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Maturation of human neonatal $CD4^+$ and $CD8^+$ T lymphocytes into Th1/Th2 effectors

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The increased susceptibility of neonates to infections has been ascribed to the immaturity of their immune system. More particularly, T cell-dependent responses were shown to be biased towards a Th2 phenotype. Our studies on the in vitro maturation of umbilical cord blood T cells suggest that the Th2 bias of neonatal response cannot be simply ascribed to intrinsic properties of neonatal T cells. Phenotypically, neonatal $CD4^+$ T cells are more immature than their adult $CD45RO^-/RA^+$ naive counterparts and they contain a subset (10–20%) of $CD45RO^-/RA^+$ $CD31^-$ cells which is very low in adults and displays some unique functional features. The activation and maturation of neonatal $CD4^+$ T cells is particularly dependent upon the strength of CD28-mediated cosignal which dictates not only the cytokine profile released upon primary activation but also the response to IL-12. Activation of adult as well as neonatal CD4+ T cells in the context of low CD28 costimulation yields to the production of low levels of only one cytokine, i.e. IL-2. In contrast, strong CD28 costimulation supports the production of high levels of type 1 (IL-2, IFNγ and TNFβ) and low levels of type 2 (IL-4 and IL-13) cytokines by neonatal T cells. The low levels of naive T cell-derived IL-4 are sufficient to support their development into high IL-4/IL-5 producers by an autocrine pathway. The ability of IL-12 to prime neonatal $CD4^+$ T cells for increased production of IL-4 (in addition to IFNγ) is observed only when CD28 cosignal is minimal. Under optimal activation conditions (i.e. with anti-CD3/B7.1 or allogenic dendritic cells) the response and the maturation of neonatal and adult naive T cells are similar. Thus the Th2 bias of neonatal immune response cannot be simply ascribed to obvious intrinsic T cell defect but rather to particular conditions of Ag presentation at priming. Unlike $CD4^+$ T cells, neonatal $CD8^+$ T cells strictly require exogenous IL-4 to develop into IL-4/IL-5 producers. Most importantly, anti-CD3/B7-activated neonatal CD8 T cells coexpress CD4 as well as CCR5 and CXCR4 and are susceptible to HIV-1 infection in vitro. © *1998 Elsevier Science Ltd. All rights reserved.*

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The neonatal immune system is generally considered to be immature and this immaturity is thought to account for the failure of the neonate to mount protective response against several pathogens. In fact the functional 'impairment' of the neonatal immune system may result from the combined effects of a number of factors including (i) true immaturity of its cellular components (e.g. impaired B cell response to polysaccharide Ags) (ii) naivety or lack of previous exposure to antigens, including normal saprophytes which not only prime T/B cells but might also generate background activation of the innate immune system favouring the functional maturation of APC and the release of Th1-promoting cytokines (iii) intra-uterine exposure to unique hormonal (e.g. progesterone) and cytokine environment (IL-4) which may favour Th2 subset development and (iv) the stress of delivery. Recent studies in the mouse have demonstrated that neonatal immunization does not lead to tolerance but rather to immune response, the strength and the nature of which depend upon (i) the dose of Ag (ii) the type of adjuvant and (iii) the type of cells presenting Ag to naive T cells[1–6]. Thus, depending upon the conditions of immunization, newborn mice may either become tolerant or develop a polarized Th2 or Th1 response as well as a balanced Th0 response.

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However, as reviewed by C.A. Siegrist in this issue, it is more difficult to induce Th1 responses in neonatal than in adult mice, and several vaccination procedures leading to protective Th1 and CTL responses in adults, induce Th2-like and inefficient CTL response in neonates[4]. In keeping with the notion that the neonatal period is associated with the preferential development of Th2 responses are the observations that healthy, as well as atopic, infants develop IgE Abs against common food antigens during the first few months of life. In the great majority of individuals, including those with the atopic predisposition, this IgE response is transient and precedes the induction of oral tolerance[7]. The latter results either from clonal deletion of Ag-specific T cells or from the development of regulatory T cells, which inhibit systemic or peripheral, but not mucosal-associated local response to the antigen[8]. Similar to food antigens, airborne allergens also induce a transient Th2-like response of small amplitude in the majority of healthy children; however this is observed after 2–4 years of life, i.e. at a time when the immune system is likely to be mature[9]. In our studies on the maturation of naive human T cells into Th1/Th2 effectors, umbilical cord blood $CD4^+$ T cells were used as a source of immunologically naive cells. In some experiments, neonatal T cells were compared to adult naive cells isolated by cell sorting of $CD4^+$ $CD45RO^-$ $CD31^+$ cells. This comparison revealed some phenotypic and functional differences, which, however, cannot be directly related to the reported Th2 bias of neonatal immune response.

