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Activated Human Neonatal CD8⁺ T Cells Are Subject to Immunomodulation by Direct TLR2 or TLR5 Stimulation

Mark McCarron²*† and Denis J. Reen*†

In conditions of optimal priming, the neonate possess competency to mount quantitatively adult-like responses. Vaccine formulations containing sufficiently potent adjuvants may overcome the neonates’ natural tendency for immunosuppression and provoke a similarly robust immune response. TLR expression on T cells represents the possibility of directly enhancing T cell immunity. We examined the ex vivo responsiveness of highly purified human cord blood-derived CD8⁺ T cells to direct TLR ligation by a repertoire of TLR agonists. In concert with TCR stimulation, only Pam₃Cys (palmitoyl-3-Cys-Ser-(Lys)₄) and flagellin monomers significantly enhanced proliferation, CD25⁺ expression, IL-2, IFN-γ, TNF-α, and intracellular granzyme B expression. TLR2 and TLR5 mRNA was detected in the CD8⁺ T cells. Blocking studies confirmed that the increase in IFN-γ production was by the direct triggering of surface TLR2 or TLR5. The simultaneous exposure of CD8⁺ T cells to both TLR agonists had an additive effect on IFN-γ production. These data suggest that a combination of the two TLR ligands would be a potent T cell adjuvant. This may represent a new approach to TLR agonist-based adjuvant design for future human neonatal vaccination strategies requiring a CD8⁺ component. The Journal of Immunology, 2009, 182: 55–62.

Immaturity of the neonatal immune system compared with adults, including possession of a T cell repertoire that is exclusively naive, is thought to contribute to the increased susceptibility to infection and the high rate of infection-related deaths during the neonatal period (1–3).

Vaccination represents an extremely efficient and cost-effective public health intervention (4), particularly when administered during the neonatal period, as access to medical care is greater at this time point than later in life in many geographical areas (5). Vaccines that are effective in the high-risk period of the first 3 mo of life and that can confer protection from “birth to death” remain an increasingly urgent unmet need. The slow induction of vaccine-mediated infant protection correlates with the gradual postnatal maturation of the infant immune system (6).

However, in certain circumstances neonates can overcome their evolutionary conserved tendency for immunosuppression and mount adult-like CD8⁺ T cell responses (7). The induction of potent CD8⁺ T cell responses is likely to be critical in the design of modern prophylactic vaccines against viruses and intracellular bacteria (8). The most convincing evidence of adult-like CD8⁺ T cell responses in human neonates are the “proof of principle” experiments provided by nature whereby functionally mature cytolytic CD8⁺ T memory lymphocytes have been detected in the cord blood of newborns with congenital infections (9, 10). This indicates that, under appropriate inflammatory conditions, the well-documented deficiencies in the neonatal immune system can be overcome. Consequently, it is thought that adjuvant choice for neonates is likely to play a key role in creating the necessary priming conditions to elicit a protective adult-like immune response (6).

At present, alum salts continue to monopolize human vaccine formulations, yet they are relatively poor adjuvants at inducing cellular immune responses (11).

TLR ligands, predominantly through their activation of innate cells, represent attractive candidates as future vaccine adjuvants (12). To date, 10 members of the TLR family have been identified (TLRs 1–10) in humans and a number of ligands for each, with the exception of TLR10, have also been characterized (13). The broad array of pathogen-derived ligands enables the innate immune system to survey the body and detect most pathogen types. Broadly speaking, TLR2 (with TLR1 or TLR6) is triggered by lipopeptides (“mimicked” by palmitoyl-3-Cys-Ser-(Lys)₄ (Pam₃Cys)₃ or fibroblast-stimulating lipopeptide-1 (FSL-1)); TLR3 by dsRNA (mimicked by poly(I:C)); TLR4 by LPS; TLR5 by flagellin monomers; TLR7 by a guanosine analog, lxoribine (7-allyl-7,8-dihydro-8-oxo-guanosine); TLR8 by ssRNA; and TLR9 by unmethylated CpG dinucleotides within particular sequence contexts (CpG motifs).

