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Human Effector CD8⁺ T Lymphocytes Express TLR3 as a Functional Coreceptor

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TLRs are evolutionarily conserved molecules that play a key role in the initiation of innate antimicrobial immune responses. Through their influence on dendritic cell maturation, these receptors are also thought to indirectly shape the adaptive immune response. However, no data are currently available regarding both TLR expression and function in human CD8⁺ T cell subsets. We report that a subpopulation of CD8⁺ T cells, i.e., effector, but neither naive nor central memory cells, constitutively expresses TLR3. Moreover, the ligation of the receptor by a specific agonist in TLR3-expressing CD8⁺ T cells increased IFN-γ secretion induced by TCR-dependent and -independent stimulation, without affecting proliferation or specific cytolytic activity. These results thereby suggest that TLR3 ligands can not only indirectly influence the adaptive immune response through modulation of dendritic cell activation, but also directly increase IFN-γ production by Ag-specific CD8⁺ T cells. Altogether, the present work might open new perspectives for the use of TLR ligands as adjuvants for immunotherapy. The Journal of Immunology, 2006, 177: 8708–8713.

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3 Abbreviations used in this paper: IRF, IFN-responsive factor; DC, dendritic cell.

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TLR3 mRNA has been found to be expressed in human CD8+ T cells (23, 24). In the absence of functional data, we sought to investigate this issue in more detail. We therefore analyzed TLR3 expression in different human CD8+ T cell subsets, and could show that TLR3 is present in effector memory and effector cells, but not in naive or central memory cells. Moreover, functional assays clearly demonstrated that the engagement of TLR3 by specific agonists increased the production of IFN-γ induced by TCR-dependent and -independent stimulation, but did not affect cytolytic activity. Taken together, these results indicate that functional TLR with costimulatory activity can be expressed not only by CD4+, but also by CD8+ T cells. These data therefore suggest that CD8+ T cells could become a new direct target of TLR-based adjuvants in T cell vaccination strategies, to maximize the activation signals provided by DC activated through their own TLR.

Materials and Methods

Cells

Sorted CD8+ T cells were kept in RPMI 1640 containing 1% nonessential amino acids, 1% sodium pyruvate, 1% l-glutamine, 0.1% 2-ME, and 8% human serum (complete medium). Viral or tumor Ag-specific CD8+ T cell clones were isolated and propagated in in vitro culture, as described elsewhere (26), and grown in complete medium plus 150 U/ml human rIL-2 (Proleukin; Chiron). The cells used in the assays described in this study were harvested at least 8 days after the last in vitro stimulation. Cell lines used as targets in cytotoxicity or APCs in cytokine production assays were: P815 (murine mastocytoma) and the HLA-A2+ human mutant cell line CEM × 721.T2 (T2) cultured, respectively, in DMEM medium and RPMI 1640 medium containing 10% FCS.

mAbs and reagents

mAbs were from BD Biosciences, except anti-CD45-ECD (Beckman Coulter) and goat anti-rat IgG allophycocyanin (Caltag Laboratories). Anti-CR3 rat IgG mAb 3D12 was provided by M. Lipp (Max Delbrück Institute, Berlin, Germany). Anti-CD3 Ab used in redirected lysis assay was LAU T3 Ab. Poly(I:C) was purchased from Amersham Biosciences and InvivoGen; poly(dI:dC) was from Roche Diagnostics.

Cell separation

Peripheral blood samples were collected from healthy donors. PBMCs were obtained by density centrifugation using Ficoll-Hypaque (Pharmacia). Our experimental procedures involve two steps designed to exclude contamination with NK cells. First, CD8+ T cells were positively enriched from cryopreserved or fresh PBMCs using anti-CD8+ coated magnetic microspheres (Miltenyi Biotec), a procedure that eliminates most NK cells because they are not efficiently retained by the magnet. Immediate reanalysis of the isolated populations revealed >99% purity (CD3+ CD8+). Second, flow cytometry-based analysis and sorting were performed on gated CD3+ CD8+ T cells, allowing the exclusion of any residual contaminating NK cells in the sorted populations (typically <0.3%) on a FACSVantage SE using CellQuest software (BD Biosciences).

