Reovirus Serotypes Elicit Distinctive Patterns of Recall Immunity in Humans

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Mammalian orthoreoviruses (reoviruses) are ubiquitous viral agents that infect cells in respiratory and enteric tracts. The frequency and nature of human cellular immunoregulatory responses against reovirus are unknown. Here we establish systems to detect and quantify reovirus-induced cytokine and chemokine recall responses using primary cultures of virus-infected peripheral blood mononuclear cells (PBMC) and two widely used reovirus serotypes, type 1 Lang (T1L) and type 3 Dearing (T3D) reexposure in vitro. In cultures from 44 healthy adults, reovirus induced exceptionally strong CD4 and CD8 T-cell-dependent gamma interferon (IFN-γ) recall responses concomitant with intense interleukin 10 (IL-10) production. These responses were elicited independently of viral replication. Surprisingly, paired analyses of subject responses to these two common serotypes revealed that while both elicit intense Th1-dominated immunity, median T3D-driven responses were 2.2-fold weaker (P = 0.0004) than those elicited by T1L. Recall responses evoked by these viral serotypes differed markedly in their mechanism of regulation. T3D IL-10 and IFN-γ responses were CD4 and CD8 dependent and blocked by interfering with CD86 costimulation but were CD80 independent. T1L responses were consistently CD28 and CD80/86 independent. Thus, despite extensive genetic and morphological similarities between reovirus serotypes, the nature and intensity of the human recall responses as well as the control mechanisms regulating them are clearly distinct.

Mammalian orthoreoviruses (reoviruses) are ubiquitous viral agents found in untreated water and raw sewage, frequently at the same levels as Escherichia coli (28, 29). Transmitted by fecal-oral routes, these viruses commonly infect respiratory and enteric tracts. Despite causing disease in many mammalian species, reovirus is not considered a human pathogen due to the absence of obvious symptoms or known clinical impact during natural or experimental infection (38, 47). While there are sporadic reports of human reovirus infection associated with illnesses, such as neonatal hepatitis and extrahepatic biliary atresia, myocarditis, meningitis, mild upper respiratory tract symptoms, and gastroenteritis in infants and children, no causal connection has been proven with human disease (21, 36, 44).

Among adults, reovirus seropositivity approaches 100% (31, 39). While few longitudinal studies have examined the natural history of reovirus infections and the capacity of assays to differentiate between infection with different serotypes is limited, multiple peaks of seroprevalence at different ages are taken to suggest that reinfection occurs from late childhood through old age (4).

Three morphologically similar groups of reovirus have been described based on genetic divergence and antigenic properties among serotypes. These occur mainly in the gene coding for the cell attachment σ1 protein (9, 18). For each serotype, the genetics and morphology have been extensively studied:

- type 1 Lang (T1L), type 2 Jones (T2J), and type 3 Dearing (T3D). T1L and T3D share approximately 25% identity in their σ1 protein, whereas other outer capsid and viral core proteins are highly conserved, with 90 to 98% identity.
- The human immune response to reovirus is poorly understood. Murine studies argue that immunity to reovirus is not serotype specific (53), as major components of B-cell immunity are cross-reactive between serotypes. Extensive serological analyses in humans confirm these findings. Neutralizing antibodies against T1L bind T3D proteins if they are specific for viral proteins other than σ1 (17). In addition, cross-reactive CD8 T-cell epitopes exist in the σ1 protein, supplementing other cross-reactive epitopes between serotypes (17, 19, 52). However, the functional impact of cross-reactivity in providing protection against other serotypes of reovirus is unclear, particularly given the high reinfection rate.

In contrast to extensive serological studies, the cell-mediated immune response to reovirus infection has not been extensively examined in humans (14). The prevalence or nature of human cytokine and chemokine responses elicited by reovirus exposure remains unknown. This need for understanding immunoregulatory responses resulting from reovirus infection is underlined by recent studies of its potential utility as an oncolytic agent (1, 45, 46).