The activation of the innate immune response via TLRs represents the traditional interface, as foreseen by Janeway (14), between pathogens and the immune system resulting in an adaptive immune response that is propagated independently of direct contact with the intact pathogen or its TLR ligands. However, the direct interaction of TLR ligands with TLR-expressing adaptive T cells is an emerging concept (15), and these cells may represent novel TLR responsive targets in future vaccine adjuvants. In a number of recent well-controlled studies, the presence of various TLRs on human CD4⁺ T cells (16, 17), CD4⁺ CD25⁺ regulatory cells (18–20), and γδ T cells (21) have been described.

Few studies have addressed the expression of TLRs on human or murine CD8⁺ T cells. The immunomodulatory action of TLR2

Abbreviations used in this paper: Pam₃Cys, palmitoyl-3-Cys-Ser-(Lys)₄; FSL-1, fibroblast-stimulating lipopeptide-1.

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on murine effector (22) and naive (23) CD8+ T cells has been demonstrated. In contrast, a recent human study failed to detect TLR2 mRNA and demonstrated only functional TLR3 expression on effector and effector memory CD8+ T cells (24).

In light of these conflicting findings, it was of interest to examine TLR expression and function on human CD8+ T cells. In particular, the use of cord blood-derived CD8+ T cells in an APC-independent model allowed a full elucidation of the TLR repertoire on human neonatal naive CD8+ T cells.

Materials and Methods

Cell purification

Venous blood from the umbilical cords of healthy babies delivered either via natural delivery or elective caesarian section was collected into endotoxin-free 50-ml polypyrrolene tubes (Corning) and anticoagulated with EDTA (10 mM; Sigma-Aldrich). In all cases informed consent was sought and obtained as directed by the local ethical institute. PBMCs were obtained by density centrifugation over Lymphoprep (Axis-Shield). A two-step purification procedure was employed to ensure the isolation to be of highly purified CD8+ T cells that were free from Ag-presenting cell contamination.

First, CD8+ T cells were negatively selected from fresh PBMCs using a negative selection Ab cocktail (human CD8+ T cell enrichment cocktail, Stemcell Technologies) containing mAb to CD4+, CD14+, CD16+, CD19+, CD56+, and glycophorin A. The Ab-labeled cells were cross-linked to magnetic nanoparticles (StemSep magnetic colloid, Stemcell Technologies) and bound to a magnetic column allowing the elution of CD8+ T cells.

The partially purified CD8+ T cells were then maintained overnight in 6-well plates (Corning) in complete medium that consisted of RPMI 1640 (Cambrex BioScience) supplemented with L-glutamine, penicillin, and streptomycin (Sigma-Aldrich) and 10% heat-inactivated filtered FBS (FBS Gold; PAA Laboratories). The following day, double-positive FACS on a BD FACSaria was performed on the CD8+ T cells based on their surface expression of CD3+ and CD8+ (BD Biosciences). Reanalysis of the sorted T cells revealed a routine purity of >99.5% CD3+CD8+ T cells. For use as a positive PCR control, monocytes were isolated by positive selection using CD14+ microbeads that magnetically retained CD14+ expressing cells in an LS column (Miltenyi Biotec).

Cell stimulation and proliferation

Highly purified human CD8+ T cells were washed in endotoxin-free Dulbecco’s PBS (Invitrogen), resuspended at 5 × 10^6/ml in complete medium and activated ex vivo in an APC-independent system using anti-CD3/CD28-coated beads (Dynabeads; Invitrogen) as surrogate APCs. For the study of effector functions, cells were activated in the presence of low dose (5 U/0.4 ng) exogenous IL-12 (R&D Systems). IL-12 acts as a third signal that is essential for the differentiation of highly purified, activated naive murine CD8+ T cells (25) and human neonatal naive CD8+ T cells (M. McCarron and D. J. Reen, manuscript in preparation) activated in the absence of APCs. T cells are unresponsive to IL-12 in the absence of cell activation. Various cell-bead ratios were used as indicated in the figure legends. With the exception of kinetic and blocking studies, TLR ligands were added at the indicated concentrations at the initiation of cultures. The following TLR stimuli were used: TLR2 agonist: Pam3Cys (EMC Microiware version 13.32). TLR4 agonist: ultra-pure LPS (Escherichia coli0111: B4; strain; InvivoGen), and the TLR5 agonist: flagellin (Salmonella thyphimurium; InvivoGen). PHA (Sigma-Aldrich) responsiveness was examined by adding PHA to a final concentration of 5 μg/ml.

