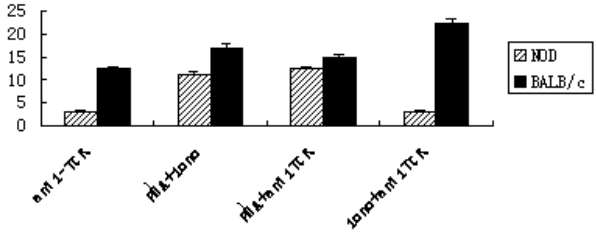

Fig. 2 Inducibility of PKC activity in NOD thymocytes.

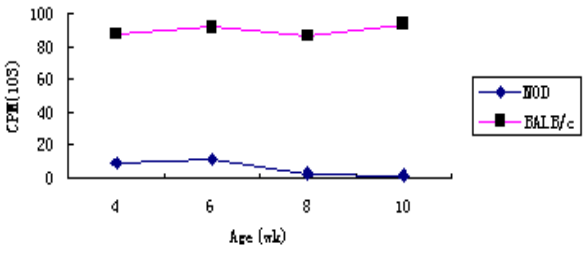

Fig 1 Age-dependent anti-TCR-induced proliferation of thymocytes.

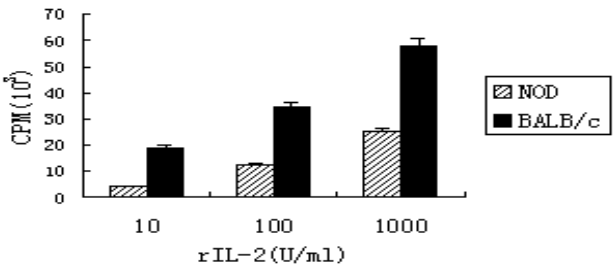

Fig. 3 Partial restoration by IL-2 of the anti-TCR-induced proliferative response of NOD thymocytes.

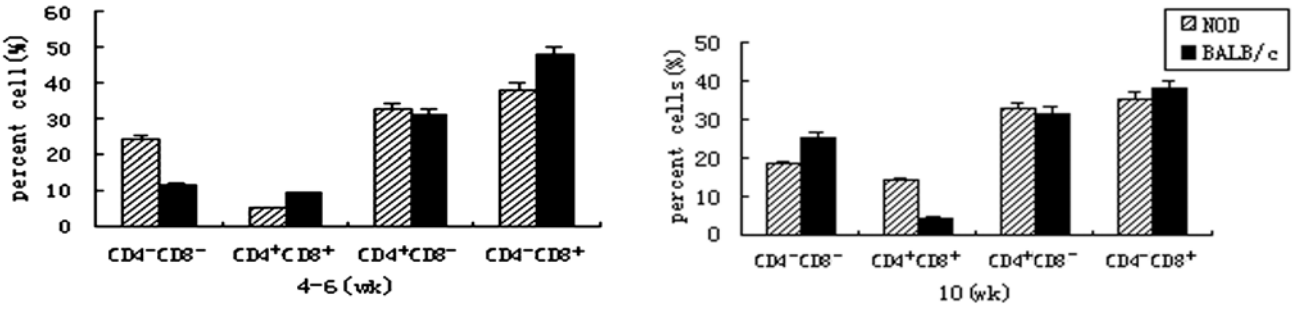

Fig. 4 Effect of IL-2 on T cell subset destribution in anti-activated thymocytes in 4-6 and 10-week-old NOD and BALB/c

DISCUSSION

Our results demonstrate that an age-related defect occurs in vitro proliferation capacity of thymocytes in NOD mice activated through the TCR/CD3 complex by either anti-TCRmAb or ConA. This thymic defect was detected in thymocytes from all NOD mice tested, beginning at 7-8-week of age, with no distinction between males and females. Besides, this defect was not apparent in thymocytes from BALB/c mice. The relatively early onset of the thymic defect in NOD mice, i.e. by 7-8 weeks of age, raises the possibility that it is caused by an inherent series of thymic events that might alter thymic T cell selection and thus culminate in anergic $CD4^+$ $CD8^-$ and $CD4^-$ $CD8^+$ thymic T cells, the positive selection of autoreactive T cells, and their migration from the thymus to the periphery in these mice.