Experimental reovirus infections have been extensively studied in murine systems (10, 27, 35). Immune protection from primary infection is associated with development of a classical Th1-biased response, mediated by both CD4 and CD8 T cells (51). However, mice lacking CD8 T cells clear reovirus infection normally (2). Delayed viral clearance is seen in B-cell-deficient mice, demonstrating the participa-
tion, but not the necessity of, humoral responses for host resistance.

Initiation of murine T-cell-mediated immune responses by dendritic cells (DC) in reovirus infection has recently been described (15, 16). In contrast to other human viral pathogens (3, 24, 37), T1L does not directly induce DC maturation or cytokine production (15). Exposure of DC to infectious T1L does not lead to productive infection, although viral σ1 protein can be detected within vesicles. Flecton et al. (15) propose that maturation of T1L-loaded DC is dependent on alternate signals, including those from infected apoptotic epithelial cells captured from the infection site. The specific signals necessary to activate and enhance antigen-presenting cell immunity during reovirus infection or reexposure remain to be determined.

Here, we develop and optimize systems enabling us to examine, for the first time, human reovirus-specific recall immune responses in short-term primary culture directly ex vivo. Virus-driven cytokine and chemokine production to two major reovirus serotypes, T3D and T1L, clearly demonstrate that despite extensive genetic and morphological similarities, cytochrome responses to these viruses differ markedly in terms of the nature and intensity of antiviral gamma interferon (IFN-γ) and interleukin 10 (IL-10) production. In addition to defining substantial biological differences in the regulation of virus-specific immunity, discovery of unique serotypic differences in antiviral recall responses provide insight relevant to improving reovirus-based therapeutic strategies for anticaner treatment.

**MATERIALS AND METHODS**

**Recruitment of participants and isolation of PBMC.** Study approval was obtained from the University of Manitoba Faculty Committee on the Use of Human Subjects in Research, and written informed consent was obtained from 44 healthy adults (age, 18 to 45 years) who were not taking any medications. Blood samples were collected, and peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density centrifugation, counted (>95% viability as determined by trypan blue exclusion), and used immediately for short-term primary culture as described below.

**Generation of viruses for cell culture.** Reovirus strains type 1 Lang (T1L) and type 3 Dearing (T3D) are laboratory stocks. They were amplified in mouse L929 monolayer fibroblasts maintained in Joklik modified minimum essential medium supplemented to contain 2.5% fetal calf serum FCS (Gibco), 2.5% VSP neonate calf serum and 100 U of penicillin per ml, 100 μg/ml of streptomycin sulfate, and 1 μg/ml of amphotericin B (11). Large amounts of virus were grown in L929 suspension cultures, extracted with Vertrel-XF, and purified in cesium chloride gradients as described previously (30). Virus bands were harvested, dialyzed extensively against 2× SSC (2× SSC is 300 mM NaCl plus 30 mM C,H$_8$NO$_7$ pH 7.0), and the number of particles was measured by reading the optical density at 260 nm. Aliquots of purified virus were inactivated by one of two methods. In one method, virus was inactivated by UV irradiation (60-min exposure at 4-cm distance from an FBTB18 transilluminator [Fisher Biotech]). Alternatively, since alkaline treatment inactivates reovirus (7), 300-μl aliquots of purified virus in 2× SSC were treated with 6 μl of 0.5 N NaOH (predetermined to raise the pH to ~12.5) overnight and then neutralized by the addition of 6 μl of 0.5 N HCl. Titers of noninactivated and inactivated viruses were determined by plaque assay on L929 monolayers as described previously (11) to confirm virus inactivation. Stock noninactivated viruses from two sets of separate purifications were determined to have particle-to-PFU ratios of 300 and 475 (T1L) and 115 and 1312 (T3D). All virus samples were then diluted in 2× SSC, aliquoted, and frozen once at −80°C until used.