For proliferation studies, cells were plated in triplicate at 5 × 10^5 per well in 96-well round-bottom plates (Corning) and 1 μCi of [3H]thymidine (specific activity 25 Ci/mmol; Amersham Biosciences) was added to each well for the last 18 h of a 72-h culture. Cells were harvested with a Tomtec Harvester 96 onto 90 × 120-mm glass fiber filters (Wallac), and [3H]thymidine uptake was determined in a 1450 MicroBeta liquid scintillation counter (Wallac). In all other assays, cells were incubated in endotoxin-free 5 ml (12 × 75-mm)-polystyrene round-bottom tubes (BD Biosciences) at 2 × 10^5 cells per tube in complete medium. This was done to promote cell and anti-CD3/CD28 bead contact.

Flow cytometry

To determine cell activation status, the purified CD8+ T cells were either left resting or stimulated by anti-CD3/CD28 beads for the indicated time, resuspended in 100 μl PBS, and stained with anti-CD25-PE (BD Biosciences). The suspension was incubated at room temperature for 10 min, followed by a wash in PBS and analysis on an LSR II flow cytometer, and the data were analyzed using BD FACSdiva software (BD Biosciences). To determine the frequency of activated CD8+ T cells responding to the TLR ligands, cells were stained intracellularly with CFSE (Invitrogen).Briefly, the cells were suspended at 10 × 10^6/ml in serum-free RPMI 1640 at a final CFSE concentration of 0.5 μM. The tube was incubated at 37°C for 5 min and unincorporated extracellular CFSE was quenched by the addition of two volumes of heat-inactivated filtered FCS followed by further 15 min of incubation. Cells were then washed twice in PBS, counted, resuspended, activated for the examination of proliferation as described above, and examined on the LSR II. To enumerate the percentage of granzyme B-producing cells, intracellular staining was performed using anti-human granzyme B-PE (Caltag Laboratories) and the Cytofix/Cytoperm kit (BD Biosciences) in accordance with the manufacturer’s recommendations. To accurately define positive events, appropriate isotype controls were used for all flow cytometric analysis.

ELISA

Cell-free supernatants that had been appropriately diluted were analyzed according to the manufacturer’s instructions for INF-γ, TNF-α, and IL-2 content using ELISA kits where the specific monoclonal capture Ab had been precoated onto microtiter wells (Invitrogen). The wells were spectrophotometrically analyzed at 450 nm using a VersaMax microplate reader (Molecular Devices). The concentration of each unknown sample was determined automatically from the standard curve and analyzed using the four-parameter algorithm on SoftMax Pro software (Molecular Devices).

RNA isolation and real-time PCR

Total RNA was extracted using TRIZol reagent (Invitrogen) in RNase-free polypyrrolene tubes (Ambion) as per the manufacturers’ instructions. The homogenates were stored at −70°C until they were processed. The RNA was then solubilized in diethyl pyrocarbonate-treated water and the residual DNA was degraded by incubation with DNase I as instructed (Invitrogen). The RNA was reverse transcribed using a SuperScript first strand synthesis system (Invitrogen) using the random primers according to the manufacturer’s instructions. Real-time quantitative PCR was performed first on an ABI Prism 7900HT sequence detection system (Applied Biosystems) using pre-designed TaqMan gene expression assays specific for human TLR2 (Hs01872448_s1) and TLR5 (Hs01920773_s1). Specific gene expression was normalized to 18S ribosomal RNA. Relative expression of TLR2 and TLR5 mRNA levels in cells activated for 24 h with restig cells was calculated using the 2^(-ΔΔCt) method of analysis (Applied Biosystems). Duplicate samples were analyzed in each experiment.

