Comparative assessment of virulence of recombinant vaccinia viruses expressing IL-2 and IL-15 in immunodeficient mice

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IL-2 and -15 belong to the four α-helix bundle family of cytokines and display a spectrum of overlapping immune functions because of shared signal transducing receptor components of the IL-2 receptor complex. However, recent evidence suggests a nonredundant unique role for IL-15 in the establishment and perhaps maintenance of peripheral natural killer (NK) cell populations *in vivo*. To explore the contribution of locally released IL-15 on peripheral NK-cell-mediated innate immune responses, we generated a recombinant vaccinia virus that expresses IL-15 and evaluated the course of vaccinial disease in athymic nude mice. Coexpression of IL-15 resulted in the attenuation of virulence of vaccinia virus, and mice inoculated with 10^5 plaque-forming units or less resolved the infection successfully. In contrast, mice inoculated with a similar dose of the control vaccinia virus failed to eliminate the virus and died of generalized vaccinial disease. Enhanced expression of IL-12 and IFN-γ as well as induction of chemokines were evident in the mice inoculated with IL-15-expressing vaccinia virus in addition to an increase in NK cells in the spleen. However, in this model system, the degree of attenuation in viral virulence attained with coexpression of IL-15 was much less than that achieved with coexpression of IL-2, suggesting that the peripheral NK-cell-mediated events are more responsive to IL-2 than to IL-15.

IL-15 is a 14- to 15-kDa polypeptide that was discovered by its ability to promote proliferation of the IL-2-dependent murine T cell line CTLL-2 (1, 2). IL-15 shares many biological properties with IL-2, despite being genetically and antigenically distinct from IL-2.

There is a growing body of evidence to implicate the importance of IL-15 within as well as outside the host immune system. Because of common signaling receptor components, IL-2 and -15 elicit a spectrum of overlapping immune functions that include T cell proliferation, activation of cytotoxic effector cells, B cell Ig synthesis via costimulation, and activation of monocytes (reviewed in refs. 3 and 4). However, evidence from recent elegant studies with targeted disruption of IL-15Rα gene (5), IL-15 gene (6), and transgene-mediated overexpression of IL-15 in mice (7, 8) indicates that IL-15 exerts a critical influence on the differentiation, proliferation, maturation, and survival of natural killer (NK), CD8, and intraepithelial lymphocyte cell populations that cannot be adequately compensated for by IL-2, thus accentuating its requirement in the homeostasis and activation of innate immune cells (9, 10). Importantly, the absence of NK cells in IL-$15^{-/-}$ and IL-15R$\alpha^{-/-}$ mice precludes delineating the contribution of IL-15 in the induction of activation and cytotoxicity in peripheral mature NK cells that is vital for the sustenance of innate immunity. Although macrophages are central to most immune responses, granulocytes are dominant in combating bacterial infections. NK cells, on the other hand, are pivotal in early host response mechanisms in eliminating viral infections, and much of what has been learned about NK cell activation, proliferation, and functions comes from viral systems (reviewed in refs. 11 and 12). Although some viruses are exquisitely sensitive to NK-cell-mediated antiviral effects, including vaccinia virus, certain other viruses, for example lymphocytic choriomeningitis virus, are relatively refractory to NK-cell-mediated attack (11, 12). NK cells display direct lytic activity toward vaccinia virus-infected cells (13), and the depletion of NK cells leads to enhanced replication of virus (11). To gain insights into how IL-15 cytokine, when present in the local microenvironment of inflammation and infection, modulates NK cell functions, we generated a recombinant vaccinia virus that expresses IL-15, evaluated the course of vaccinial disease in immunodeficient athymic nude mice, and compared the effects to those of a recombinant virus expressing functionally related IL-2.

Materials and Methods

Mice. Female athymic congenic nude (nu/nu) mice 8–12 weeks old, bred under specific pathogen-free conditions, were obtained from the Veterinary Resources Program, National Institutes of Health (Bethesda, MD).

Cells and Reagents. The CV-1, 143B, and CTLL-2 cell lines were obtained from American Type Culture Collection. CV-1 and 143B cells were used for vaccinia virus growth, and the CTLL-2 cell line was used for detection of IL-2 and -15 bioactivity. ELISA kits to detect human IL-2, human IL-15, and murine IFN-γ as well as monoclonal and polyclonal antibodies to human IL-15 were purchased from R & D Systems. Rhodamine-conjugated anti-mouse and anti-goat antibodies were purchased from Chemicon. Fc Block-2.4G2, FITC-conjugated 145–2C11, and phycoerythrin-conjugated DX5 antibodies were purchased from PharMingen.