**Primary cell culture.** Freshly isolated PBMC were suspended in complete medium (RPMI 1640 with 10% heat-inactivated fetal calf serum, penicillin/streptomycin/amphotericin B [Fungizone], and 50 μM 2-mercaptoethanol) and 5 × 10$^5$ cells in 200 μl were cultured in each well in 96-well U-bottom plates. Duplicate cultures were treated with 2 × 10$^9$ PFU of T1L or T3D or equivalent amounts of inactivated T3D, each in 100 μl for a final multiplicity of infection of 4 PFU/cell. Streptokinase (Aventis Behring, Marburg, Germany), a ubiquitous bacterial antigen, was used at 5,000 U/ml as a recall antigen. In some experiments, anti-CD4 (2 μg/ml), anti-CD8 (4 μg/ml), anti-HLA-DR (2 μg/ml), anti-CD80 (1 μg/ml), isotype controls immunoglobulin G1 (IgG1) (4 μg/ml and IgG2a (10 μg/ml) (BD Pharmingen, San Diego, CA), anti-HLA-ABC (10 μg/ml) (Immunotech, Marseille, France), and cytokote-T lympoocyte-associated antigen 4 (CTLA-4) Ig (5 μg/ml; a gift from P. Nickerson, University of Manitoba) were used to evaluate activation requirements. Based on data obtained in preliminary experiments, supernatants were harvested for analysis after 6 days of culture, and the times of peak reovirus-driven cytokine responses for the cytokines and chemokines were evaluated.

**Human cytokine ELISAs.** As a safety precaution, culture supernatants were UV irradiated for 1 hour to inactivate residual infectious virus prior to enzyme-linked immunosorbent assay (ELISA) analysis. Preliminary experiments (data not shown) demonstrated that this had no impact on the sensitivity or precision of the assays used to evaluate cytokine concentrations. Anticytokine antibodies (capture and biotinylated detection) were purchased from BD Pharmingen (Mississauga, Ontario, Canada), Endogen (Woburn, MA), Biolegend (San Diego, CA), or R&D Systems (Minneapolis, MN), and recombinant cytokine standards were obtained from BD Pharmingen, Endogen, or Peprotech (Rocky Hill, NJ). Antibodies and standards were used as previously described (8, 40). At a minimum, duplicate samples were evaluated. The concentration of cytokine in each supernatant was calculated from a minimum of three points falling on the linear portion of titration curves, which were calibrated against recombinant cytokine standards serially diluted on each plate. Standard errors typically ranged from 3 to 10%.

**Real-time PCR.** A real-time PCR was developed to quantify reovirus genomic RNA using the L1 gene relative to the endogenous control human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) within infected human PBMC and mouse L929 fibroblasts. Total viral and cellular RNA was extracted from cells by using the Tri reagent-chloroform extraction method (Tri reagent from Sigma; chloroform, isopropanol, and ethanol from Fisher Scientific) according to the manufacturer’s protocol. RNA pellets were resuspended in diethyl pyrocarbonate- treated water for 15 min at 65°C. A total of 0.68 μg of RNA was used for RNase-free DNase I digestion (Pharmingen) prior to cDNA synthesis (49). RNA was reverse transcribed into cDNA by using Superscript II reverse transcriptase (Invitrogen) with unlaabeled sequence-specific primers for GAPDH (endogenous control) and reovirus L1 gene (target gene) (Table 1) in a 40-μl reaction mixture. Real-time PCR amplification mixtures (25 μl) contained 2.5 μl cDNA template, 0.2 μl each primer, and 10 μl of 2× SYBR Green Master Mix (Invitrogen) and water up to 20 μl. Reovirus genomic RNA standards were serially diluted on each plate. Standard errors typically ranged from 3 to 10%.
Reovirus does not productively replicate in PBMC. To evaluate whether reovirus-driven cytokine responses were associated with viral replication in our system, we determined reovirus end-point titers and expression of viral RNA in our PBMC cultures. In time course experiments, ranging from 0, 24, 72, and 244 h postinfection after reovirus T1L and T3D exposure to PBMC, no increase in progeny virus was detectable by viral titer methods. More specifically, 5- to 10-fold-fewer viruses were detected at 6 days postinfection compared to time zero. To determine whether virus replication occurred without secretion of infectious viral particles, we assessed viral genome replication by real-time PCR. Quantification of reovirus genomic RNA using the L1 gene (viral polymerase) relative to the cellular endogenous control GAPDH was performed by real-time PCR using the \( \Delta{\Delta}C_T \) analysis method. Shown is a time course experiment, ranging from time 1, 24, 72, and 244 h after reovirus infection of PBMC compared to infected mouse L929 cells, which are permissive for productive reovirus replication and serve as a positive control. The values are mean values plus standard errors of the means (error bars) for duplicate samples from two individuals.