Flow cytometry

Cells were stained with appropriate mAbs in PBS, 0.2% BSA, and 50 μM EDTA for 20 min at 4°C and either directly analyzed by flow cytometry with a FACSscan or sorted with a FACSVantage SE into defined populations. Both are equipped with CellQuest software (BD Biosciences). For intracellular staining, cells were first labeled with mAbs to cell surface markers, fixed and permeabilized in PBS/1% formaldehyde/2% glucose/5 mM Na-azide for 20 min at room temperature, and incubated with mAbs to intracellular proteins in PBS/0.1% saponin for 20 min at 4°C.

Cytolytic activity

Sorted CD8+ T cell subsets were kept overnight in complete medium and used in mAb-directed 51Cr release assays at a defined lymphocyte to target cell ratio (10:1). FeCr-bearing P815 target cells were labeled with Na51CrO4 and used in the presence or the absence of anti-CD3 mAb (LAU T3, various concentrations). Cytolytic activity of Ag-specific CD8+ T cell clones was assessed using target cells (T2) labeled with 51Cr for 1 h at 37°C and washed twice. Labeled target cells (1000 cells in 50 μl) were incubated in presence of various concentrations of cognate peptide (50 μl) for 10 min at room temperature before the addition of effector cells. Chromium release was measured after incubation for 4 h at 37°C. The percentage of specific lysis was calculated as: 100 × (experimental − spontaneous release)/(total − spontaneous release). Radioactivity was measured with TopCount (PerkinElmer Life and Analytical Sciences).

Cytokine analysis

Cell-free supernatants were analyzed for IFN-γ content using an ELISA kit (BioSource International), according to the manufacturer’s instructions.

RNA isolation and RT-PCR

Total RNA was isolated from sorted CD8+ T cells or Ag-specific CTL clones using an RNA Microkit Prep (Stratogene). The DNase treatment step (20 min at 37°C) was performed before column elution. Reverse transcription was performed using Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies). Amplifications were performed with platinum TaqDNA polymerase (Invitrogen Life Technologies) for 38 cycles: 30 s at 94°C; 45 s at 58°C; 60 s at 72°C. The amplification products were resolved in a 2% agarose gel containing ethidium bromide (Sigma-Aldrich). The primer pairs used for PCR analysis were as follows: TLR3 forward, 5'-TGGAGATGTGCCTCTATTTGTC-3' and reverse, 5'-ATGCAACAGCATCCTTAAAG-3'.

Results

TLR3 mRNA expression by activated CD8+ T cells

To investigate TLR expression by defined human T lymphocyte subsets, we sorted CD4+ and CD8+ T cells and evaluated TLR expression patterns by RT-PCR. CD8+ T cells, but not CD4+ T cells, expressed TLR3, but not TLR2, TLR4, or TLR9 (data not shown). To determine whether TLR3 expression was linked to CD8+ T cell differentiation, we next isolated by magnetic and flow cytometry-based cell sorting highly purified CD8+ T cell subsets based on their cell surface expression of CD45RA and CCR7 (Fig. 1A). As previously reported, simultaneous staining for CD45RA and CCR7 defines four functionally distinct subsets of CD8+ T cells, which are designated as naive (C45RA+CCR7+), effector (CD45RA+CCR7−), effector memory (CD45RA−CCR7−), and central memory (CD45RA−CCR7+), respectively (33). RT-PCR analysis of total RNA isolated from the four different CD8 subsets clearly revealed expression of TLR3 mRNA in effector and effector memory cells, but not in naive or central memory cells (Fig. 1B). Both the inclusion of cDNA dilutions and the use of CD8 and GAPDH primers allowed to exclude artifacts due to a higher mRNA content in the effector and effector memory subsets. Purified CD8+ T cell subsets from six healthy donors showed the same expression pattern (data not shown). Thus, TLR3 expression in human CD8+ T cells appears to depend on their differentiation status.

