

Functional assessment of CD2, CD3 and CD28 on the surface of peripheral blood T-cells from infants at low versus high genetic risk for atopy

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Recent studies from several laboratories suggest that the rate of postnatal maturation of T-cell function(s) associated with *in vitro* activation may be slower in children at high genetic risk for atopy (HR), compared to their normal (low risk; LR) counterparts. The present study compared the *in vitro* activity of the function-associated surface molecules CD2, CD3 and CD28 in panels of 27 HR and 13 LR infants, with a reference panel of 10 adults, employing assay systems involving T-cell stimulation with MoAbs against these molecules. The response maxima induced by saturating levels of the MoAbs were equivalent in all 3 groups, but T-cells from the HR infants required 10–50 fold higher levels of anti-CD3 stimulation to attain their maximum response, relative to adults ($p=0.02$); T-cells from LR infants were also less responsive to anti-CD3 than adults, but these differences were smaller and did not attain statistical significance. It is suggested that these differences are attributable to varying proportions of competent T-memory cells (which respond to low levels of anti-CD3) in PBL from these populations, the postnatal accumulation of which proceeds slowest in the HR group.

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An association between severe atopic disease in childhood and a generalized depression in *in vitro* T-cell function has been recognized for many years (reviewed in 1), but the precise nature and significance of this putative immunological “defect” in atopic children remains to be elucidated. However, recent studies from a number of laboratories suggest that postnatal maturation of T-cell cytokine production capacity, in particular IFN γ (2, 3), may be differentially delayed in children with atopic family history, relative to their counterparts with no atopic history. These differences also appear to be detectable in cord blood (4, 5), indicating that they stem from intrinsic variations in the kinetics of maturation of immune function, rather than from events occurring after birth.

The depressed IFN γ -secreting capacity of T-cells from children genetically at high risk for atopy was found to be associated with reduced *in vitro* cloning efficiency (2). This suggests that aspects of the initial activation process, and/or downstream cellular

processes associated with growth (and clonal expansion), may be defective in T-cells from HR neonates.

The present study focusses upon the early phase of T-cell activation, in particular on the functional capacity of TcR complex-associated CD3, and the co-stimulator molecules CD2 and CD28.

Material and methods

Subjects

Two groups of children under 1 yr of age were recruited with parental consent and classified as “high risk” (HR) for development of atopy (≥ 1 first degree relative with atopic history), as determined via a modified American Thoracic Society questionnaire) or “low risk” (LR; no family history), as in our earlier study (2). The HR group mean age was 25.8 ± 15.2 weeks ($n=27$), and the LR group was 30.3 ± 13.8 weeks ($n=13$). 10 normal healthy adult laboratory volunteers were included for comparison (none were under treatment for atopic disease).

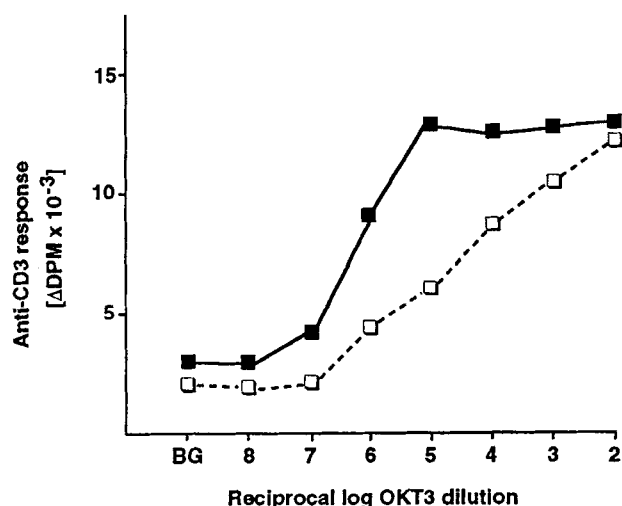


Fig. 1. Dose response for anti-CD3 stimulation of adult and neonatal T-cells. Examples of dose curves for an individual adult (closed squares) and an individual HR child (open squares). Data represent mean DPM/culture of PBMC following stimulation with log₁₀ fold dilutions of soluble MoAb OKT3 (BG: background medium control).