Generation of Recombinant Vaccinia Viruses. Vaccinia virus recombinants expressing the human IL-15 gene were generated by standard procedures by using pSC11 as the transfer vector (14). One recombinant virus generated expressed IL-15 with its native signal sequence and was designated IL-15(NS) vaccinia. A second recombinant was also created in which the cognate signal sequence of human IL-15 was replaced by the murine Ig κ-chain signal sequence (METDTLLLWVLLLWVPGSTGDY), and this virus was designated IL-15(IS) vaccinia.

Confocal Microscopy. CV-1 cells were infected with recombinant vaccinia viruses at a multiplicity of infection of 0.1. Eighteen hours after infection, the cell monolayer was fixed with a mixture of methanol and acetone (1:1 mixture) and intracellular IL-15 was detected with an anti-human IL-15 antibody followed by a rhodamine-labeled second antibody. Fluorescent images were

Abbreviations: RPA, ribonuclease protection assay; pfu, plaque-forming units; NK, natural killer.

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visualized and recorded with an Olympus Confocal Imaging System (New Hyde Park, NY).

Ribonuclease Protection Assay (RPA). Mice were killed by cervical dislocation, and internal organs, namely, spleen, liver, lung, and brain, were harvested and flash-frozen immediately. Approximately 10 mg of tissues from each organ was processed for total RNA isolation by addition of TRIzol reagent (Life Technologies, Grand Island, NY) and homogenized according to the manufacturer's instructions. Ten micrograms of total RNA was used to determine the levels of expression of a select set of cytokines and chemokines by a sensitive RPA, as described (15). Cytokine and chemokine riboprobe template panels were purchased from PharMingen.

Detection of Viral DNA by PCR. Cellular DNA was isolated from infected tissues by using a commercially available kit (Boehringer Mannheim) according to the manufacturer's instructions. Five micrograms of cellular DNA was used in PCR amplification of the *Escherichia coli* β-galactosidase DNA sequences with *Pfu* Turbo DNA polymerase (Stratagene). One-tenth of each reaction mixture was then electrophoresed in a 1.5% agarose gel and Southern blotted before probing with an internal primer labeled with ^{32}P. The sequences of the primers were as follows: 5′-actatcccgaccgccttact; 3′-gctggtttccatcagttgct; internal probe, ttcaacatcagccgctacag.

Flow Cytometry. Spleens were harvested from mice infected with vaccinia virus recombinants, and single-cell suspensions were made for flow cytometric analysis. Cells were first incubated with Fc Block-2.4G2 (PharMingen) antibody to block Fcγ III/II receptors before staining with a specific antibody against pan-NK cell marker (phycoerythrin-conjugated DX5). After 30-min incubation on ice, the cells were washed three times in FACS buffer (PBS with 3% FCS plus 0.05% NaN_3), fixed with 1% paraformaldehyde, and analyzed on a FACScan by using CELL QUEST software (Becton Dickinson).

Results and Discussion

Characterization of Recombinant Vaccinia Viruses. The presence of IL-15 mRNA that is constitutively expressed in many tissues without demonstrable protein has implicated a predominantly posttranscriptional regulatory control for this cytokine operating most likely at the level of translation and translocation (reviewed in ref. 3). Transfection studies as well as *in vitro* translation studies have defined many regulatory elements that contribute to the apparent discordance in transcription and translation of IL-15. The 48-aa signal sequence of IL-15, which is unusual not only in length but also in the topological distribution of hydrophobic and hydrophilic amino acid residues for a classical eukaryotic signal sequence, has been suggested to be a formidable impediment for efficient translation of IL-15 mRNA (3, 16). Thus, in creating a vaccinia recombinant to express IL-15, recognizing the potential difficulties that the native signal sequence may pose for efficient synthesis of IL-15, we generated two different versions, one with the 48-aa native signal sequence [IL-15(NS) vaccinia] and another with the signal sequence of the Ig κ light chain replacing the native signal sequence [IL-15(IS) vaccinia].