Poly(I:C) costimulates IFN-γ secretion by activated Ag-experienced CD8+ T cells

To explore the functional role, if any, of TLR3 in effector CD8+ T cells, we used the synthetic copolymer polyinosinic-polycytidylic acid, poly(I:C), which mimics dsRNA, a well-defined TLR3 agonist. Highly purified CD8+ T cells (>99% CD8+/CD3+) were sorted into naive, effector, and effector memory subsets (with a purity ≥95%) upon reanalysis of sorted cells by flow cytometry. After overnight rest in culture medium without cytokines, individual T cell subsets were stimulated with various doses of PHA, a mitogen that binds to a number of glycoproteins expressed on the plasma membrane and can activate strong T cell responses. The IFN-γ content in cell-free supernatants harvested 48 h after PHA stimulation was measured by ELISA. Correlating with TLR3 expression, only effector and effector memory CD8+ T cells secreted IFN-γ in response to short-term PHA stimulation (Fig. 2). Addition of the TLR3 agonist poly(I:C) significantly increased the quantity of IFN-γ released by effector and/or effector memory CD8+ T cells in response to PHA, in a dose-dependent manner.
(Fig. 2, B–D). However, poly(I:C) by itself did not detectably induce IFN-γ release by any of the purified CD8^+ T cell subsets even at the highest concentration tested (Fig. 2, B–D). Moreover, addition of poly(I:C) during stimulation of naive CD8^+ T cells with PHA did not induce any detectable IFN-γ secretion (Fig. 2A). The costimulatory effect of poly(I:C) was specific, as indicated by the lack of activity, even at high concentrations, of the control poly(dI:dC), which does not engage TLR3 (Fig. 2, B–D).

**Poly(I:C) has no detectable effect on CTL activity**

Effector CD8^+ T cells exert cytolytic activity and kill target cells after productive TCR ligation. For freshly isolated bulk CD8^+ T cells, triggering of lytic function can be achieved by cross-linking with anti-CD3 Abs in a redirected lysis assay. As expected, anti-CD3 Ab triggered readily detectable lytic activity among CD8^+ T cells (Fig. 3). Addition of poly(I:C), at concentrations that co-stimulate IFN-γ secretion, had no effect on CTL activity (Fig. 3A). Moreover, no effect was observed when a pool of sorted effector and effector memory CD8^+ T cells was used as a CTL source (Fig. 3B). Likewise, addition of the control poly(dI:dC) did not induce any changes in the levels of CTL activity (Fig. 3).

**Exposure to poly(I:C) increases IFN-γ secretion, but does not affect the lytic activity of Ag-specific cloned CD8^+ T cells**

To test the function of TLR3 in well-defined Ag-specific T cells, we used HLA-A2-restricted CTL clones isolated from healthy donors or melanoma patients. TLR3 transcripts could be amplified in all six Ag-specific CD8^+ T cell clones tested. These included clones specific for immunodominant epitopes from influenza, EBV, and CMV (data not shown). Addition of poly(I:C) during Ag triggered specific lysis assay (Fig. 4A) or during redirected lysis assay...
Poly(I:C) did not detectably modulate target cell lysis. Titration curves for both antigenic peptide and cross-linking anti-CD3 Ab were identical in the presence or absence of graded amounts of poly(I:C). In contrast, IFN-γ concentrations in 48-h coculture supernatants were significantly enhanced (data not shown). To better quantitate this effect, CD8+ T cells from the same clone were stimulated with specific peptide-pulsed APCs at a 1:1 ratio during 48 h. As expected, cloned CD8+ T cells secreted IFN-γ in a peptide concentration-dependent manner. In contrast to lytic activity, addition of poly(I:C) during the stimulation period increased the quantity of IFN-γ released in a dose-dependent fashion (Fig. 4 C).

It is noteworthy that T2 cells used as APCs in this experiment expressed TLR3 transcripts, as assessed by RT-PCR. However, they did not produce detectable IFN-γ when incubated in the same assay conditions, except for the inclusion of CD8 T cells (data not shown).

Pretreatment of CD8+ T cells with poly(I:C) does not affect IFN-γ secretion or cytolytic activity

Purified CD8+ T cells were preincubated with 100 μg/ml poly(I:C) or with medium during 48 h before stimulation with PHA. Poly(I:C)- or medium-preincubated CD8+ T cells secreted comparable amounts of IFN-γ in response to PHA at any concentration (Fig. 5 A). However, further addition of graded amounts of poly(I:C) during the assay did increase IFN-γ secretion by both populations (Fig. 5 B). Moreover, preincubation of cloned CD8+ T cells with 100 μg/ml poly(I:C) for 20 h had no effect on their lytic activity (Fig. 5 C).