![Graph showing cytokine response to T1L and T3D](image)

**FIG. 1.** Reovirus serotypes T1L and T3D do not show evidence of replicating viral genome in PBMC. Quantification of reovirus genomic RNA using the L1 gene (viral polymerase) relative to the cellular endogenous control GAPDH was performed by real-time PCR using the \( \Delta{\Delta}C_T \) analysis method. Shown is a time course experiment, ranging from time 1, 24, 72, and 244 h after reovirus infection of PBMC compared to infected mouse L929 cells, which are permissive for productive reovirus replication and serve as a positive control. The values are mean values plus standard errors of the means (error bars) for duplicate samples from two individuals.

\( 2\times \) Sybr Green I master mix buffer (12.5 µl) (Applied Biosystems), and 0.1 µM forward and reverse primer for L1-specific reactions (0.4 µM forward and reverse primer for GAPDH-specific reactions [Table 1]). Reactions were run on an ABI 7500 real-time PCR system (Applied Biosystems). The cycling conditions comprised a polymerase activation step (10 min at 95°C) and 42 cycles, with 1 cycle consisting of 15 s at 95°C and 60 s at 61°C. Each assay included (in duplicate): a positive control of reovirus cDNA from infected L929 fibroblasts, a no-template control, and 1/2 dilution of each test cDNA. Relative quantification of L1 RNA versus GAPDH RNA was performed as previously described (50), using the \( \Delta{\Delta}C_T \) analysis method.

**Statistical analysis.** Associations between antigen-driven responses were determined using two-tailed Wilcoxon tests (paired, nonparametric data). Fisher’s exact test was used to compare differences in the frequencies of responsive individuals.

**RESULTS**

Reovirus-specific cytokine production in humans. To examine the frequency and nature of reovirus-driven cytokine production, we developed a short-term, in vitro primary culture system using PBMC isolated directly ex vivo from adults. PBMC were cultured with infectious and inactivated T3D for 6 days, the time point found in preliminary experiments with five subjects (data not shown) to yield maximum production of the recall immunity cytokines and chemokines of interest. To compare these responses to another prototypic reovirus serotype, parallel cultures were simultaneously set up using blood samples from the same individuals within this population and stimulated with the same titer of infectious T1L. For a positive control of immune responsiveness, PBMC were cultured with streptokinase, a ubiquitous bacterial antigen that elicits T-cell-dependent cytokine and chemokine responses in most adults.

As seen in Fig. 2, primary culture with streptokinase revealed readily detectable type 1 (IFN-γ, CXC chemokine ligand 9 [CXCL9], and CXCL10) and type 2 (IL-5, IL-13, and chemokine [C-C motif] ligand 17 [CCL17]) recall responses in short-term primary culture for most of the individuals tested, demonstrating the sensitivity of this approach. Very strong IFN-γ responses with median values of ~77 U/ml (8,900 pg/ml, based on the WHO standard) were seen following stimulation with 4 PFU/cell infectious T1L. Equivalent amounts of infectious T3D elicited median IFN-γ responses in these paired cultures that were substantially less intense (\( P < 0.001 \)) (Fig. 2). With few exceptions, patient-paired analysis of T3D- and T1L-driven responses revealed that T1L was a stronger stimulator of IFN-γ (Fig. 3). Similar results were seen irrespective of the T1L and T3D particle-to-PFU ratios of two separate viral preparations, which ranged from 300 to 475 for T1L and from 115 to 1,312 for T3D.

Simultaneous comparison of live versus noninfectious T3D as stimuli revealed that inactivated virus was markedly weaker in its capacity to elicit IFN-γ production, as UV-inactivated virus decreased IFN-γ responses by ~10-fold.