Reagents

The following monoclonal antibodies (MoAbs) were used: anti-human CD28 (anti-Leu 28 from Becton Dickinson, San Jose, CA), anti-human CD2 (T11 from Coulter Immunology, Hialeah, FLA), and anti-human CD3 (culture supernatant from the OKT3 cell line, obtained initially from ATCC, USA). Affinity-purified sheep-anti-mouse Ig was prepared in-house.

Cell cultures

Mononuclear cells were separated from heparinized peripheral blood (PBMC) samples and divided into two aliquots. One was used as unfractionated PBMC, and the other was used to prepare purified T-cells via dual cycle erythrocyte rosetting, as detailed previously (2). This procedure yields $\geq 95\%$ pure T-cells. The functional capacity of surface CD2, CD3 and CD28 was assessed in two different culture systems:

PBMC – Replicate cultures of 5×10^4 PBMC were established in RB microplate wells containing a total volume of 100 μ l RPMI 1640 medium supplemented with L-glutamine, 10% heat-inactivated FCS, and 20 U/ml recIL-2, together with serial log-fold dilutions of anti-CD3 (OKT3) MoAb over the dilution range 10^{-2} – 10^{-8} . The cultures were labelled with ^{3} H-thymidine at 48 h and harvested at 72 h for determination of ^{3} H-DNA synthesis (as DPM per culture).

In parallel, replicate cultures were established containing a 10^{-2} dilution of OKT3 supernatant, to-

gether with anti-CD28 (50 ng/ml). These represented optimal (saturating) levels of respective MoAbs, as determined in preliminary experiments employing normal adult PBMC; these involved serial titrations of both OKT3 supernatant and the anti-CD28 MoAb, which produced curves comparable to the adult samples in Fig. 1 – “saturating” antibody levels selected for further experiments were in the middle of the plateau region of these curves. Anti-CD2 alone was not stimulatory to PBMC, but was highly effective with purified T-cells stimulated with low levels of anti-CD3 (see below).

Purified T-cells – In these experiments, suboptimal stimulating levels of anti-CD3 (10^{-4} dilution of OKT3, determined in preliminary titration experiments similar to those above) were conjugated to the surface of the microplates via linkage to plate-bound sheep-anti-mouse Ig, by a modification of the method in (6). 5×10^4 T-cells in the medium detailed above was added to replicate wells, in the presence or absence of 100 ng/ml anti-CD2 or 50 ng/ml anti-CD28. ^{3} H-DNA synthesis was determined as above. The level of anti-CD2 employed was chosen on the basis of preliminary experiments with adult cells, as above.

Data analysis – Data analysis was performed by “t” test, providing the F test verified the lack of difference between population SDs. If the latter were significantly different, analysis was performed by Mann Whitney test.

Table 1. Functional capacity of cell surface CD2, CD3 and CD28 on T-cells from HR and LR infants in accessory cell-independent (purified T-cells) and accessory cell-dependent (PBMC) culture systems

MoAb stimulation	HR	LR	Adults
(i) Purified T-cells			
CD28	29,088 ± 4,359 (ns)	31,278 ± 7,535 (ns)	11,614 ± 3,344
CD2	27,949 ± 4,370 (ns)	31,470 ± 8,322 (ns)	13,461 ± 4,653
CD28 + CD2	45,290 ± 5,121 (ns)	40,847 ± 7,861 (ns)	21,004 ± 6,650
	————— p = 0.044 ———		
(ii) PBMC			
CD3	7,497 ± 1,419 (ns)	8,719 ± 2,943 (ns)	4,974 ± 1,689
CD28	6,982 ± 911 (ns)	7,092 ± 1,925 (ns)	8,119 ± 2,951

Purified T-cells were stimulated with submaximal levels of immobilized anti-CD3, with or without soluble anti-CD2/CD28 at supramaximal concentrations; PBMC cultures contained supramaximal soluble anti-CD3, with or without soluble anti-CD2/CD28. Data are shown ($\bar{x} \pm$ sd) for CD2 and CD28 indicate the increase in ^{3} H-DNA synthesis attributable to the addition of anti-CD2 or anti-CD28, obtained after subtraction of figures obtained for respective cultures containing only anti-CD3; figures for the later are shown for PBMC, and for purified T-cells the low level CD3 accounted for <2,000 DPM per culture throughout.

