Epstein-Barr Virus IL-10 Engages IL-10R1 by a Two-step Mechanism Leading to Altered Signaling Properties*§

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Background: ebvIL-10 is a viral mimic of human IL-10 (hIL-10).

Results: ebvIL-10 assembles the ebvIL-10-IL-10R1 complex in a two-step process that alters its signaling properties relative to hIL-10.

Conclusion: Distinct receptor engagement by ebvIL-10 enhances its bioactivity on cells with high IL-10R1 levels.

Significance: Elucidating differences in hIL-10 and ebvIL-10 function is critical to understand EBV immune evasion.

Human IL-10 (hIL-10) is a pleiotropic cytokine that is able to suppress or activate cellular immune responses to protect the host from invading pathogens. Epstein-Barr virus (EBV) encodes a viral IL-10 (ebvIL-10) in its genome that has retained the immunosuppressive activities of hIL-10 but lost the ability to induce immunostimulatory activities on some cells. These functional differences are at least partially due to the ~1000-fold difference in hIL-10 and ebvIL-10 binding affinity for the IL-10R1:IL-10R2 cell surface receptors. Despite weaker binding to IL-10R1, ebvIL-10 is more active than hIL-10 in inducing B-cell proliferation. To explore this counterintuitive observation further, a series of monomeric and dimeric ebvIL-10:hIL-10 chimeric proteins were produced and characterized for receptor binding and cellular proliferation on TF-1/hIL-10R1 cells that express high levels of the IL-10R1 chain. On this cell line, monomeric chimeras elicited cell proliferation in accordance with how tightly they bound to the IL-10R1 chain. In contrast, dimeric chimeras exhibiting the highest affinity for IL-10R1 exhibited reduced proliferative activity. These distinct activity profiles are correlated with kinetic analyses that reveal that the ebvIL-10 dimer is impaired in its ability to form a 1:2 ebvIL-10-IL-10R1 complex. As a result, the ebvIL-10 dimer functions like a monomer at low IL-10R1 levels, which prevents efficient signaling. At high IL-10R1 levels, the ebvIL-10 dimer is able to induce signaling responses greater than hIL-10. Thus, the ebvIL-10 dimer scaffold is essential to prevent activation of cells with low IL-10R1 levels but to maintain or enhance activity on cells with high IL-10R1 levels.

Human IL-10 (hIL-10)² is a dimeric immunomodulatory cytokine involved in the complex regulation of innate and adaptive immunity to self-antigens and foreign antigens (1). As a potent anti-inflammatory cytokine, hIL-10 limits immune responses mounted against pathogens to prevent irreversible damage to the host. hIL-10 suppresses inflammation by inhibiting the production of proinflammatory cytokines (e.g. IFNγ, IL-1, TNF, and IL-12) produced by numerous cell types and the expression of MHC class II and B7 on antigen-presenting cells (2–7). hIL-10 also mediates several immunostimulatory functions by promoting the proliferation or differentiation of thymocytes, mast cells, and B-cells (8–11).

hIL-10 biological activity requires the assembly of a cell surface receptor heterodimer consisting of IL-10R1 and IL-10R2 chains (12, 13). hIL-10 binding to the extracellular domains of IL-10R1-IL-10R2 activates intracellular Jak family tyrosine kinases, Jak1 and Tyk2, and subsequently intracellular signaling pathways (e.g. STAT3) leading to cellular responses (14, 15).

The Epstein-Barr virus (EBV) is a γ-herpesvirus carried by a high percentage of the human population as a life-long asymptomatic infection (16). EBV induces infectious mononucleosis and is tightly linked to the emergence of B- and epithelial cell malignancies (17). To successfully establish latent infections, EBV has evolved several immune evasion strategies that disrupt host antiviral immune responses (2). One strategy used by EBV is to produce a “viral IL-10” protein (ebvIL-10) upon infection that shares 83% amino acid sequence identity with hIL-10 (18) (Fig. 1). Functional studies demonstrate that ebvIL-10 suppresses proinflammatory cytokine production like hIL-10 and also inhibits MHC class II expression on monocytes. However, unlike hIL-10, ebvIL-10 does not stimulate the proliferation of thymocytes and mast cells and does not enhance the expression of MHC class II on B-cells (10, 11, 19, 20). These studies suggest that ebvIL-10 has been altered, relative to hIL-10, to efficiently disrupt host immune responses to EBV.