To verify that the recombinants express IL-15, CV-1 cell monolayers were infected with each of the recombinant viruses. The input virus inoculum was adjusted to obtain a multiplicity of infection of 0.001 so that when measuring the levels of secreted IL-15 in the culture supernatant, potential carryover of IL-15 in the original inoculum was minimized. Secreted IL-15 was monitored in the culture supernatants by ELISA assay. As depicted in Fig. 1, not only was secreted IL-15 detectable in the supernatants, but also the kinetics of expression were rapid. Impressively, within 8 h after infection, secreted IL-15 levels exceeded 100 pg for both recombinants and continued to rise even up to 48 h when cytopathic effects were evident in over 75% of the infected monolayer. Equally important was the fact that the recombinant virus bearing the native signal sequence [(IL-15(NS) vaccinia] was very efficient in producing secreted IL-15, although it consistently lagged behind slightly in total yields in multiple repeats of CV-1 cell infections. Similar yields and expression kinetics for the two recombinants were also observed in 143B human osteosarcoma cells and HeLa cells (data not shown). Moreover, supernatants from monolayers infected with the control vaccinia virus failed to yield any meaningful amounts of IL-15. It should be emphasized that the control vaccinia virus used in this experiment was created in an identical manner, except that the transfer vector pSC11 used for recombination into WR strain of vaccinia contained no cloned insert. Moreover, the control virus and the IL-15-expressing recombinants were indistinguishable in plaque morphology and replicating efficiency in a number of different cell lines tested, indicating that the expression of IL-15 did not affect vaccinia replication *in vitro*, and thus implying an absence of any direct antiviral effects for IL-15 (data not shown).

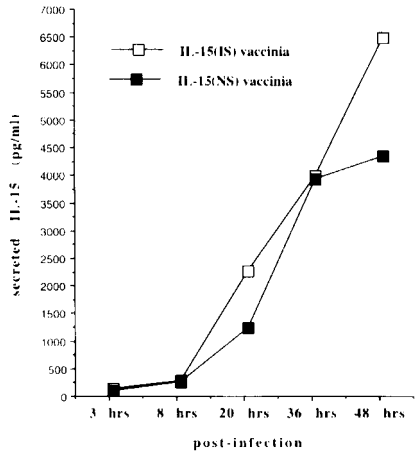

Fig. 1. Secretion of IL-15 by recombinant vaccinia viruses. Recombinant vaccinia viruses with the coding region of human IL-15 gene under the control of vaccinia virus P7.5 early–late promoter were created as described in *Materials and Methods*. In IL-15(IS) vaccinia virus, the signal sequence of murine IgG κ light chain mediates the secretion of IL-15, whereas in IL-15(NS) vaccinia virus, the secretion is mediated by the native signal sequence. CV-1 cells were infected with the recombinant viruses at a multiplicity of infection of 0.001, and the secreted IL-15 in culture supernatants was measured by ELISA.

Intracellular trafficking and subcellular compartmentalization of IL-15, as dictated by its signal peptide, have been studied in transient transfection assays previously (3, 16). However, the availability of two vaccinia virus recombinants, IL-15(NS) vaccinia and IL-15(IS) vaccinia, allowed us to compare the cellular distribution profiles of IL-15 having its cognate 48-aa atypical signal peptide versus the distribution profile of IL-15 carrying a prototypical secretary signal sequence. Fig. 2 represents a confocal micrograph of CV-1 cells examined 18 h after infection. The intracellular distribution profiles of the two IL-15 versions were clearly different, IL-15 with its cognate signal sequence being more punctate and distributed throughout the cytoplasm, whereas the Ig-signal sequence bearing IL-15 was predominantly confined to a perinuclear vicinity likely to be the Golgi region. Clearly more work is needed to elucidate the mechanistic aspect of intracellular trafficking and secretion of IL-15, and the recombinant vaccinia viruses we generated would likely be valuable tools in these endeavors.

The biological activity of the vaccinia-expressed IL-15 was

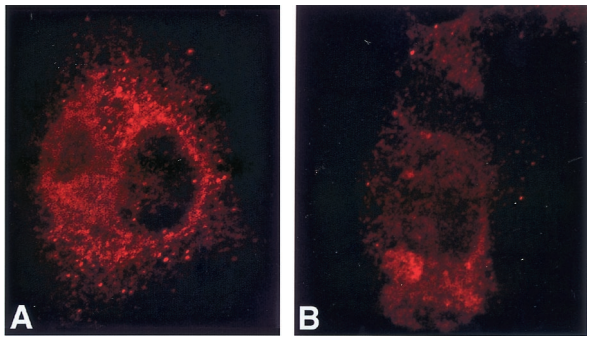

Fig. 2. Intracellular distribution of IL-15 in CV-1 cells infected with recombinant vaccinia viruses. CV-1 cells were infected with the respective recombinant viruses, and the expression profile of IL-15 was examined by confocal microscopy 18 h after infection. In *A*, cells were infected with IL-15(NS) vaccinia virus, and in *B*, cells were infected with IL-15(IS) vaccinia virus.