Altogether, these results indicate that poly(I:C) exposure does not sensitize CD8+ T cells to subsequent stimulation. Instead, poly(I:C) has to be present at time of stimulation to exert its effect on IFN-γ secretion.

Additional experiments showed that neither Ag- nor mitogen-driven CD8+ T cell proliferation was modified by inclusion of poly(I:C) in the culture medium even at concentrations as high as 100 μg/ml (data not shown). This lack of effect

FIGURE 4. Poly(I:C) stimulation increases IFN-γ secretion, without modulation of cytolytic activity, by Ag-specific CD8+ T cell clones. A, Specific killing of 51Cr-labeled T2 target cells by a CD8+ T cell clone was determined using graded amounts of specific peptides, at 10:1 E:T cell ratio for 4 h in the absence or presence of poly(I:C) (closed symbol) or poly(dI:dC) (open symbol). B, Redirected killing by a CD8+ T cell clone was investigated using anti-CD3 (LAU T3 Ab, at different concentrations) and 51Cr-labeled P815 target cells at a 10:1 E:T cell ratio for 4 h in the absence or presence of poly(I:C) (closed symbol) or poly(dI:dC) (open symbol). C, A CD8+ T cell clone was cocultured with specific peptide-pulsed APCs for 48 h at a 1:1 CD8+ T cell to APC ratio. IFN-γ concentration in cell-free supernatant was measured by ELISA. All samples were assayed in duplicates.

FIGURE 5. Pretreatment with poly(I:C) does not change CD8+ T cell IFN-γ secretion or cytolytic capacity. A, Purified CD8+ T cells were preincubated in IL-2-containing culture medium in absence (□) or in presence of 100 μg/ml poly(I:C) (■), for 48 h. After treatment, cells were stimulated during 48 h with graded amounts of PHA. IFN-γ concentration in cell-free supernatant was measured by ELISA. B, Purified CD8+ T cells were cultured during 48 h in IL-2-containing culture medium in absence (□) or in presence of 100 μg/ml poly(I:C) (■). After treatment, cells were stimulated during 48 h with 10 μg/ml PHA and graded amount of poly(I:C). IFN-γ concentration in cell-free supernatant was measured by ELISA. C, Cloned CD8+ T cells were preincubated in IL-2-containing culture medium in absence or in presence of 100 μg/ml poly(I:C) for 20 h. After treatment, specific killing of 51Cr-labeled T2 target cells by a CD8+ T cell clone was assessed using graded amounts of specific peptides at a 10:1 E:T cell ratio.
was documented using purified CD8\(^+\) T cell subsets freshly isolated from PBLs as well as Ag-specific CD8\(^+\) CTL clones propagated by serial restimulation in vitro.

**Discussion**

The present results demonstrate that human effector CD8\(^+\) T cells express functional TLR3, which can enhance TCR-induced IFN-\(\gamma\) secretion upon engagement with specific agonists.

The study of TLR expression and functions has initially mainly focused on innate immune cells, as these cells are the first line of defense against pathogen infection. However, it is becoming increasingly clear that TLR can also be expressed by adaptive immune cells such as T lymphocytes, and that they can modulate the functions of these cells (17–20, 34). The physiological relevance of these observations is not clear yet, but expression of functional TLR3 in CD8\(^+\) T cells is reminiscent of that of other germline-encoded activatory receptors such as NKG2D, which is involved in NK-mediated antiviral innate immunity (35) and which can also be expressed by cells of the adaptive immune system such as CD8\(^+\) T cells (36, 37). However, in contrast with NKG2D, TLR3 directly recognizes viral components, whereas NK-activating receptor ligands are expressed by target cells upon viral infection, cellular stress, or transformation (35, 38). Interestingly, because functional TLR3 is also present in NK cells (39, 40), both NKG2D and TLR3 may represent complementary arms of the antiviral arsenal developed by the innate immune system. Our results suggest that this might also be true for adaptive immunity, if TLR3 and NKG2D can be simultaneously engaged in CD8\(^+\) T cells. This issue remains to be addressed.