The abbreviations used are: hIL-10, human IL-10; ebvIL-10, Epstein-Barr virus IL-10; Bicine, N,N-bis(2-hydroxyethyl)glycine; RU, response units; SPR, surface plasmon resonance; NT, N terminus; A8, AB loop; CD, CD loop; CT, C terminus.

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The mechanistic basis for the distinct biological activities of hIL-10 and ebvIL-10 remains poorly understood. Cell binding studies have shown that ebvIL-10 exhibits ~1000-fold lower affinity for IL-10R1 than hIL-10 (21). The reduced affinity of ebvIL-10 for IL-10R1 makes ebvIL-10 unable to induce signaling in cells with low levels of IL-10R1, which are still responsive to hIL-10 (22). However, as pointed out by Ding et al., once sufficient IL-10R1 levels are expressed on cells, there does not seem to be a strict correlation between affinity, receptor densities, and signaling responses (22). For example, ebvIL-10 is more potent than hIL-10 in proliferating activated human B-cells (8, 21).

To further define differences in ebvIL-10 and hIL-10 receptor engagement, a series of ebvIL-10/hIL-10 chimeric proteins were generated (Fig. 1), and their receptor binding and cell proliferative activities were determined. We show here that the ebvIL-10 dimer engages IL-10R1 by a two-step binding mechanism, where the first binding event mimics an ebvIL-10 monomer interaction, which allows the molecule to discriminate between low and high levels of IL-10R1. In contrast, the hIL-10 dimer efficiently binds IL-10R1 by a simple one-step binding mechanism with high affinity. The distinct mechanism of IL-10R1 engagement by ebvIL-10 provides a biochemical/structural model for the complex biological activities of ebvIL-10 on various cell types.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis, Expression, and Purification of IL-10M1**—The ebvIL-10 or cIL-10 gene was mutated either by PCR amplification with primers containing mutated sequences or by the QuikChange site-directed mutagenesis kit (Stratagene). The variants of hIL-10 and ebvIL-10 were overexpressed using Escherichia coli strain BL21 (DE3) or Rosetta2 (DE3), and purified as previously described for hIL-10 (23, 24).

**Expression, Purification, and Biotinylation of IL-10R1 and IL-10R1/IL-10R2 Constructs for SPR**—SIL-10R1_T213C was expressed in Drosophila S2 cells and purified using an hIL-10 affinity column (25). The receptor was biotinylated with polyethylene oxide-maleimide-activated biotin (Pierce) as described previously (26).

IL-10R1^WZ and IL-10R1/IL-10R2^WZ were made by generating three different plasmids, 10R1WZB1, 10R2WZA2Avi, and WZA2Avi. 10R1WZB1 consists of IL-10R1 residues 21–234 (Uniprot code Q13651), a 13-residue linker, followed by the WinZip B1 coiled-coil domain described by Arndt et al. (27). 10R2^WZA2 consists of IL-10R2 residues 20–220, a 13-residue linker, the WinZip A2 coiled-coil (27), and a 15-residue AviTag (Avidity) sequence (28). Finally, the WZA2Avi plasmid contains the WinZip A2 coiled coil followed by the 15-residue AviTag sequence (28). Immediately before the coiled-coil in each plasmid, a glycine-cysteine-glycine sequence (GCC) is encoded in each plasmid, which forms a disulfide bond, to covalently couple the WinZip B1/A2 coils and associated receptors to one another.

IL-10R1/IL-10R2^WZ and IL-10R1^WZ were expressed by co-transfecting plasmids 10R1WZB1 + 10R2WZA2Avi or 10R1WZB1 + WZA2Avi in Drosophila S2 cells. All transfections were performed by calcium phosphate precipitation as described by the manufacturers and included the hygromycin resistance vector, pCoHYGRO (Invitrogen), for selection using hygromycin for 3 weeks. Protein expression was induced with 0.5 mM CuSO₄ in serum-free Insect-XPRESS medium (BioWhittaker).