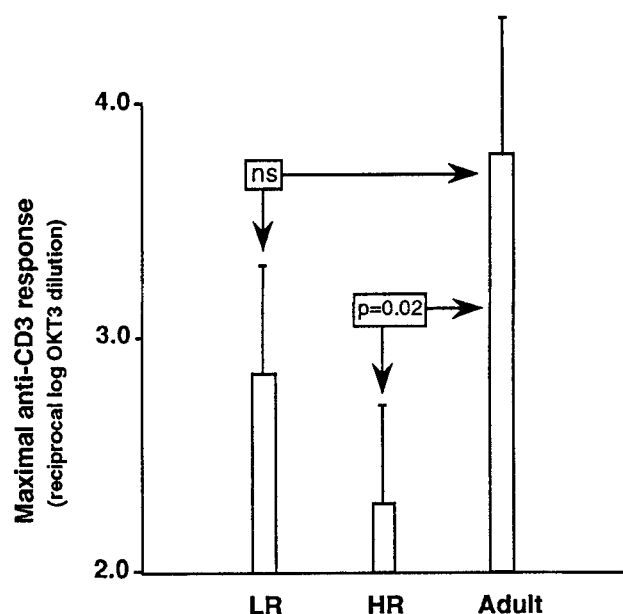


Fig. 2. Comparative maximum response thresholds for anti-CD3 stimulation. Data from each individual subject's anti-CD3 dose curve (as per Fig. 1) were examined, and respective anti-CD3 concentrations recorded (as reciprocal $\log_{10}$ dilution) which resulted in maximal "plateau" stimulation. The results shown are $\bar{x} \pm \text{S.E.}$ of these figures, for each population.

Results

Stimulation of purified T-cells via CD2 and CD28

Direct stimulation of purified T-cells with anti-CD2 or anti-CD28 elicits significant proliferative responses, but the maximal response capacity of each activation pathway is only revealed if subthreshold CD3 stimulation is applied simultaneously. Due to lack of accessory cells in the preparations, anti-CD3 MoAb does not provide a significant activation stimulus unless it is first immobilized on a solid phase (the base of the microplate wells), and this procedure was accordingly followed here. This procedure thus provides data on CD2 and CD28 function on T-cells, independent of any effects due to accessory cells.

As can be seen in Table 1 (top section), the level of short-term activation achievable via optimal stimulation through these pathways was comparable in the LR and HR children, and was equivalent to (or exceeded) that of adults.

Stimulation of PBMC

In parallel experiments, PBMC from the LR and HR donors, plus those from an adult reference panel, were tested for responses to the same MoAbs. Two approaches were followed. Firstly, dose response experiments were performed employing varying dilutions of OKT3 culture supernatant (i.e. soluble anti-CD3), as illustrated in Fig. 1, in order to deter-

mine the magnitude of anti-CD3 responses in each group, and the nature of the respective dose response curves. Under these experimental conditions, maximal anti-CD3 response means for the three groups ranged between approximately $5-8 \times 10^3$ DPM, and differences between the groups were not significant (Table 1; bottom). However, variations were observed in the shape of the dose response curves. The nature of these variations are illustrated in the examples in Fig. 1, which shows a curve from a typical adult, compared to one from a HR child; although both ultimately attain a comparable maximum response, the levels of anti-CD3 required to achieve this maximal stimulation threshold are respectively at OKT3 supernatant dilutions of 10^{-5} for the adult, versus 10^{-2} for the infant. Comparison of the overall data for the three populations in Fig. 2 indicates that the mean threshold concentration of anti-CD3 required to achieve maximal stimulation is significantly higher (i.e. it occurs at a lower dilution of OKT3 supernatant) in HR children compared to adults, whereas the smaller difference between LR and adults did not achieve statistical significance.