TLR studies

For blocking experiments, cells were activated by anti-CD3/CD28 beads (cell-bead ratio of 8:1) in the presence of IL-12 (5 U/0.4 ng) for 24 h followed by the addition of either anti-human TLR2 Ab (InvivoGen), anti-human TLR5 Ab (InvivoGen), or control IgG at the indicated graded concentrations. The activated cells were incubated with the blocking Abs for 1.5 h. Pam,Cys or flagellin was then added and supernatants were collected after a further 48-h incubation in the presence of the agonists and stored at −70°C until examined.

For the cooperative studies, CD8+ T cells (5 × 10^5/ml) were activated as before and either left untreated or treated with the TLR agonists alone or in combination. Proliferation was examined as described above and supernatants were obtained and stored at −70°C.

Statistical analysis

Results are expressed as means with SD. Statistical significance was determined using a Student’s paired t test. Only p-values <0.05 were considered statistically significant. Sample sizes are indicated in the figure legends. Student’s paired t test was performed with Minitab statistical software version 13.32.

Results

Highly purified human neonatal CD8+ T cells, isolated independently of contaminating TLR-expressing cells, are activated by artificial surrogate APCs

Human umbilical cord blood contains a relatively homogeneous population of resting naive neonatal CD45RA+CD8+ T cells (26). CD3+CD8+ T cells were purified by a combination of column and FACSari cell sorting resulting in a routine final purity of >99.5%.

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Upon activation, human neonatal CD8$^+$ T cells become responsive to TLR2 or TLR5 stimulation

The addition of the TLR2/TLR1 agonist Pam$_{3}$Cys to anti-CD3/CD28 beads increased cell proliferation, with maximal proliferation being obtained at 1 μg/ml (Fig. 2A). The TLR2/TLR6 agonist FSL-1 provoked a similar proliferation pattern to Pam$_{3}$Cys (data not shown). More strikingly, the TLR5 agonist flagellin induced a dose-dependent augmentation of CD8$^+$ T cell proliferation (Fig. 2B). This was most potently observed at 0.1 μg/ml and this concentration was used in subsequent experiments. The TLR4 ligand ultrapure LPS did not alter the proliferation pattern of the anti-CD3/CD28-activated T cells over the range of concentrations tested (Fig. 2C). Similarly, activated T cells were found to be unresponsive to agonists to TLR3, TLR7, TLR8, and TLR9 over a range of concentrations (data not shown). There was no cell division or detectable cytokine secretion in human neonatal CD8$^+$ T cells when TLR agonists were added in the absence of anti-CD3/CD28 activation of the cells.

Flagellin and Pam$_{3}$Cys independently regulate activated human neonatal CD8$^+$ T cell division and activation

Following activation, CD8$^+$ T cells undergo cellular division before differentiation and acquisition of effector functions (29). We examined the effect of TLR2 and TLR5 agonists on the cell division of activated CD8$^+$ T cells using CFSE.

The addition of flagellin and Pam$_{3}$Cys to anti-CD3/CD28-activated CD8$^+$ T cells significantly decreased the percentage of undivided cells from 62.8% (63.2 ± 3.4%) to 23.9% (23.1 ± 1.7%) and 42.4% (43.8 ± 2.6%), respectively (Fig. 3A). Flagellin proved a more potent costimulator, driving 16% (16.7 ± 3.2%) of cells into a third division compared with 3.9% (4.3 ± 1.2%) of cells activated in the presence of Pam$_{3}$Cys and 1.5% (1.6 ± 0.4%) by cells activated by anti-CD3/CD28 alone.