IL-10R1^WZ and IL-10R1/IL-10R2^WZ were purified using a hIL-10 affinity column as described previously (25). SDS-PAGE analysis of purified IL-10R1^WZ and IL-10R1/IL-10R2^WZ confirmed that the IL-10R1 and IL-10R2 chains are linked by disulfide bonds. Biotinylation of IL-10R1^WZ and IL-10R1/IL-10R2^WZ was performed with bovine-protein ligase (birA) according to the manufacturer’s instructions (Avidity). 160 µl of 34 µM protein samples in 10 mM Tris, pH 8.0, were mixed with 3 µl of 1 mg/ml birA, 20 µl of 0.5 M Bicine, pH 8.3, and 20 µl of 100 mM ATP, 100 mM MgOAc, and 500 µM d-biotin. The reaction proceeded at room temperature for 2 h. Protein biotinylation was verified by Western blotting with HRP-conjugated streptavidin. The reaction mixture was dialyzed against 20 mM Tris, pH 8.0, 150 mM NaCl.

Biotinylated IL-10R1^WZ and IL-10R1/IL-10R2^WZ (IL-10R1^WZ-Br and IL-10R1/IL-10R2^WZ-Br, respectively) were puri-
Two-step IL-10R1 Binding Mechanism by ebvIL-10

fied by a monomeric avidin column (column volume, 2 ml; Pierce) following the manufacturer’s protocol. Briefly, samples were loaded on the monomeric avidin column equilibrated with PBS, and biotinylated proteins were eluted with 2 mM biotin in PBS after column washing with PBS. The purified proteins were dialyzed against 10 mM Hapes, pH 7.4, 150 mM NaCl.

Monomer SPR Analysis—Biacore 2000 and 3000 instruments were used to determine the affinity of monomeric hIL-10 and ebvIL-10 chimeras for IL-10R1 T213C-Bt, IL-10R1 W2Z-Bt, and IL-10R1-IL-20R1 WZ-Bt. Streptavidin (Pierce) in 10 mM sodium acetate (pH 5.0) was immobilized at the level of 2000–3600 RU by amine-coupling chemistry on CM5 chips (Biacore), which had been activated with a 1:1 mixture of 0.1 M N-hydroxysuccinimide and 0.1 M 3-(N,N-dimethylaminopropyl)-N-ethylcarbodiimide. IL-10R1 T213C-Bt, IL-10R1 W2Z-Bt, and IL-10R1-IL-10R2 WZ-Bt were captured on streptavidin-coupled CM5 chips to reach a final density of 77-236 RU, 178-212 RU, 262-307 RU, respectively. The control surfaces were coupled with streptavidin alone. IL-10M1 chimeras were injected in a random order at a flow rate of 50 μl/min. Surfaces were regenerated by the injection of 2 M MgCl2 (pH 7.4) for 1 min followed by 20 mM EDTA for 1 min. Kinetic data were prepared by the method of double referencing as described by Rich and Myszka (29). Using BiaEvaluation version 3.2 software, sensorgrams were globally fit to 1:1 binding models.

Proliferation Assay—A proliferation assay was performed using TF-1/IL-10R1 cells as previously described (26). Briefly, IL-10s were diluted in an assay medium (RPMI 1640, 10% FBS, 2 mM l-glutamine, 100 units/ml penicillin, 100 μM streptomycin, 50 μM β-mercaptoethanol) and dispensed into 96-well microplates. Subsequently, 5000 TF-1/IL-10R1 cells were added to each well, and the plates were incubated for 2 days at 37 °C with 5% CO2. To assess viable cell density, alamarBlue (Molecular Probes) was added to each well, and the plates were incubated for 2 days at 32 °C/H9262 in a 5% CO2 incubator. IL-10 dimers were injected over the IL-10R1-FC surface at 100 μl/min. The resulting sensorgrams were fit to 1:1, and bivalent analyte models, using the Biacore T-200 evaluation software version 1.0.