Unlike the situation with accessory cell-depleted purified T-cell preparations, assessment of CD2 and CD28 function in intact PBMC can be carried out by addition of supra-optimal stimulatory levels of soluble anti-CD2 or anti-CD28, into cultures containing saturating levels of anti-CD3.

As shown in Table 1 (bottom section), the degree of stimulation obtained with anti-CD2 or anti-CD28, over that achieved with anti-CD3, was comparable in T-cells from HR and LR infants and both were $\geq$ adults.

Discussion

The precise status of T-cell function in human newborns and infants, has not been clearly established. On the one hand, the reactivity of cord blood (CB) cells to polyclonal mitogens is reportedly high when measured in short term cultures (7, 8), whereas the prolonged growth of these cells (after initial stimulation) to form clones is markedly reduced relative to adults, particularly in HR children (2). In attempts to gain more detailed information on the nature of the differences in T-cell function between adults and children, a number of laboratories have employed T-cell activation assays which permit analysis of individual activation or co-stimulator pathways, in particular the use of MoAb against TcR complex-associated molecules, which mimic their respective ligands.

This approach has yielded conflicting results. In particular, responses to anti-CD3 have been reported as both normal (9, 10) or reduced (11-13). This dichotomy may be explicable in part by the use of

different assay systems that are dependent to varying degrees on the contribution of accessory cells, which are themselves known to be functionally deficient in human neonates (14, 15). In order to account for possible variations arising from the latter, the present study employed both accessory-cell dependent (soluble anti-CD3) and independent (solid-phase bound anti-CD3) assay systems, and moreover included supplementation with recIL-2, which has been shown to compensate for any functional deficiencies in PBMC accessory cells in activation assays employing neonatal T-cells (10, 12). Additionally, previous studies have employed single or a limited range of anti-CD3 concentrations, thus limiting the potential results to information upon maximum response capacity. Accordingly, the present experiments included full dose-response experiments with anti-CD3, in order to obtain additional data upon stimulation thresholds.

The principal findings from this study are as follows. It is clear that, as illustrated in Figs 1 and 2, the feature discriminating the anti-CD3 response between neonates and adults is the shape of the dose response curve, as opposed to simply the magnitude of the overall response. In the extreme example shown in Fig. 1, the CD3 complex on adult T-cells is 2–3 logs more sensitive to stimulation, than that upon neonatal cells, and this difference is more consistent between adults and HR (as opposed to LR) children.

Recent studies on purified populations of "naive" versus "memory" T-cells from adult humans, offer a plausible explanation for these results. The latter clearly demonstrate that the potential maximum anti-CD3 response in both naive and memory cells were within 10% of one another, whereas triggering of the maximum response in mature memory cells required at least 100 fold less purified anti-CD3 MoAb, relative to their naive counterparts (16). It is known that the T cell population in the peripheral blood of infants is comprised almost 100% of naive cells at birth, and the percentage of T-memory cells increases progressively over the first 2–3 years of life (17). Thus, these differences between the anti-CD3 dose-response curves of T-cells from adults and infants are likely to reflect differing proportions of the two cell types. By extension, the more consistently lower CD3-responsiveness in the HR sample may indicate a slower accumulation of memory cells within this group. This suggestion is consistent with earlier findings indicating that the efficiency of clonal expansion of T-cells (one of the limiting steps in memory cell generation) is less efficient in HR children, relative to LR.

The experiments with CD2/CD28 stimulation indicate that the overall response maxima in both groups of children were essentially equivalent, and

were $\geq$ adults (although the levels of inter-subject variation mitigated against the differences attaining statistical significance). It has recently been shown that analogous to the dose curve for anti-CD3, the maximal response of T-memory cells to anti-CD28 is reached at much lower MoAb levels than that required for naive cells (16); however, the available sample sizes were insufficient for us to pursue this question in detail in the present study, and further experiments will be required to ascertain whether T-cells from LR and HR children differ in this respect.

As noted above, the design of these experiments was such that T-cell function (s) could be assessed independent of effects due to accessory cells. While our results suggest that intrinsic differences in CD3 function may exist between HR and LR children, it will be important in future experiments to ascertain whether these differences are widened or narrowed, when accessory-cell dependent assay systems are used.

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