The thymic T cell anergy noted in NOD mice can potentially result from a defect in accessory cell function and/or an intrinsic defect in T cells. In activated T cell clones, TCR occupancy by a specific Ag in the absence of a costimulatory signal results in T cell anergy in vitro, i.e., these T cells are unable to proliferate in response to a further antigenic or mitogenic stimulus[4].T cell anergy also occurs in vivo in the thymus and periphery and was identified as a possible mechanism that controls T cell-mediated tolerance [5]. Our data demonstrate that subsets of both NOD and BALB/c thymic APC stimulate an equivalent anti-TCRmAb-induced high response of BALB/c thymic T cells (Table 1). In contrast, NOD thymic T cells exhibit a low response in the response of thymic APC. This data support the notion that thymic T cells from> 8-week-old mice are intrinsically defective in their ability to be stimulated for a high proliferative response. This defect does not appear to be due to a failure of NOD thymic APC to provide a costimulatory signal to NOD thymocytes. Thus, the mechanism responsible for thymic T cell anergy, which we observed in NOD mice is likely to be different from that of the in vitro induced T cell anergy. It is possible that the anergy in the thymus of NOD mice originates from a genetic defects that resides within a bone-marrow-derived precursor T cell. Our observations of an equivalent ConA-induced proliferative response and a twofold reduction of anti-TCRmAb-stimulated response of NOD vs BALB/c splenic T cells, compared with the large differences observes for the corresponding respective thymic T cell responses (data not shown), suggest that some anergic T cells indeed exit from the thymus to the periphery.

We observed that the majority of anti-TCRmAb-induced thymocyte blast population was composed of mature $CD4^+CD8^-$ and $CD4^-CD8^+$ T cells. The reduction in the frequencies of $CD4^+CD8^-$ and $CD4^-CD8^+$ anti-TCRmAb-induced thymocytes in 10 -week -old NOD mice indicates that the T cell defect in these mice affects the function of both these thymic T cell subsets.The unresponsivenees of NOD thymocytes to anti-TCRmAb could be partially recersed by the addition of rIL-2 but neither IL-1, IFN-γ , nor TNF$-\alpha$,when each was added independently to the thymocyte culture (date not shown).A similar percentage (20to25%) of thymic T cellblasts from 8- to 12-week-old NOD and BALB/c mice express IL-2Rα $-$andβ $-$chains after actication by ConA[6]. Thus, NOD thymocytes can synthesize and express a functional high affinity IL-2R, as demonstrated by the ability of rIL-2 to significantly enhance the proliferative responses of NOD $CD4^+CD8^-$ and $CD4^-CD8^+$ thymocytes but not to the level of control BALB/c thymic T cells. Similar effects of IL-2 on reversing T cell anergy were recently obtained by others [7]. It remains to be determined whether thymic T cell unresponsiveness to anti-TCRmAb(or ConA) in NOD mice results from a specific block in IL-2 production.

PLC activation after antigenic or mitogenic T cell stimulation leads to the generation of IP3 and DAG,

which elicit Ca^{2+} mobilization from intracellular stores and the activation of PKC, respectively[8]. These events evoke lymphokine production and cell proliferation[9]. PMA and ionomycin stimulate T cell proliferation by bypassing the requirement for early signaling events, by direct activation of PKC and an increase in Ca^{2+}, especctively[10]. Several evidence suggest that thymocyte anergy in 8-12-week-old NOD mice is not due to uncoupling betweenTCR/CD3 and PLC. Reports also demonstrated a normal generation of IP3 after ConA activation in an anergy-induced T cell clone[11]. Second, these NOD thymocytes responded normally to the combination of PMA plus anti-TCRmAb or PMA plus ionomycin but failed to respond to anti-TCRmAb plus iomomycin. Thus, PLC activation in NOD thymocytes is probably sufficient to induce the production of IP3 to support Ca^{2+}, elevation but may be insufficient to induce the amount of DAG required for PKC activation. Hence, a defect upstream of PKC avtivation that leads to a failure of NOD thymocytes to proliferate is likely to exist in these mice after 7 weeks of age.