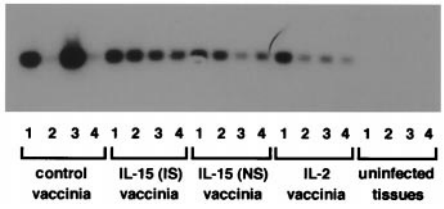

Fig. 3. Quantitative assessment of viral DNA in tissues of infected mice. Nude mice were inoculated i.v. with 10^6 pfu of the respective recombinant vaccinia viruses, and tissues were harvested 72 h after infection. Genomic DNA was then isolated from lung (lanes 1), spleen (lanes 2), brain (lanes 3), and liver (lanes 4). Each sample represents tissues pooled from three animals, and the recovery of DNA was first standardized to the cytochrome-*c* oxidase II gene content in each sample before PCR amplification of the β-galactosidase gene.

confirmed by using the murine thymoma cell line CTLL-2, which requires IL-2 or -15 for proliferation (data not shown). It should be noted that, although many T cell lines are relatively resistant to vaccinia virus infection (17), CTLL-2 cells supported lytic replication of vaccinia, and therefore prior inactivation of vaccinia viral infectivity was necessary when assessing the IL-15 biological activity in CV-1 cell culture supernatants from infected monolayers. Psoralen treatment, as previously reported (18), very effectively eliminated vaccinia viral infectivity in the culture supernatants.

Course of Vaccinial Disease in Athymic Nude Mice. To assess whether the expression of IL-15 cytokine would modify the course of the disease *in vivo*, we infected immunodeficient athymic nude mice with the recombinant vaccinia viruses. Initially, mice (five per group) were inoculated i.p. with increasing concentrations of the virus and observed for a period of 6 weeks. Table 1 summarizes the mortality results of three independent experiments. Mice inoculated with the control vaccinia virus displayed 100% mortality in all virus doses tested [10^4 plaque-forming units (pfu) through 10^7 pfu], whereas in both IL-15(NS) vaccinia and IL-15(IS) vaccinia virus-inoculated groups, no mortality was seen with 10^4–10^5 pfu doses. However, in mice injected with 10^6 pfu of IL-15(IS) vaccinia, significant mortality occurred during the observation period (86.6% $\pm$ 11.5). Curiously, less mortality (20% $\pm$ 20) resulted in mice inoculated with the same dose of IL-15(NS), although at 10^7 pfu, all mice succumbed regardless of whether they received IL-15(IS) or IL-15(NS). However, at doses where deaths occurred with IL-15-expressing vaccinia infections, a longer time lapse before death was observed consistently in these mice compared with mice infected with comparable doses of the control virus. A vaccinia recombinant that expresses human IL-2 gene (a generous gift from Bernard Moss, National Institute of Allergy and Infectious Diseases, Bethesda, MD), generated in a manner similar to that of IL-15-expressing recombinants (designated here as IL-2 vaccinia for easy comparison of the recombinant viruses), not only served as an important control in these experiments but also allowed direct comparisons to be made between these two functionally related cytokines. The important findings that emerged from the above experiment are: (*i*) coexpression of IL-15 resulted in attenuation of the virulence, enabling the immunodeficient nude mice to tolerate and recover from an $\approx$100-fold higher vaccinia virus challenge, and (*ii*) attenuation of virulence was far superior with the coexpression of IL-2 than with IL-15.

Viral Load Assessments in Organs of Infected Nude Mice. Innate host responses to viral infections become operational quite early in the infection to curtail the spread and replication of the invading virus (reviewed in refs. 11 and 12). Hence, we evaluated the impact of cytokine coexpression on the viral load in lung, spleen, brain, and liver after i.v. inoculation of respective vaccinia recombinants. Because all recombinant vaccinia genomes used in this study contain the *E. coli* β-galactosidase gene, the viral load that is a direct correlate of the amount of virus present in each organ was assessed by PCR amplification of this gene from the tissue DNA extracted 72 h after infection. As shown in Fig. 3, extensive replication of the control vaccinia virus in the brain was consistent with the neurotropic characteristic of this virus. More importantly, the viral loads generally paralleled the degree of virulence attenuation observed in Table 1, such that IL-2 vaccinia displayed the least amounts of viral DNA in all organs examined.