The expression of IL-2R and the production of IL-2 for autocrine consumption simultaneously promote clonal expansion and survival of activated T cells (30). We examined the expression of...
the IL-2R α-chain (CD25), a central component of the IL-2 responsive receptor and prototypical cell activation marker, on CD8⁺ T cells activated in the presence of the two TLR ligands. Both flagellin and Pam₃Cys significantly enhanced the percentage of activated CD8⁺ T cells with CD25 surface expression at 48 and 72 h following activation (Fig. 3B).

Additionally, CD8⁺ T cells activated in the presence of either TLR agonists produced IL-2 that was 4-fold greater in the presence of flagellin and 3-fold greater in the presence of Pam₃Cys than that produced by CD8⁺ T cells activated in the absence of the TLR agonists following 72 h of incubation (Fig. 3C).

Collectively, these results indicate that the TLR agonists are capable of enhancing clonal expansion by enhancing CD25 expression on activated CD8⁺ T cells as well as significantly increasing secretion of IL-2.

Flagellin monomers and Pam₃Cys independently enhance the effector functions of activated human neonatal CD8⁺ T cells

Following cell differentiation, CD8⁺ T cells express a range of effector molecules that mediate defense against pathogens or tumors (31).

Both flagellin and Pam₃Cys enhanced IFN-γ (Fig. 4A) and TNF-α (Fig. 4B) production at various cell-bead ratios examined. The effect of TLR2 and TLR5 ligation on the cytolytic capacity of activated CD8⁺ T cells was studied by examining the percentage of CD8⁺ T cells expressing intracellular granzyme B at 24, 48, and 72 h in the presence of the ligands. Both flagellin and Pam₃Cys sustained significantly higher levels of granzyme B in cells activated for 72 h compared with cells activated by anti-CD3/CD28 alone (Fig. 4C).

Human neonatal CD8⁺ T cells express TLR2 and TLR5 mRNA, and TLR ligand-mediated IFN-γ production occurs in a TLR2- or TLR5-specific manner

We next examined highly purified human neonatal CD8⁺ T cells for both TLR2 and TLR5 mRNA expression before and after
Flagellin and Pam₃Cys mutually enhance human neonatal CD₈⁺ T cell activation

A recent area of intense research in APC biology is the examination of the combinatorial activation of TLRs to uncover the additive, synergistic, or antagonistic effects on various cellular functions in the hope of tailoring adjuvant mixtures to elicit stronger and more durable immune responses (32). To date, an examination of this on TLR-expressing T cells has not been undertaken. Consequently, we examined the effect of dual TLR stimulation on activated CD₈⁺ T cells with Pam₃Cys and flagellin. The exposure of activated CD₈⁺ T cells to both agonists significantly enhanced human CD₈⁺ T cell clonal expansion compared with stimulation with each agonist alone (Fig. 6A). More strikingly, there was an additive effect on IFN-γ production when both TLR agonists were present (Fig. 6B).

Thus, a considerable enhancement of a key effector molecule in human neonatal CD₈⁺ T cells was achieved by dual stimulation
with flagellin and Pam₃Cys compared with each agonist alone. The complementary use of these agonists as adjuvants in CD8⁺ T cell vaccination strategies may prove advantageous.

Discussion
This study further demonstrates that TLRs can act downstream of their traditional role in the inflammatory response, providing another link in the previously bifurcated fields of innate and adaptive immunity.

Our results show that activated neonatal naive CD8⁺ T cells are functionally responsive to direct stimulation by TLR2 or TLR5 agonists. Both resting and activated neonatal naive CD8⁺ T cells were unresponsive to ligands directed against the remaining TLRs. Flagellin and Pam₃Cys functioned directly to enhance cellular activation, clonal expansion, and cell effector function beyond that which was achieved by normal cellular activation. In general, flagellin tended to be a more potent agonist than Pam₃Cys. Significantly, the combined stimulation of neonatal T cells with both TLR ligands resulted in increased clonal expansion and an additive effect on IFN-γ secretion. These results suggest a significant costimulatory role for TLR2 and TLR5 in human CD8⁺ T cell function.