RESULTS

Kinetics of IL-10R1 Binding to ebvIL-10 and hIL-10 Monomers—Prior studies revealed high quality hIL-10/sIL-10R1 kinetic constants could be obtained using an engineered hIL-10 monomer (hIL-10M1 (26)). Surface plasmon resonance (SPR) assays were performed by injecting hIL-10M1 over a biacore chip coupled with biotinylated IL-10R1 (sIL-10R1 T213C-Bt) (Fig. 2). To compare hIL-10-IL-10R1 and ebvIL-10-IL-10R1 binding kinetics, an ebvIL-10 monomer was engineered (ebvIL-10M1), and IL-10R1 binding was determined using the same SPR assay (Fig. 2). The binding constants (Table 1), determined by global fitting of the sensorgrams, reveal that hIL-10M1 and ebvIL-10M1 exhibit essentially identical on-rates (k_{on}) but markedly different off-rates (k_{off}, 200-fold; Table 1). The off-rates correspond to half-lives (t_{1/2}) of 44 min for the hIL-10M1-IL-10R1 complex, compared with 13 s for the ebvIL-10M1-IL-10R1 interaction. The rate constants were used to determine equilibrium binding constants (K_{D} = k_{off}/k_{on}) for the hIL-10M1-IL-10R1 (K_{D} = 0.46 nM) and ebvIL-10M1-IL-10R1 (K_{D} = 121 nM) interactions, which differ from one another by 263-fold. SPR analysis of hIL-10M1-IL-10R1 and ebvIL-10M1-IL-10R1 interactions were repeated using a coiled-coil domain to couple IL-10R1 (IL-10R1 WZ-Bt) to the chip surface (Fig. 2). As shown in Table 1, the method used to couple IL-10R1 to the biacore chip did not significantly alter hIL-10M1 or ebvIL-10M1 binding kinetics.

Kinetic Analysis of IL-10R1 Binding to EBV/Human IL-10M1 Chimeras—Sequence differences between hIL-10 and ebvIL-10 are located in four regions of the molecule, corresponding to the N terminus (NT; 18 residues), AB loop (AB; 3 residues), CD loop (CD; 3 residues), and C terminus (CT; 3 residues) (Fig. 1). To identify residues responsible for the distinct IL-10R1 binding properties of ebvIL-10 and hIL-10, ebvIL-10M1 NT, AB, CD, and CT sequences were replaced with human IL-10 amino acids to generate four ebvIL-10M1 chimeras: ebvIL-10M1 NT, ebvIL-10M1 AB, ebvIL-10M1 CD, and ebvIL-10M1 CT (Fig. 1). The chimeras were purified and subjected to SPR analysis on the IL-10R1 WZ-Bt surface. The binding studies reveal that ebvIL-10M1 AB and ebvIL-10M1 CD exhibit 11.5- and 6.7-fold higher affinity for IL-10R1 compared with ebvIL-10M1, whereas ebvIL-10M1 NT and ebvIL-10M1 CT exhibited increases of 3.2- and 1.8-fold, respectively (Table 2 and supplemental Table S1). Thus, sequence differences between ebvIL-10 and hIL-10 in the AB and CD loops play a larger role in IL-10R1 binding than residues located in the N and C termini (Table 2).

Three single residue AB loop chimeras (ebvIL-10M1 T39M, ebvIL-10M1 E42Q, and ebvIL-10M1 V43L) and one CD loop chimera (ebvIL-10M1 A87I) were created to identify the contributions of specific residues within the loops to IL-10R1 binding (Fig. 1). The analysis of the chimeras by SPR revealed the V43L substitution in the AB loop and A87I substitution in the CD loop were responsible for most of the IL-10R1 affinity increases observed for the ebvIL-10M1 AB and ebvIL-10M1 CD chimeras. The single site ebvIL-10M1 V43L and ebvIL-10M1 A87I chimeras bound IL-10R1 4.3- and 6.3-fold tighter than ebvIL-10M1, which corresponds to ~38 and ~98% of the binding increases observed for ebvIL-10M1 AB and ebvIL-10M1 CD. This result is consistent with the A87I mutation being the single most important residue for receptor binding on cells (20). Interestingly, both Val-43 and Ala-87 pack into the hydrophobic core of the molecule and do not make direct contacts with IL-10R1.