Comparison of Cytokine Expression Profiles in Tissues of Infected Mice. Because both IL-2 and -15 are devoid of any direct antiviral effects, the attenuation of vaccinia viral virulence associated with the coexpression of these cytokines is likely to be secondary to the induction of cytokines with inherent antiviral activity or recruitment and activation of cellular components of the innate immune system that restrict viral replication and rapidly eliminate vaccinia infected cells. More potent attenuation of virulence observed with IL-2 vaccinia in comparison to IL-15-

Table 1. Effects of coexpression of IL-15 or -2 on viral virulence in athymic nude mice

	Percent mortality after i.p. inoculation of virus at dose (pfu)			
Virus	10^4	10^5	10^6	10^7
Control vaccinia	100	100	100	100
IL-15(IS) vaccinia	NM	13.3 $\pm$ 11.5	86.6 $\pm$ 11.5	100
IL-15(NS) vaccinia	NM	NM	20.0 $\pm$ 20.0	100
IL-2 vaccinia	NM	NM	NM	NM

Groups of five mice were inoculated with the indicated virus dose and observed for 6 weeks. The data shown are the geometric means $\pm$ SD from three separate experiments. NM, no motality observed.

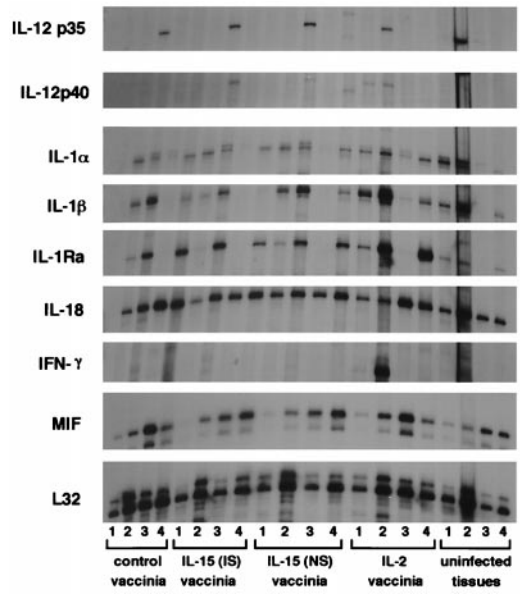

Fig. 4. Cytokine expression in tissues of infected mice. Nude mice were inoculated i.v. with 10^6 pfu of the respective recombinant vaccinia viruses and tissues were harvested 36 h after infection. Total cellular RNA was then isolated from lung (lanes 1), spleen (lanes 2), brain (lanes 3), and liver (lanes 4), and the cytokine mRNA levels were determined by RPA as described in *Materials and Methods*. Each sample represents tissue RNA pooled from three animals. The expression levels of ribosomal gene L32 served as an internal control for RNA integrity. Data are representative of two similar experiments performed.

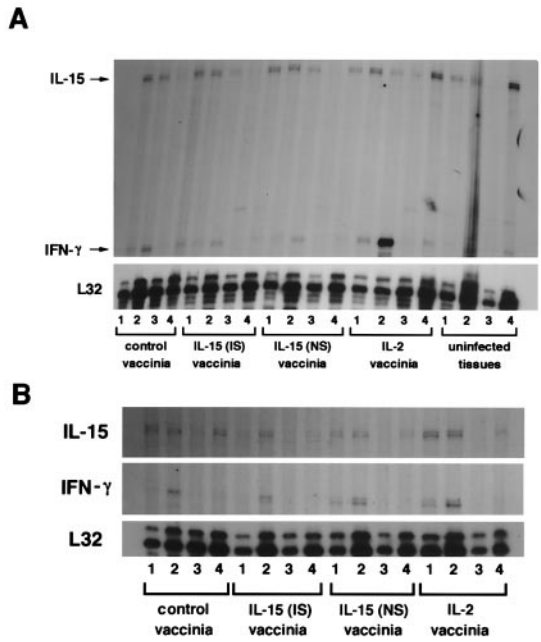

Fig. 5. Effects of recombinant vaccinia virus infection on endogenous IL-15 expression. Nude mice were inoculated i.v. with 10^6 pfu of the respective recombinant vaccinia viruses, and tissues were harvested 36 h (*A*) and 10 days (*B*) after infection. Total cellular RNA was then isolated from lung (lanes 1), spleen (lanes 2), brain (lanes 3), and liver (lanes 4), and IL-15 mRNA levels were determined by RPA as described in *Materials and Methods* by using a riboprobe fragment specific for murine IL-15. Each sample represents tissue RNA pooled from three animals. IFN-γ represents a gene that is modulated early in vaccinial infection. The expression levels of ribosomal gene L32 serve as an internal control for RNA integrity. Data are representative of two similar experiments performed.

expressing recombinants further strengthens the notion that, despite their many similar biological activities, qualitative and/or quantitative differences between IL-2 and -15 exist.