Other cytokines characteristic of a Th1-biased immune response were also evident. Approximately half the population demonstrated detectable CXCL10 responses to either inactivated or infectious T3D (Table 2 and Fig. 2). In contrast, only a quarter of individuals produced detectable levels of this chemokine following stimulation with infectious T1L (\( P = 0.02 \) by Fisher’s exact test).

Th2 immunity-associated reovirus responses were sporadically detectable, with roughly a third of the population exhibiting detectable but low-level IL-13 recall responses and much lower frequencies of IL-5 or CCL17 production (Table 2). Overall, there was no evidence of differences in the intensity or prevalence of type 2 immunity-associated cytokine and chemokine production following T3D versus T1L stimulation. Thus, human reovirus recall responses are dominated by strong Th1-like responses, with minimal frequencies of low-level Th2-biased activation.

Interestingly, the Th1-biased memory response was consistently paralleled by expression of very strong virus-driven IL-10 responses (13). Stimulation with infectious T3D elicited detectable IL-10 responses in a lower frequency of adults than...
T1L stimulation did (82% versus 100%; \( P < 0.0116 \) by Fisher’s exact test). The median T1L-specific IL-10 response was intense (400 pg/ml) and on average threefold stronger than that elicited by T3D (Fig. 3). IL-10 production in response to inactivated T3D was readily detected but at substantially lower levels and frequency (49%) than responses seen with live virus (data not shown).

IL-10 is conventionally described as having opposing effects to IFN-\( \gamma \), and the production of IL-10 and IFN-\( \gamma \) to a wide range of immunologic stimuli is frequently inversely proportional. Therefore, we sought to determine the relationship between virus-driven IFN-\( \gamma \) and IL-10 production. Contrary to expectation, a strongly positive correlation between IFN-\( \gamma \) and IL-10 levels was seen upon T3D stimulation (Fig. 4A).

Thus, as a whole, humans demonstrate strongly Th1-biased recall responses to reovirus that are most pronounced upon live virus stimulation, weak Th2-associated immunity, and strong virus-dependent IL-10 responses. The T3D serotype consistently elicits weaker induction of IFN-\( \gamma \) and IL-10 in recall responses than does T1L stimulation (Fig. 4).

FIG. 2. Reovirus serotypes T3D and T1L induce Th1-biased recall responses. Antigen-dependent recall responses to streptokinase (SK), UV-inactivated T3D (UV-T3D), T3D, and T1L compared to medium alone are shown. IFN-\( \gamma \) (A), CXCL9 (B), CXCL10 (C), IL-5 (D), IL-13 (E), and CCL17 (F) were measured by ELISAs of supernatants from PBMC cultures grown for 6 days. The black horizontal bars represent the median responses from 44 individual healthy adults (small black circles). Significant differences in cytokine production compared to medium alone or as indicated by the bars over two groups above the graph are indicated as follows: ***, \( P < 0.001 \); **, \( P < 0.01 \).

FIG. 3. T3D consistently elicits weaker IFN-\( \gamma \) and IL-10 responses compared to T1L. IFN-\( \gamma \) (A) and IL-10 (B) were measured by ELISAs. \( P \) values represent significant differences in cytokine production following T3D versus T1L stimulation in 44 individuals stimulated with the two serotypes in paired cultures. ***, \( P < 0.001 \).
Costimulatory requirements of T-cell-mediated, reovirus-dependent cytokine production. To assess the roles of CD4 and CD8 T-cell populations in reovirus-specific recall responses, PBMC were cultured with live T3D or T1L in the presence of blocking anti-CD4, anti-CD8, anti-major histocompatibility complex (anti-MHC) class II or class I antibodies (Fig. 5 and 6) or isotype control antibodies (data not shown). As previously, T3D-specific responses were less intense than those elicited by T1L. IFN-γ responses to both serotypes were dependent on both class I and II antigen presentation and activation of CD4 and CD8 T cells (Fig. 5A and B). In marked contrast, IL-10 in T3D-stimulated cultures was T cell dependent, but there was no evidence of T-cell requirements for T1L-driven IL-10 production (Fig. 6A and B).