To date, the investigation of TLR expression on CD4⁺ T cells and T regulatory cells has provided the impetus for research in this area (15). Consequently, there is a dearth of studies examining TLR expression on either murine or human CD8⁺ T cells.

Two studies have emerged with regard to TLR2 expression and function on murine CD8⁺ T cells. Sobek et al. found that a murine CD8⁺ cytolytic T effector cell line was functionally responsive to TLR2 stimulation (22). Cottalorda et al. extended this observation to purified murine naive CD8⁺ T cells demonstrating that, following TCR sensitization, Pam₃Cys enhanced cellular activation and effector functions (23). Taken together, these studies demonstrated that activated naive and effector murine CD8⁺ T cells are responsive to TLR2 stimulation, and this in turn enhances their activation and increases IFN-γ production. However, studies on TLR2 expression in human CD8⁺ T cells are less clear. Komai-Koma et al. first suggested that, similar to human naive CD4⁺ T cells, cord blood CD8⁺ T cells expressed TLR2 mRNA and protein (16). However, these data were not demonstrated. A more recent study found that freshly purified human CD8⁺ T cells express mRNA for TLR3 but do not express mRNA for TLR2 (24).

Our observations conclusively demonstrate that human CD8⁺ T cells express TLR2 mRNA and, following TCR stimulation, are functionally responsive to the most commonly used TLR2 agonist, Pam₃Cys. We did not find naive cells to be responsive to a TLR3 agonist. However, Tabiasco et al. found that following sorti purification of CD8⁺ T cell subsets, TLR3 expression was restricted to effector and effector memory T cell subsets and, in agreement with our study, was not present on the human naive CD8⁺ T cells (24). This may indicate differential regulation between T cell subsets depending on their differentiation state, which is commonly the case in the APC compartment (33). It remains unclear why Tabiasco et al. failed to detect TLR2 mRNA in naive CD8⁺ T cells.

Functional expression of TLR5 on human CD4⁺ and T regulatory cells has been described (18). The human Jurkat cell line is also responsive to flagellin (34). However, data on TLR5 expression on T cells is sparser in other nonhuman species. In two murine studies, there are conflicting views on whether naive CD4⁺ T cells possess TLR5 mRNA (35, 36). Additionally, neither study attempted to examine the responsiveness of the cells to flagellin stimulation. To our knowledge, the only studies to examine the functional responsiveness of nonhuman T cells to flagellin stimulation are a mouse and rat study. In the mouse study, TLR5 was not detected and, in particular, anti-CD3⁺-stimulated mouse naive CD8⁺ T cells were functionally unresponsive to flagellin stimulation (23). In the rat model, TLR5 mRNA was detected in the CD4⁺ and T regulatory cells, but the anti-CD3⁺-activated cells were unresponsive to flagellin stimulation (37). Collectively, these studies are conflicting on whether murine CD4⁺ T cells possess TLR5 mRNA. Moreover, the two functional studies on anti-CD3⁺-activated murine T cells imply that, if present, TLR5 is nonfunctional.

This raises the intriguing possibility that human naive CD8⁺ T cells uniquely express functional TLR5, which, as described in this study, can act as an extremely potent costimulatory receptor. It is already known that human but not murine dendritic cells express functional TLR5 (38). Perhaps this differential expression should not be surprising given the vastly different microbial challenges that the human species must encounter compared with the murine species and the large number of immunological differences between the species, which have already been described and reviewed by Mestas and Hughes (39).

There is growing evidence that TLR signaling on both murine and human T cells is physiologically important. In two murine pathological models, direct TLR costimulation of murine T cells was implicated in a MyD88-dependent colitis model (40) and a TLR9-dependent experimental autoimmune myocarditis model (41). In a murine model of Candida infection (20) and a tumor model (19), the direct action of TLR2 and TLR8 ligands, respectively, on T regulatory cells temporally reversed T regulatory cell suppression and reduced Candida infection load and tumor size. The direct action of TLR9-triggered murine CD4⁺ T cells in enhancing humoral immunity by causing increased Ig titers has also been described (42). An indirect association with human disease has also been posited through the demonstration of alterations in the expression of various TLRs on T cell subsets between healthy and ill humans during filarial (43), hepatitis C (44), and HIV (45) infections.