We next examined the expression profiles of a select set of cytokines that are likely to be involved in innate immunity by virtue of their recognized antiviral effects or influence on effector cells. Total cellular RNA prepared either 36 h or 10 days after infection from lung, spleen, brain, and liver after i.v. administration of vaccinia recombinants were evaluated by a sensitive RPA. As shown in Fig. 4, when expression profiles of IL-12, -1α, -1β, -1Ra (receptor antagonist), -18, IFN-γ, and macrophage migration inhibitory factor were assessed 36 h after infection, striking differences were apparent in the IL-2 vaccinia-infected mice, particularly in the spleen where dramatic induction of IFN-γ (20-fold), as well as significant up-regulation of IL-1 β (4-fold) and IL-1Ra (5-fold), were seen. In addition, elevated IL-1Ra was evident in the liver of these mice. Up-regulation of inducible IL-12 p40 in the spleen was seen in animals inoculated with IL-2 vaccinia, IL-15(IS) vaccinia, and IL-15(NS) vaccinia but not in the spleens of animals inoculated with the control vaccinia virus. Dramatic early induction of IFN-γ expression in the spleen that was unique to IL-2 vaccinia infection was not sustained throughout the course of infection. As shown in Fig. 5 (compare *A* and *B*) in RNA samples from tissues harvested day 10 after infection, IFN-γ expression levels, particularly in the spleen, were comparable in animals inoculated with any of the recombinant viruses including the control vaccinia virus. The constitutive expression of murine IL-15 in a wide range of tissues was also evident in Fig. 5, which was not modulated by vaccinia viral infection although certain viral agents have been implicated in up-regulating the expression of this cytokine (19, 20). Nonetheless, when we compared the expression levels of tumor necrosis factor (TNF)-α, TNF-β, lymphotoxin-β, and transforming growth factor-β1, no significant changes were noted in tissues harvested 36 h after infection or day 10 after infection (data not shown).

Comparison of Chemokine Expression Profiles in Tissues of Infected Mice. Chemokines are pivotal in orchestrating the innate immune responses to invading pathogens and their ability to recruit and activate effector cells of the innate immune system, especially in certain viral infections (21–23) such as murine cytomegalovirus, is indispensable for the survival of the host (24). To explore whether coexpression of IL-2 or -15 during vaccinia infection modulates the expression of chemokines that may account for the differences in attenuation of virulence, mRNA levels for a select set of chemokines were measured by RPA. Fig. 6*A* illustrates the expression profiles of chemokines in tissues harvested 36 h after infection, whereas in Fig. 6*B*, tissues were harvested 10 days after infection. Although at 36 h after infection, RANTES expression in the spleen was only slightly more in mice infected with the cytokine-expressing recombinants, especially the IL-2 vaccinia virus, by day 10 after infection, its expression was markedly elevated not only in the spleen but also in the lung. In contrast, in mice infected with the control virus, the expression levels of RANTES not only were relatively low in the spleen but remained undetectable in the lung, a tissue that vaccinia virus targets. Also, it should be noted that other chemokines such as lymphotactin, macrophage inflammatory protein-1β, TCA-3 (I-309), and monocyte chemoattractant protein-1 were induced particularly in the splenic tissues in vaccinia infection, although no differences were observed in the levels of expression of these chemokines among the recombinant viruses.