Phenotype of neonatal $CD4^+$ T cells

When compared to adult naive $CD4^+$ T cells, neonatal T cells express less CD4RA and CD31 but more CD38 Ags. After a few days of culture on fibroblast monolayer or in IL-4-supplemented medium, neonatal cells upregulate the expression of CD45 RA and CD31 to reach similar levels as their adult counterparts[10,11]. The CD31 adhesion molecule is a differentiation marker which is selectively expressed on adult naive $CD4^+$ T cells; CD31 is progressively but irreversibly lost during repetitive stimulation of adult or neonatal naive CD4+ T cells[12]. Interestingly, a significant proportion of umbilical cord blood $CD4^+$ T cells (10–20%) are $CD31^-$; this subset of cells (i.e. $CD31^-$ $CD45RO^-/RA^+$) is hardly detectable in adults and might correspond to immature T cells. Indeed, after 4–6 days of culture in the presence of IL-4 these cells express adult levels of CD31 and CD45RA. Neonatal $CD31^-$ RO^- $CD4^+$ T cells display distinct functional features. Unlike their $CD31^+$ RO^- counterparts, they differentiate into IL-4 and IFNγ producers after long-term culture in IL-4 and IL-12 supplemented cultures[11]. Although this observation was the first to demonstrate that IL-12, a potent inducer of Th1 response, may also favour the development of IL-4 producing cells, its biological significance remains to be explored.

Naive $CD4^+$ T cells release IL-4 at priming

The polarization of the immune response is generally determined at an early stage and it has been demonstrated that Th1/Th2 effectors may be derived from the same precursor naive T cell. Several factors regulate Th subset development including: (i) the intensity and the nature of TCR-mediated activation signal[13,14], (ii) the strength and the nature of costimulatory signals delivered by APC[15–18], (iii) the cytokine and hormonal milieu in which T cells are primed[19,20] and perhaps also (iv) the composition of the extracellular matrix at the site of T cell priming. These factors do not act independently, the effect of one signal being dependent upon the others. The integration of these signals according to the genetic background of the naive T cells dictates Th subset development[21]. Among all the factors controlling T cell development, cytokines appear to be most important, with IL-4 and IL-12 promoting Th2 and Th1 responses, respectively[19]. The other factors may act by altering the endogenous production of cytokines at priming. For example, and as discussed hereafter, the CD40/CD40L costimulation pathway plays an important role in the development of IFNγ producing effectors because it is required for the production of IL-12 by dendritic cells during Ag presentation to naive T cells[16].

The cellular origin and the mechanisms regulating the early production of IL-12 before or at the time of T cell priming have been determined. Thus, several pathogens elicit a protective type 1 response by directly activating cells of the innate immune system (macrophages, NK cells γ/δ T cells) for the early production of large amounts of IL-12, IFNγ and perhaps also IFNα and TGFβ[22]. In addition, IL-12 is also produced by dendritic cells at the time of Ag presentation to naive $CD4^+$ T cells[16,23]. We first reported that CD40 ligand expressed an activated $CD4^+$ but not $CD8^+$ T cells triggers IL-12 production by CD40 bearing APC[24]. The levels of IL-12 released by dendritic cells upon Ag presentation to T cells depends upon their maturation and activation stage and is tightly regulated by the cytokine environment[25] as well as by a number of T cell-associated surface molecules including OX40 and LAG-3 Ags (Ohshima and Delespesse; Demeure and Delespesse, unpublished observations). This T cell-dependent production of IL-12 is likely to play a critical role in the development of Th1 responses to soluble Ags devoid of intrinsic adjuvant activity, such as auto-antigens. Although it has been established that IL-4 is the strongest driving force for the induction of Th2 responses[20], the cells responsible for the early production of this cytokine at the site of T cell priming have not yet been clearly identified. Depending upon the experimental system different types of cells have been proposed as an early source of IL-4, including γ/δ T cells, natural T cells (some of which expressing NK cell markers such as NKI-1 Ag) and basophils[26,27]. However, none of these types of cells was shown to be required for the development of Th2 response to single protein Ags. We reported that upon repetitive stimulation by anti-CD3 mAb immobilized on CD32 (FcγR2)-B7.1 transfected L fibroblasts, in the absence of exogenous cytokine, naive human $CD4^+$ T cells acquire a typical Th2 lymphokine-producing phenotype[28]. The default Th2 development of repetitively stimulated $CD4^+$ T cells was observed with neonatal T cells (unfractionated as well as cell-sorted $CD31^+$ $CD45RO^-$ or $CD31^-$ $CD45RO^-$ subsets) as well as adult naive T cells. Assuming that IL-4 is required for the acquisition of a Th2 phenotype, these observations