The induction of anergy in thymic T cells and its partial expression (in the presence of anti-CD3) in the periphery of NOD mice observed at7-8-week-old could subsequently result in the loss of regulatory T cells that are crucial for the immune regulation of potentially self-reactive T cells [12]. It is also possible that the NOD thymic T cell defect has a role in the later loss of a regulatory mechanism that leads to development of overt diabetes. This loss of suppression is supported by the finding of a diminished ability of exceed 8-week-old NOD thymocytes to confer protection from IDDM in cell transfer experiments[13]. The defects observed in peripheral T cells might be due to a change in the peripheral T cell repertoire,owing to the possible failure of anergy $CD4^+CD25^+$ anergic regulatory T cells either to exit the thymus or to function normally in the periphery in over 8-week-old NOD mice. Further experiment upon is required to determine whether such a relationship exists.

REFERENCES

1. Asselin-Paturel C, Megherat S, Vergnon I, et al. Differential ef fect of high doses versus low doses of interleukin-12 on the adoptive transfer of human specific cytotoxic T lymphocyte in autologous lung tumors engrafted into severe combined immunodeficiency disease-nonobese diabetic mice: relation with interleukin-10 induction. Cancer, 2001, 91(1):113-22.
2. Chiu PP, Jevnikar AM, Danska JS. Genetic control of T and B lymphocyte activation in nonobese diabetic mice. J Immunol, 2001, 167(12):7169-179.
3. Nelson BH.Interleukin-2 signaling and the maintenance of self-tolerance. Curr Dir Autoimmun, 2002, 5:92-112.
4. Arreaza GA, Cameron MJ, Jaramillo A, et al. Neonatal activation of CD28 signaling overcomes T cell anergy and prevents autoimmune diabetes by an IL-4-dependent mechanism.J Clin Invest, 1997, 100(9): 2243-2253.
5. Bodey B, Body B Jr, Kaiser HE. Apoptosis in the mammalian thymus during normal histogenesis and under various in vitro and in vivo experimental conditions. In Vivo, 1998, 12(1): 123-33.
6. Ramsdell R, Fowkles BJ. Clonal deletion versus clonal anergy: the role of the thymus in inducing self tolerance. Science,1990, 248:1335.
7. Nelson BH.Interleukin-2 signaling and the maintenance of self-tolerance.Curr Dir Autoimmun.2002, 5:92-112.
8. Irvine RF. Nuclear lipid signaling. Sci STKE, 2002 (150): RE13. Review.
9. Simon VR, Moran MF. SERCA activity is required for timely progression through G1/S. Cell Prolif, 2001, 34(1):15-30.
10. Galron D, Tamir A, Altman A, et al.Inhibition of PMA-induced human T cell proliferation by bryostatin is associated with enhanced degradation of conventional protein kinase C (cPKC): Ca^{2+} signals restore mitogenic activity without abrogating enhanced cPKC degradation. Cell Immunol, 1994, 158 (1):195-207.
11. Nieland JD, Kruisbeek AM. A T cell lymphoma can provide potent co-stimulatory effects to T cells that are not mediated by B7-1, B7-2, CD40, HSA or CD70. Int Immunol, 1995, 7 (11):1827-1838.
12. Rose NR. Mechanisms of autoimmunity. Semin Liver Dis, 2002, 22(4):387-394
13. Buschard K, Hanspers K, Fredman P, et al. Treatment with sulfatide or its precursor, galactosylceramide, prevents diabetes in NOD mice. Autoimmunity, 2001, 34(1):9-17.