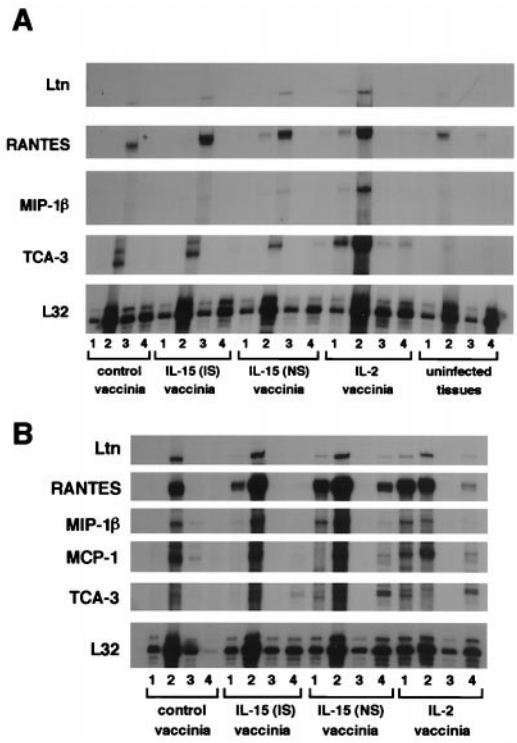

Fig. 6. Effects of recombinant vaccinia virus infection on chemokine expression in tissues of infected mice. Nude mice were inoculated i.v. with 10^6 pfu of the respective recombinant vaccinia viruses and tissues were harvested 36 h (*A*) and 10 days (*B*) after infection. Total cellular RNA was then isolated from lung (lanes 1), spleen (lanes 2), brain (lanes 3), and liver (lanes 4), and the chemokine mRNA levels were determined by RPA as described in *Materials and Methods*. Each sample represents tissue RNA pooled from three animals. The expression levels of ribosomal gene L32 serve as internal controls for RNA integrity. Data are representative of two similar experiments performed.

Quantitation of Serum Cytokine Levels. In cell culture infections, when the levels of secreted IL-15 or -2 were measured in parallel, IL-15(IS) consistently yielded slightly more IL-15 than -15(NS), and the yield of IL-2 from IL-2 vaccinia was comparable to that seen with IL-15 recombinants (see Table 2). However, as evident from the data in Table 1, consistently somewhat better attenuation occurred *in vivo* with IL-15(NS). This observation was unexpected, because if IL-15 contributes to the attenuation of vaccinia infection, one would have expected IL-15(IS) to provide greater protection as it produces more secreted IL-15 on the basis of *in vitro* secretion data. Alternatively, although the atypical cognate signal sequence carrying IL-15 is slightly sluggish in being secreted from *in vitro*-infected established cell lines, one could speculate that *in vivo* in certain infected cells, the cognate signal sequence might still perform more optimally. If this were the case, then one might find more serum IL-15 in mice infected with IL-15(NS) vaccinia virus than in mice infected with IL-15(IS) vaccinia. As shown in Table 2, when serum IL-15 levels were measured in mice 5 days after infection following i.v. administration of 10^6 pfu virus, this indeed was proven to be true. Furthermore when organ cultures made from lung, liver, spleen, and brain of nude mice were infected *ex vivo* with the same input virus titers and the secreted IL-15 was measured, IL-15(NS) vaccinia yielded more IL-15 in every organ consistently (data not shown). Thus we are left to conclude that, despite its atypical length and amino acid composition, the signal peptide of IL-15 functions optimally *in vivo* perhaps in a subset of cell populations with a secretion efficiency similar to that of a prototype signal sequence.

Table 2. Recombinant vaccinia virus expressed cytokines measured by ELISA

Recombinant virus	Cytokine expressed	Culture supernatant*	Infected animal serum†
IL-15(IS) vaccinia	IL-15	2.4 ± 0.35 ng/ml	72.3 ± 6.4 pg/ml
IL-15(NS) vaccinia	IL-15	1.5 ± 0.21 ng/ml	83.0 ± 6.6 pg/ml
IL-2 vaccinia	IL-2	1.4 ± 0.25 ng/ml	76.0 ± 5.6 pg/ml

*CV-1 cells were infected with recombinant viruses at a multiplicity of infection of 0.01 and secreted cytokines were measured 36 h after infection. The means of triplicate samples ± SD are shown.

†10^6 pfu of respective recombinant viruses were inoculated intravenously, and serum was collected 5 days after infection and assayed for cytokines by ELISA. The values shown are the means ± SD from three individual animals.

Previous studies have clearly established that recombinant vaccinia viruses expressing either murine or human IL-2 are eliminated in immunoincompetent mice (25, 26), and the induction of host IFN-γ is pivotal in this process. Prior administration of anti-IFN-γ antibodies or in mice genetically made deficient in IFN-γ or its receptor, the IL-2-mediated attenuation of virulence was absent (17, 27, 28). Not only was induction of IFN-γ reaffirmed in the present study, but we also demonstrated that this relatively rapid (within 36 h after infection) induction of IFN-γ mRNA primarily, if not exclusively, occurs in the spleen (see Figs. 4 and 5*A*). Moreover, when serum levels of IFN-γ were measured 5 days after infection by ELISA, mice inoculated with the cytokine-expressing vaccinia recombinants had significant amounts of circulating IFN-γ, whereas no measurable IFN-γ was detected in sera from control vaccinia-infected animals. Specifically, in IL-2 vaccinia-infected animals 128.7 ± 7.0 pg/ml; IL-15(NS) vaccinia-infected animals 48 ± 4.7 pg/ml; and IL-15(IS) vaccinia-infected animals 22.3 ± 3.1 pg/ml of serum IFN-γ were detected (values are the means of IFN-γ from three individual mice per group ± SD). Equally important was a correlation between the levels of circulating IFN-γ induced by a particular recombinant vaccinia with the degree of attenuation in virulence that virus displayed. For example, IL-2 vaccinia was most robust in inducing IFN-γ and displayed the highest attenuation, whereas IL-15(IS) vaccinia that induced the lowest levels of IFN-γ displayed the least attenuation in virulence (compare with Table 1). Previous studies examining the role of IFN-γ in mice infected with IL-2-expressing vaccinia virus in mediating the survival of these immunodeficient mice that lack functional T cells to survive an otherwise lethal infection have implicated NK cells as the primary source of induced IFN-γ (17, 27). It is important to note that extrathymically derived γδ-T cells present in these nude mice are also likely to be a source of IFN-γ (29). The fact that both γδ-T cells and monocyte/macrophage cells are functionally augmented by these cytokines (4) suggests that these cell populations, too, may contribute to the mitigation of virulence. However, elimination of NK cells from nude mice results in lethal infection when inoculated with IL-2 expressing vaccinia virus (17, 27).