To better understand the mechanism underlying reovirus-driven recall responses, we assessed the requirement for costimulation and putatively differential dependence on CD80 versus CD86 costimulation. PBMC were stimulated by virus in the presence of blocking antibodies to CD80 or CD86 or with CTLA-4 Ig. CD86-dependent costimulation is necessary for the majority of T3D-stimulated IFN-γ and IL-10 production, but consistently, it had no detectable impact on T1L-driven cytokine production (Fig. 5C and D).

Thus, costimulatory requirements for virus-induced T-cell-dependent cytokine production are markedly different for reovirus serotypes.

DISCUSSION

In contrast to increased understanding of human serological responses to reovirus (25, 39, 43, 47), no information is available on the presence or nature of the human immunoregulatory cytokine response to this ubiquitous virus. Here, we demonstrate that T3D and T1L, both shown in epidemiologic studies to lead to high levels of infection, seroconversion, and reinfection in the general population, elicit clearly distinct T-cell-dependent cytokine responses, which are independent of viral replication in PBMC. Both viruses require classical CD4 and CD8 T-cell activation for IFN-γ induction; however, clearly different costimulatory pathways are involved in activation of reovirus-specific memory responses to the two serotypes. Among adults, T3D is a consistently stronger inducer of chemokine CXCL10 and a weaker inducer of both IFN-γ and IL-10 production. These differential immune responses elicited by T3D versus T1L are similarly evident whether measured by the frequency of responding individuals or by the intensity of the cytokine response induced.

Reovirus strain-dependent differences in innate cytokine responses from the human monocytic cell line THP-1 have been previously described (14). In contrast to viral replication-dependent cytokine expression and production in THP-1 cells (14), reovirus-infected PBMC did not demonstrate increased L1 RNA expression or production of progeny virus, despite evidence of serotype-specific cytokine production. A caveat of the inability to detect reovirus L1 RNA expression is that this does not preclude the possibility that some level of reovirus gene expression (and perhaps translation) may occur in reovirus-infected PBMC, which could contribute to differences in immune responses between replication-competent and inactivated reoviruses.

In murine models of reovirus infection, the protective antiviral immune response is viewed as a Th1-biased immune response mediated by both CD4 and CD8 cells (51). CD8 T-cell

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>No. of subjects exhibiting a response/total no. of subjects (%)</th>
<th>P value</th>
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<tr>
<td>IFN-γ</td>
<td>44/44 (100) / 44/44 (100) / NS</td>
<td></td>
</tr>
<tr>
<td>CXCL9</td>
<td>1/44 (2.3) / 2/44 (4.5) / NS</td>
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<td>CXCL10</td>
<td>21/39 (54) / 10/39 (26) / 0.02</td>
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<tr>
<td>IL-5</td>
<td>1/44 (2.3) / 0/44 (0) / NS</td>
<td></td>
</tr>
<tr>
<td>IL-13</td>
<td>16/44 (36) / 7/44 (16) / NS</td>
<td></td>
</tr>
<tr>
<td>CCL17</td>
<td>2/44 (4.5) / 4/44 (9) / NS</td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>32/39 (82) / 39/39 (100) / 0.0116</td>
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* Data are the numbers of subjects exhibiting a response following in vitro reexposure to infectious T3D and T1L serotypes to the total numbers of subjects. A response was defined as cytokine production greater than the value for medium alone plus two times the standard deviation.

b P values were derived from Fisher’s exact test. NS, not significant.

FIG. 4. A positive correlation between T3D-dependent IFN-γ and IL-10 responses is not seen in response to T1L stimulation. Correlation between virus-specific IFN-γ and IL-10 in T3D-stimulated cultures (A) and T1L-stimulated cultures (B) is depicted. The black lines represent best-fit slope (r) of IFN-γ/IL-10 pairs from 39 individual healthy adults (small black circles). NS, not significant.
cytotoxicity is dependent on CD4 T-cell priming, as depleting MHC class II antigen-presenting cells diminishes CD8 T-cell responses (26). Here, we demonstrate that Th1-type cytokine production likely dominates human immunoregulatory responses against reovirus reexposure or reinfection. Induction of IFN-γ by either reovirus serotype is dependent on interaction with cells expressing CD4 and CD8, as demonstrated by experimental blocking of these TcR coreceptors and their respective MHC ligands. Whether this reflects cooperation between CD4 and CD8 T cells, as seen in many viral responses, or acquisition of CD4 by CD8 T cells upon activation (22) is currently under investigation. Intracellular IFN-γ staining of CD4+ versus CD8+ cells in reovirus-stimulated cultures was technically inadequate to reliably distinguish the phenotype of IFN-γ-producing cells, likely due to the very low frequency of antigen-specific cells within the PBMC of healthy individuals (data not shown).