Although residue changes at positions Val-43 and Ala-87 in the ebvIL-10 sequence increase IL-10R1 binding affinity (K_{D} values of 22 and 15 nM, respectively) more than other residue positions tested, these chimeras do not reconstitute “hIL-
10M1-like IL-10R1 binding properties ($K_D = 0.28 \text{ nm}$; Table 2). To further increase IL-10R1 affinity, four additional chimeras were constructed (ebvIL-10M1NT,AB, ebvIL-10M1NT,AB71, ebvIL-10M1AB,AB71, and ebvIL-10M1V43L,AB71) that contain two hIL-10 sequence regions in ebvIL-10M1 (Fig. 1). Analysis of these chimeras by SPR, on the IL-10R1WZ-Bt surface, once again demonstrated the importance of human AB and CD loop sequences (Fig. 1) for high IL-10R1 binding. The chimera with the greatest IL-10R1 affinity was ebvIL-10M1AB,A87I ($K_D = 0.93 \text{ nm}$; Table 2), which regained all but $\sim$3-fold of the binding energy observed for hIL-10M1. Binding parameters obtained for single segment replacements (e.g. ebvIL-10M1NT = 3.2-fold $K_D$ decrease and ebvIL-10M1AB71 = 6.3-fold $K_D$ decrease) could be used to predict the binding parameters of the double segment replacement (e.g. ebvIL-10M1NT,AB71, calculated $K_D$ decrease $= 3.2 \times 3.2 = 20.2$-fold versus observed $K_D$ decrease $= 22.4$; Table 2). Thus, residue changes in these chimeras are essentially independent. The greatest differences between observed and predicted IL-10R1 $K_D$ increases were observed for chimeras containing the AB loop region. Overall, these results reveal that ebvIL-10 alters IL-10R1 binding through a series of subtle sequence changes dispersed between the AB and CD loop of the molecule.

**Biological Potency of Monomeric IL-10 Chimeras Is Proportional to IL-10R1 Binding Affinity**—The IL-10M1 chimeras described above exhibit IL-10R1 binding affinities that differ by

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**TABLE 1**

| SPR parameters for hIL-10/IL-10R1 and ebvIL-10/IL-10R1 interactions | $k_{on}$ ($s^{-1}$) | $k_{off}$ (s) | $t_{1/2}$ ($s$) | $k_{on2}$ | $k_{off2}$ (s) | $t_{1/22}$ ($s$) | $K_{D1}$ | $K_{D2}$ |
|---|---|---|---|---|---|---|---|---|
| hIL-10M1 | $5.7 \times 10^5$ | $2.6 \times 10^{-4}$ | 2665 | 6.5 $\times 10^{-4}$ | 0.46 |
| hIL-10M1H | $7.9 \times 10^5$ | $2.2 \times 10^{-4}$ | 3150 | $1.7 \times 10^{-3}$ | 0.28 |
| hIL-10M1V43L | $8.5 \times 10^5$ | $0.9 \times 10^{-4}$ | 7700 | 14 |
| hIL-10M1NT | $1.8 \times 10^6$ | $4.4 \times 10^{-4}$ | 1575 | 14 |
| ebvIL-10M1NT | $4.3 \times 10^5$ | $5.2 \times 10^{-2}$ | 13.3 | 232 |
| ebvIL-10M1NT,AB | $4.7 \times 10^5$ | $4.4 \times 10^{-2}$ | 15.8 | 14 |
| ebvIL-10M1NT,AB71 | $7.1 \times 10^5$ | $1.0 \times 10^{-2}$ | 69.3 | 5.6 |
| ebvIL-10M1NT,AB,AB71 | $3.1 \times 10^5$ | $7.3 \times 10^{-2}$ | 9.6 | 408 |

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$a$ Parameter derived from the IL-10R1T213C-Bt surface.

$b$ Parameter derived from the IL-10R1WZ-Bt surface.