Because of our observation that the most dramatic induction of IFN-γ expression occurred in the spleen after IL-2 vaccinia infection, we examined the NK cell content in this organ 3 days after i.v. inoculation of vaccinia virus by using an antibody (DX5) against a murine pan-NK cell marker by flow cytometry. The highest percentage of NK cells was present in the spleens of mice inoculated with IL-2 vaccinia (13%), whereas in mice infected with IL-15-expressing vaccinia recombinants, the percentage of NK cells in the spleen was appreciably lower (≈9%), although it was still higher than the value obtained in mice inoculated with the control vaccinia virus (≈5%). Thus, NK cell content in the spleen after infection with recombinant viruses parallels both IFN-γ mRNA levels in the spleen as well as serum IFN-γ levels.

For example, infection with IL-2 vaccinia resulted in the greatest percentage of splenic NK cells, serum IFN-γ levels and most abundant IFN-γ mRNA in the spleen. Our findings are consistent with the ability of both IL-15 and -2 to augment NK cell activity as well as to produce IFN-γ in these cells (4, 30, 31). IFN-γ production in murine NK cells is enhanced via CD28-mediated events and IL-15 has been shown to induce the expression of CD28. Thus, IL-15 is likely to enhance the production of IFN-γ in mouse NK cells via both direct as well as indirect pathways (reviewed in ref. 4). In addition to IFN-γ-mediated antiviral effects, the direct lytic activity of NK cells toward virally infected cells constitutes an important antiviral host defense mechanism. Recent *in vitro* studies with human peripheral blood mononuclear cells (PBMCs) have indicated that a wide variety of viruses that infect human PBMCs induce expansion of NK cells with heightened lytic activity that can be effectively abrogated by using antibodies against IL-15, further supporting an important role for IL-15 in NK-mediated antiviral activity (19, 20, 32, 33).

It is intriguing to note that in every parameter we evaluated that was likely to reflect peripheral NK cell activity, IL-2 appears to outperform IL-15. In fact, we failed to observe any mortality even when we administered 10^9 pfu of IL-2 vaccinia i.v. to nude mice (data not shown). Although IL-15 is indisputably pivotal in the establishment and perhaps in the maintenance of peripheral NK cell populations (5, 6), our data suggest that in the periphery, IL-2, when present, may be more effective in stimulating NK cell functions. In this regard, although IL-2 is not constitutively produced like IL-15, recent evidence suggesting its presence in the extracellular matrix bound to glycosaminoglycans like heparan sulfate in a functionally active form even in young pathogen-free mice (34) is likely to indicate a potential mechanism underlying the IL-2 influence early in innate immune responses before the onset of T and B cell-dependent adaptive responses. The ability of IL-2 to enhance the functional activity of both NK cells and monocytes/macrophages that are important cellular components of innate immunity further illustrates the potential role of IL-2 in innate immunity (35, 36).

In conclusion, by using an *in vivo* infection model system in which T cells are absent and where the primary host defense mechanisms depend on NK cells of the innate immune system, we have examined the effects of locally secreted IL-15 cytokine that is essential for NK cell biology. Our data are consistent with the notion that IL-15 augments the innate host defense mechanisms that are likely to involve: (*i*) induction of IL-12 and IFN-γ secretion with ensuing effects both locally and systemically, (*ii*) induction of chemokines such as RANTES that also influence NK cell functions, and (*iii*) NK cell chemotaxis to sites of inflammation.

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