Surprisingly, costimulation requirements for T3D- and T1L-driven IFN-γ responses differ markedly. T3D-driven IFN-γ production is dependent upon CD86-mediated costimulation and independent of CD80. Similarly, while IL-10 production from other viral stimuli may or may not be dependent on CD28-CD80/CD86 pathways (6, 23), T3D-driven IL-10 production is highly dependent on CD86 and independent of CD80 costimulation. This observation is similar to the differential use of CD80 versus CD86 seen in influenza virus infection where virus-specific IFN-γ production is dependent on CD86 and not on CD80 (6).

Strikingly, blocking the classical CD80/86 pathway with antibodies to CD80 or CD86 or using CTLA-4 Ig consistently failed to inhibit either T1L-driven IFN-γ or IL-10 immune responses to reovirus, indicating a distinctive pathway of regulation for T3D versus T1L serotypes. Although upregulation of costimulatory molecules, such as CD80 and CD86, on antigen-presenting cells can be induced by double-stranded RNA via multiple pathways (20), the functional impact of these costimulatory molecules is clearly distinct in T1L- versus T3D-stimulated responses. Indeed, recent studies indicating that T1L does not directly activate murine DC (15, 16) may actually reflect a lack of use of the CD28-CD80/CD86 pathway in T1L-specific T-cell activation and resulting in IFN-γ production, rather than an inability for reovirus to initiate DC maturation and T-cell activation.

Differential dependence of type-specific immune responses on costimulation has been identified for influenza viruses. H1N1 induces CD4 T-cell-dependent cytotoxic T-lymphocyte responses that require CD86 costimulation, while H3N2 elicits cytotoxic T-lymphocyte responses independent of CD80/CD86.
Whether reovirus T1L responses are independent of CD80/CD86 costimulation or are independent of the CD28 pathway but reliant upon alternative costimulatory pathways, such as ICOS, CD40, OX40, or 41BB (5, 42, 54), remains to be determined. Regardless, both reovirus and influenza virus provide examples where induction of T-cell-dependent cytokine responses are differentially regulated between viral serotypes. The clinical impact of this finding is currently under investigation.

The generally perceived innocuous clinical impact of natural reovirus infection, combined with recent observations that reovirus preferentially kills tumor cells while sparing normal cells, raises possibilities for therapeutic use of this virus (12, 33, 34, 41, 48). Its efficacy in killing disseminated tumors in rodent models argues for its potential in systemic treatment of metastases, either by direct oncolytic activities, or by potentiating protective immune responses against tumor antigens. To date, the great majority of published work has been with T3D, the serotype that in our hands elicits markedly weaker responses. Phase I and II clinical trials are under way (32). The marked differences in human recall responses demonstrated in this study suggest the importance of evaluating different serotypes to maximize potential therapeutic benefits, as each yields quite different immune responses in humans and these differences might be clinically relevant.

In summary, this study is a novel contribution to our understanding of human immunoregulatory cytokine response to reovirus. Despite extensive genetic and morphological similarities between reovirus serotypes T3D and T1L, the control mechanisms and resulting responses elicited by each virus serotype are unique. T3D is characterized by T-cell-dependent IFN-γ and IL-10 cytokine production that clearly requires CD86-mediated costimulation. In contrast, T1L elicits consistently stronger IFN-γ and IL-10 responses, both of which are independent of classical CD28 and CD80/CD86 costimulatory requirements. This research impacts the emerging use of reovirus as an anticancer agent and expands the immunological information base that will be needed to construct appropriate and safe therapies.

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REFERENCES