$c$ Parameter derived from the IL-10R1-FC surface.

$d$ Parameter derived from the IL-10R1-FC surface. $K_{D1}$ and $K_{D2}$ derived from $k_{off}/k_{on}$ and $k_{off2}/k_{on2}$ respectively.

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**FIGURE 2.** Receptor constructs and SPR analysis of hIL-10M1 and ebvIL-10M1. Shown is a schematic diagram of IL-10R1 (A) and IL-10R1-IL-10R2 (D) chip surfaces used to characterize IL-10 monomers. Observed (black) and final calculated (red) sensorgrams were obtained by injecting hIL-10M1 (B) and ebvIL-10M1 (C) over the IL-10R1WZ-Bt chip surface. Residual errors in the fitting are shown below each sensorgram. Sensorgrams were obtained by injecting hIL-10M1 (E) and ebvIL-10M1 (F) over the IL-10R1-IL-10R2WZ-Bt surface.
Two-step IL-10R1 Binding Mechanism by ebvIL-10

TABLE 2

Kinetic parameters and biological activity of monomeric IL-10 chimeras

|     | R1WZ-Bt surface | R1/R2WZ-Bt surface | Bioassay (TF-1 cells) |
|-----|-----------------|--------------------|----------------------|
|     | K_D (nM)        | Increase           | K_D (nM)             | Increase |
|     | nM              | -fold              | nM                   | -fold    | ng/ml       | -fold |
| ebvIL-10M1 | 94            | 1.0                 | 14                    | 1.0          | 9.8         | 1.0 |
| NT   | 29             | 3.2                 | 63                    | 2.2          | 9.7         | 1.0 |
| AB   | 8.2            | 11.5                | 0.82                  | 17.1         | 0.79        | 12.3 |
| CD   | 14             | 6.7                 | 3.5                   | 4.0          | 3.3         | 3.0 |
| CT   | 51             | 1.8                 | 8.3                   | 1.7          | 2.4         | 4.0 |
| T39M | 142            | 0.7                 | 18                    | 0.8          | 4.6         | 2.1 |
| E43Q | 56             | 1.7                 | 7.0                   | 2.0          | 2.9         | 3.4 |
| V35L | 22             | 4.3                 | 1.9                   | 7.4          | 1.2         | 6.3 |
| A87I | 15             | 6.3                 | 2.5                   | 5.6          | 2.6         | 3.8 |
| NT/AB| 1.4            | 67.1                | 0.36                  | 38.9         | 0.84        | 11.7 |
| NT/A87I | 4.2            | 22.4                | 0.93                  | 15.1         | 2.0         | 5.0 |
| AB/A87I | 0.93          | 101.1               | 0.21                  | 66.7         | 0.33        | 29.8 |
| V35L/A87I | 2.5          | 37.6                | 0.36                  | 38.9         | 0.65        | 15.2 |
| hIL-10M1 | 0.28          | 335.7               | 0.11                  | 127.3        | 0.37        | 26.4 |

~336-fold. To quantify the impact of IL-10R1 affinity on IL-10 cell proliferative responses, dose-response curves were generated for each chimera using a TF-1/IL-10R1 cell proliferation assay (Fig. 3) (21). TF-1/IL-10R1 cells are a myeloid progenitor cell line that has been transfected with the human IL-10R1 chain by Liu et al. (21, 32). The cell line provides a robust quantitative assay for characterizing IL-10-mediated cell proliferation that mimics the behavior of hIL-10 and ebvIL-10 on primary activated human B-cells (8). The EC_{50} value derived in the assay defines the biological potency of a particular IL-10 molecule for subsequent comparisons against their measured IL-10R1 affinities. For example, ebvIL-10M1^{AB,A87I}, which exhibits the highest IL-10R1 affinity (K_D = 0.93 nM versus 0.28 nM for hIL-10M1), showed essentially equivalent potency with hIL-10M1 (EC_{50} = 0.37 ng/ml), whereas ebvIL-10M1 and ebvIL-10M1^{NT} displaying low IL-10R1 affinity exhibited the weakest potency with EC_{50} = 9.9 ng/ml (Table 2). A plot of EC_{50} values versus IL-10M1-IL-10R1 K_D results in a regression line with a positive slope and a goodness of fit (R^2) of 0.74. These experiments demonstrate that the strength of the IL-10M1-IL-10R1 interaction is proportional to the biological potency of the IL-10 monomer in this assay. Interestingly, the 336-fold difference in IL-10M1 versus IL-10R1 K_D values causes no more than a 26-fold difference in biological potency.