imply that naive T cells, of neonatal or adult origins, are capable of producing IL-4 at some time during these repetitive stimulation cycles. Three series of observations indicate that indeed IL-4 is released within the first 72 h of naive T cell activation. First, addition of anti-IL-4 neutralizing Ab during the first 3 days of primary cultures, results in the development of effectors producing much less IL-4/IL-5 and increased levels of INFγ[28]. Second, IL-4 mRNA is detected after 48 h of naive CD4 T cell stimulation with a combination of soluble anti-CD3 and anti-CD28 mAbs[18]. Third, IL-4 protein can be measured in the supernatant fluids of priming cultures performed in the presence of blocking anti-IL-4 receptor mAb (to prevent IL-4 consumption by activated T cells)[18]. To exclude the possibility that the IL-4 released in priming culture could be derived from a minority subset of cells (NK1.1$^+$-like T cells, γ/δ T cells, basophils or memory T cells) contaminating the preparation of naive CD4$^+$ T cells, these were directly cloned by limiting dilution, immediately after purification from cord or adult blood[28,29]. Single naive T cells were grown on CD32 B7.1L transfectants in the presence of anti-CD3 and IL-2 and were examined for cytokine production. These experiments revealed that, each clone produced IL-4 and IL-5 at generally high levels, regardless of its neonatal or adult origin. Given the very high efficiency of the cloning procedure, these results indicate that each single naive CD4 T cell is capable of differentiating into a high IL-4/IL-5 producer when primed and restimulated in the absence of exogenous IL-4 as well as IL-4 inhibitors. Most interestingly, addition of saturating concentration of anti-IL-4+ anti-IL-4 receptor mAbs to single cell cultures leads to the development of clones producing much lower but still detectable levels of IL-4/IL-5, suggesting that IL-4 enhances, but might not be absolutely required for, the development of IL-4 producing effectors. Taken collectively, these observations are taken to demonstrate that human naive CD4$^+$ T cells of adult or neonatal origin release small but functionally significant levels of IL-4 at priming. Naive T cell-derived IL-4 not only primes T cells for increased IL-4 and decreased IFNγ production by a direct autocrine pathway, but also downregulates IL-12 production by dendritic cells. The production of IL-4 during the interaction of naive T cells with dendritic cells is regulated by a number of costimulatory molecules expressed on dendritic cells including B7.1, B7.2 and OX40 ligand[18,29]. It is completely suppressed by TGFβ, partly inhibited by saturating concentrations of IFNγ and IFNα and most interestingly, it is not affected by IL-12.

Naive T cells produce a large spectrum of cytokines

It is generally accepted that naive CD4$^+$ T cells produce mainly (if not exclusively) IL-2, which is used as an autocrine growth and differentiation factor[30]. These cells lack effector functions and are much more dependent on costimulatory signals than memory/effector T cells for optimal activation. We found that anti-CD3/B7.1 stimulated neonatal CD4$^+$ T cells release high levels of type 1 cytokines (IFNγ, IL-2, TNFα and TNFβ) together with low levels of IL-4 and IL-13. The production of these cytokines is, however, strictly dependent upon appropriate CD28 mediated costimulation, explaining why it was not detected in earlier studies. Most importantly, a similar cytokine profile is released when neonatal cells are stimulated in more physiological conditions, i.e. by allogenic dendritic cells. Naive T cell-derived cytokines may regulate Th subset development either directly via an autocrine pathway (IL-4, IFNγ) or indirectly by controlling the function of dendritic cells (IL-4, IL-13 and IFNγ). The very high levels of TNFβ production at priming might be involved in the germinal centre formation, which is known to be more pronounced in primary than secondary T cell-dependent Ab responses. The production of high levels of type 1 cytokines at priming contrasts with the default development of repetitively stimulated naive T cells into Th2 effectors and underlines the predominant role of IL-4 in the regulation of Th subset development.