The emerging consensus in the literature appears to be that T cells require activation as a prerequisite to TLR responsiveness (15). This activation-dependent checkpoint has been extended to human CD8⁺ T cells in our study. A requirement for prior TCR activation would presumably prevent nonspecific activation of T cells by exogenous or endogenous ligands. However, this may not accurately reflect the full effect of TLR ligands on T cells, as a TCR-independent effect has since been replicated in a murine study (47). Recently, the ability of Pam₃Cys to induce homotypic aggregation in human total CD4⁺ T cells independently of TCR sensitization has been observed (45). A similar effect on neonatal naive CD8⁺ T cells was observed during the course of our study (data not shown). Collectively, these results may indicate that T cells, or at least subsets within the T cell compartment, are sensitive to TLR ligand stimulation and modulation of certain functions in the absence of TCR stimulation. Furthermore, the possibility that human neonatal CD8⁺ T cells possess mRNA or surface expression of additional TLR receptors that are not responsive to their respective ligands based on traditional T cell activation parameters has not been precluded by this study. The ability of these receptors to modulate chemotaxis or the costimulatory effect of the TLR2 and TLR5 agonists should be addressed in future studies.
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The generation of a protective immune response is the therapeutic goal of vaccination. It was elegantly demonstrated recently that IL-2 signaling during a primary CD8+ T cell response is critical for generating a robust recall response upon restimulation (48). Both flagellin and PAM3Cys caused significantly more IL-2 to be produced than did cells activated by anti-CD3/CD28 alone. Moreover, they prolonged the increase in IL-2 secretion for up to 72 h, whereas IL-2 production in the absence of the TLR ligands began to wane after 48 h. This indicates that, despite the ongoing consumption of IL-2, the TLR agonists enhanced sufficient quantities of IL-2 to sustain cellular expansion and would presumably facilitate generation of a robust recall response through the paracrine action of excess IL-2.

The requirement for more potent adjuvants has also created a new momentum in APC biology and underpinned the recent discoveries that dual TLR stimulation of APCs with various ligand combinations can unlock activation programs that stimulation with a single ligand is unable to achieve. Depending on the combination, this can result in additive (49), synergistic (50), and even negative (51) outcomes on cell cytokine secretion profiles. Our results demonstrate for the first time with T cells that dual stimulation of activated neonatal CD8+ T cells with two TLR agonists can enable clonal expansion and have an additive effective on cellular IFN-γ secretion.

TLR ligands can now be added to the list of factors such as cytokines and cell surface receptors that can alter an ongoing and specifically activated T cell response, allowing integration of local signaling and appropriate modulation of cellular function. This may be similar to the signal integration model that is emerging for dendritic cells (52). For instance, flagellar breakage, inefficient capping, spill-over during flagella construction, and the active secretion of monomeric flagellin by Salmonella can account for the secretion of flagellin monomers at sites of infection (53, 54). The flexibility of CD8+ T cells to directly detect high concentrations of flagellin at a site of infection would allow the T cells to enhance their activation and effector functions until the threat had subsided.

The generalized immunosuppression in the neonatal immune system precludes the use of most of the current vaccines, with the exception of the bacillus Calmette-Guérin (55), from being given at birth. This remains a serious problem, particularly in developing countries where neonatal infection remains a major killer. Future vaccines incorporating adjuvants or a combination of adjuvants that could elicit a stronger immune response are likely to be able to overcome this limitation in neonates (6).

Collectively, these data demonstrate the direct response of neonatal naive CD8+ T cells to two TLR ligands and suggest that the combined and sustained dual stimulation of this cell type may represent an attractive new avenue in adjunct design for future neonatal vaccination strategies requiring a CD8+ component.

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Disclosures

The authors have no financial conflicts of interest.

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