Role of IL-10R2 in IL-10M1 Receptor Affinity—IL-10 must engage IL-10R1 as well as the IL-10R2 chain to induce biological responses (12). To determine if IL-10R2 significantly alters IL-10 affinity, a soluble IL-10R1-IL-10R2 heterodimer (R1/R2WZ-Bt) was designed and coupled to a biacore chip for SPR analysis of the IL-10M1 chimeras (Fig. 2D). The presence of the IL-10R2 chain increased the affinity of all of the IL-10M1 chimeras by 2.5–11.6-fold (Table 2). However, no correlation was observed between enhanced IL-10R2 binding and IL-10M1 chimeric biological potency. Whereas the chimeras exhibited a 336-fold difference in affinity for IL-10R1, the affinity differences of the chimeras for IL-10R1-IL-10R2 heterodimer were 127-fold. Plotting IL-10R1-IL-10R2 affinity versus IL-10 biological potency slightly improved the linearity of the regression line from 0.74 for IL-10R1 alone to 0.84 for the IL-10R1-IL-10R2WZ-Bt heterodimer (Fig. 4A). Overall, the IL-10R2 chain did not make a significant difference in the relationship between receptor binding strength and biological potency.

Biological Potency of Dimeric IL-10 Chimeras Is Inversely Proportional to Receptor Binding Affinity—IL-10 exists as an intercalated V-shaped dimer (33, 34). Assembly of a 1:2 IL-10-IL-10R1 complex (35, 36) on the cell surface could significantly alter the signaling properties of the molecule. To characterize IL-10 dimer signaling, the biological potency of four dimeric EBV/human IL-10 chimeras (ebvIL-10^{NT}, ebvIL-10^{NT,A87I}, ebvIL-10^{AR,A87I}, and ebvIL-10^{AR}) previously studied in monomeric form (Table 2), were characterized on TF-1/IL-10R1 cells (Table 3). All IL-10 dimers studied exhibited 3.8–987-fold greater potency than equivalent monomeric IL-10 chimeras. However, in contrast to the IL-10 monomers, the hIL-10 dimer, which exhibits the highest affinity for IL-10R1, exhibited the lowest potency (EC_{50} = 0.097 ng/ml), whereas ebvIL-10, with the lowest affinity for IL-10R1, was the most potent molecule tested (EC_{50} = 0.01 ng/ml; Table 3). Further analysis revealed that IL-10 dimer biological...
potency was inversely correlated with its IL-10R1 binding affinity (Table 3 and Fig. 4B).

**hIL-10 and ebvIL-10 Dimers Engage IL-10R1 Differently**—The IL-10 dimer assembles a complex containing two IL-10R1 and two IL-10R2 chains to induce its biological activity (35, 36). To elucidate kinetic differences in IL-10 dimer binding to IL-10R1, hIL-10 and ebvIL-10 dimers were injected over an IL-10R1-FC surface that allows the formation of 1:2 IL-10R1 complexes (Fig. 5). Although hIL-10 formed 1:2 hIL-10-IL-10R1 complexes on the surface of the SPR chip, the hIL-10+IL-10R1-FC sensorgrams were best fit to a 1:1 binding model (Fig. 5A). In contrast, ebvIL-10-10R1 sensorgrams were globally fit to a bivalent analyte kinetic model (Fig. 5B). The resulting binding constants reveal an initial ebvIL-10-10R1 interaction of 232 nM ($K_{D1}$), with a second apparent binding constant, $K_{D2}$, of 5.6 nM (Table 1). The $K_{D1}$ value for ebvIL-10 is very similar to the $K_{D1}$ value of ebvIL-10M1 (94 nM; Table 1). Thus, the binding kinetics between ebvIL-10M1 and IL-10R1 closely mimic the initial engagement of the ebvIL-10 dimer by IL-10R1. In addition to demonstrating a two-step recognition process for ebvIL-10 binding to IL-10R1, the initial ebvIL-10-10R1