One of the main consequences of TLR3-induced signaling in cells of the innate immune system is the secretion of massive quantities of type I IFNs (10, 11), which are antiviral cytokines. Because effector CD8\(^+\) T cells are involved in the clearance of viral infections, it is of interest that they express receptors endowed with the capacity to detect viral components. It is indeed conceivable that upon CTL-induced lysis, virally infected cells release viral genetic material such as dsRNA in the extracellular space. As suggested by our results, detection of dsRNA by TLR3 expressed in effector CD8\(^+\) T cells would then enhance their IFN-\(\gamma\) production, which can in turn induce a strong antiviral state (41, 42) propagating to the cells in the vicinity of the CTL-infected target cell conjugates. This scenario would constitute an efficient amplification system of the overall antiviral effect that can be mediated by Ag-specific CD8\(^+\) T cell responses. The understanding of the precise involvement of TLR3 expressed by CD8\(^+\) T cells in viral infections will obviously need further investigations.

Viral infections require a Th1 type of response to be cleared. Most TLR ligands have the capacity to promote cell-mediated immunity through their action on DC (14, 16). Interestingly, several IRF, which are downstream targets of TLR signaling (8), have been reported recently to influence the differentiation of Th cells through their expression in both APCs and T lymphocytes themselves (43, 44). As shown in this study, TLR3 expression in CD8\(^+\) T cells is restricted to Ag-experienced cells, and thus seems to be associated with a particular state of differentiation. IRF3, which is strongly activated by TLR3 signaling, is also involved in the regulation of TLR3 expression (45, 46). It could therefore be interesting to investigate the role of IRF3 in CD8\(^+\) T cell differentiation, and to correlate its expression with that of TLR3.

The subcellular localization of TLR3 has not been investigated in this study, but it is generally believed to be mainly intracellular in immune cells, including resting human T lymphocytes (34, 47). The costimulatory effect of poly(I:C) observed in our study and in others (34) was induced by exogenous addition of the TLR3 ligand. As in other reports, we used the same range of poly(I:C) concentrations, and transfection was not used. Thus, it is conceivable that effective concentration of ligand at the endosomal compartment is likely to be much lower and closer to physiologically relevant levels. Interestingly, TLR2 has been shown to relocalize to the cell surface of CD4\(^+\) T cells upon stimulation with anti-CD3Ab and IFN-\(\alpha\) (17). Similarly, TLR3 was detected at the cell surface of \(\gamma\)\(\delta\) and \(\alpha\)\(\beta\) T lymphocytes after activation (34). It remains to be determined whether such a relocalization of TLR3 occurs upon CD8\(^+\) T cell activation. However, because the binding of dsRNA to TLR3 was shown to require an acidic microenvironment (48), it is more likely to occur in TLR3-containing intracellular vesicles. The elucidation of the mechanisms leading to the delivery of dsRNA to these structures awaits further investigations.

Wesch et al. (34) recently suggested that the moderate increase of cytokine production observed after stimulation of \(\alpha\)\(\beta\) T cells with poly(I:C) might be explained by the responsiveness of only a subset of this population. We indeed provide strong evidence in the present work that among CD8\(^+\) T cells, only the effectors, but not their naive or central memory counterparts, express TLR3 and are functionally responsive to stimulation with poly(I:C).

TLR ligands raise a great deal of interest in the field of cancer immunotherapy, because their immunostimulatory properties could be exploited to increase the efficacy of cancer vaccines (49–51). For instance, recently, TLR9 ligands have been successfully used in conjunction with peptide-based vaccines to induce strong Ag-specific CTL responses in cancer patients (28). Although not yet tested in humans, TLR3 ligands are also promising in this respect, as they have been shown to promote Ag cross-presentation, to favor Th1 type of responses, and to activate NK cells (29, 30, 40). Murine tumor models using immunization with poly(I:C) as adjuvant have demonstrated the potency of this compound to enhance both primary and memory CD8\(^+\) T cell responses (31, 32). Our results suggest that, in addition to their well-characterized effects on DCs, TLR3 agonists might also directly influence some CD8\(^+\) T cell effector functions. The increased IFN-\(\gamma\) production provided by TLR3 signaling in CD8\(^+\) effector T cells could be beneficial in therapeutic vaccines, and may lead to better responses against tumors or chronic viral infections.

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**Disclosures**

The authors have no financial conflict of interest.

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