Role of IL-12

In vivo and in vitro studies in the mouse have established that IL-12 promotes the development of IFNγ-dominated responses and often, but not always, inhibits the development of IL-4 producing effectors[22,31,32]. IL-12 primes naive T cells for increased IFNγ production via a direct effect on T cells involving two signals: one mediated by IL-12 itself and the other by IL-12 induced IFNγ. In the mouse the latter mechanisms (i.e. IFNγ) is required to maintain the expression of high affinity IL-12 receptor on primed cells and is thus essential for the development and persistence of Th1 effectors[33]. In the human system, it was reported that IFNα, but not IFNγ, prevents the spontaneous downregulation of high affinity IL-12 receptors and therefore plays an essential role in Th1 cell development[34]. The mechanisms whereby IL-12 inhibits the development of IL-4 effectors is less clear and depends upon several factors, including the presence of other cytokines at priming (IL-4) and the genetic background of the mice. As mentioned above, IL-12 does not inhibit IL-4 production in priming cultures of naive human CD4$^+$ T cells. However, and most interestingly, it strongly inhibits their production of IL-13 and markedly enhances that of IL-2 and IFNγ. The effect of IL-12 on the acquisition of IL-4 producing capacity are quite variable and depends upon the origin of naive T cells (neonatal/adult) and the experimental conditions (i.e. the intensity of CD28-mediated cosignal). Thus, IL-12 regularly inhibits the development of IL-4 producing cells when added to cultures of naive T cells stimulated with anti-CD3/B7.1 or allogenic dendritic cells, regardless of whether the naive cells are of neonatal or adult origin[23]. In contrast and most interestingly, addition of IL-12 to naive T cells activated by anti-CD3 mAb immobilized on CD32-single L transfectants expressing very low but functional levels of mouse B7.1, has a differential effect on neonatal and adult naive CD4$^+$ T cells[35]. Neonatal, but not adult naive T cells, primed in these conditions display an enhanced IL-4 producing capacity. The biological significance of this finding is however unclear and deserves further investigation. Since neonatal as well as adult naive T cells are primed by dendritic cells known to express high levels of costimulatory molecules, it is unlikely that the above observations may account for the Th2 bias of neonatal

immune responses unless neonatal dendritic cells are more immature and express less costimulatory molecules than their adult counterparts.

Maturation of neonatal CD8 T cells

Unlike $CD4^+$ T cells, neonatal $CD8^+$ T cells do not produce IL-4 at priming and do not acquire a Th2 phenotype upon repetitive stimulation with anti-CD3 mAb immobilized on CD32 B7.1 (or B7.2) L tranfectants[36]. Exogenous IL-4 and IL-2 are absolutely required for their development into IL-4/IL-5 producers. Contrary to $CD4^+$ T cells, the effects of IL-12 dominate over those of IL-4 in the Th1/Th2 polarization of neonatal $CD8^+$ T cells. Thus IL-12 completely inhibits the effect of IL-4 on the acquisition of IL-4 producing capacity by $CD8^+$ T cells. However, and most interestingly, IL-12 increases the enhancing activity of IL-4 for IL-5 production by $CD8^+$ T cells. Neonatal $CD8^+$ T cells coexpress low to moderate levels of CD4 upon activation with allogenic dendritic cells or anti-CD3 plus anti-CD28 mAbs[37]. Most importantly, activated neonatal CD8 T cells can be productively infected by macrophage-tropic, but not T cell-tropic, strains of HIV-1. Given that macrophage-tropic HIV-1 is responsible for disease transmission and since $CD8^+$ T cells are potent inhibitors of HIV-implication, these observations are relevant to the rapid progression of HIV disease in a large proportion of perinatally infected patients.

CONCLUSION

Our findings confirm and extend the notion that neonatal human $CD4^+$ T cells are more immature than adult naive $CD4^+$ T cells. This immaturity is evidenced by their phenotype and some functional features. First, neonatal, but not adult, naive T lymphocytes contain a subset $CD4^+$ $CD31^-$ $CD45RO^-$ cells which differentiate into IL-4 (and IFNγ) producers after long-term culture in IL-4+ IL-12 supplemented medium. Second, IL-12 primes neonatal (but not adult) naive T cells activated under infraoptimal conditions (low CD28 cosignal), for increased IL-4 production. Further investigations are, however, clearly required to examine whether this particular response of neonatal T cells to IL-12 may account for the preferential induction of Th2 responses in the neonatal period. Indeed, neonatal and adult naive T cells do not differ with regard to (i) their ability to produce IL-4 at priming and to develop into Th2 effectors upon repetitive stimulation in neutral conditions, (ii) the production of high levels of type 1 cytokines at priming and (iii) their response to IL-12 under optimal activation conditions. Therefore the reported Th2 bias of neonatal immune response cannot be directly related to $CD4^+$ T cell immaturity only; perhaps it may result from the immaturity (or naivety) of several other types of cells than T cells, including dendritic cells and cells of the innate immune system.

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