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**TABLE 3**

| Kinetic parameters and biological activity of dimeric IL-10 chimeras |
|-------------------------------------------------|
| SPR analysis                                       |
| R1Wz-EB surface                                 | R1-FC dimer surface                     | Bioassay (TF-1 cells) |
| $K_D$ Increase | $K_D1$ Increase | $K_D2$ Increase | $EC_{50}$ Increase |
|----------|----------------|----------------|------------------|
| ebvIL-10 | 100 | 1.0 | 232 | 5.6 | 0.010 | 1.0 |
| NT       | 30 | 3.3 | 0.028 | 0.36 |
| NT/A87I | 4.2 | 23.8 | 0.033 | 0.30 |
| AB/A87I | 0.90 | 111.1 | 0.047 | 0.21 |
| A87I     | 14 | 7.1 | 0.020 | 0.50 |
| hIL-10   | 0.28 | 357.1 | 0.097 | 0.10 |

|                         |                         |                         |
|-------------------------|-------------------------|-------------------------|
| a R1 SPR data obtained using the monomeric constructs. | | |
**Two-step IL-10R1 Binding Mechanism by ebvIL-10**

**A**

![Graph A](image)

**B**

![Graph B](image)

FIGURE 6. **Cellular proliferation of hIL-10 and ebvIL-10 monomers and dimers in the presence of the neutralizing anti-IL-10R1 antibody (3F9).** A, EC\textsubscript{50} values for hIL-10M1 and ebvIL-10M1 in the presence of increasing anti-IL-10R1 antibody, 3F9. B, EC\textsubscript{50} values for hIL-10 and ebvIL-10 dimers in the presence of increasing anti-IL-10R1 antibody, 3F9.

$K_D$ value (232 nM; Table 1) differs from the hIL-10:IL-10R1 $K_D$ ($K_D = 0.24$ nM; Table 1) by 967-fold, which agrees well with the ~1000-fold difference between hIL-10 and ebvIL-10 $K_D$ values determined on cells (21, 22).

**Influence of IL-10R1 Levels on IL-10 Biological Potency—** IL-10R1 receptor levels have been shown to distinguish cells that respond to only hIL-10 versus those that can respond to hIL-10 and ebvIL-10 (22). To further evaluate this observation, the biological potency of hIL-10 and ebvIL-10 monomers and dimers was evaluated in the presence of increasing concentrations (10 and 100 μg/ml) of the neutralizing anti-IL-10R1 antibody, 3F9 (Fig. 6). 3F9 was used in the assay to reduce IL-10R1 available for IL-10 signaling on the cells. Thus, to a first approximation, increasing 3F9 concentrations should make the TF-1/IL-10R1 cells mimic cells with lower endogenous IL-10R1 levels.

As expected, all IL-10 molecules tested exhibited reduced potency in the presence of 3F9. The addition of 3F9 in the presence of hIL-10M1 and ebvIL-10M1, reduced the potency (increased the EC\textsubscript{50}) of each molecule in a linear fashion (Fig. 6B). Thus, the relative activities of the hIL-10M1 and ebvIL-10M1 in the absence of 3F9 remained the same with increasing 3F9 concentrations. This same trend was not observed upon analysis of the IL-10 and ebvIL-10 dimers (Fig. 6B). As shown in Fig. 3, the biological activity of ebvIL-10 is approximately 1 log more potent than hIL-10 in the proliferation assay. However, upon the addition of 10 μg/ml 3F9, the potency of hIL-10 is slightly greater than ebvIL-10 (EC\textsubscript{50} = 0.25 ng/ml versus 0.34 ng/ml). When 100 μg/ml 3F9 was added to the assay, hIL-10 was considerably more potent than ebvIL-10. Thus, the relative potencies of hIL-10 and ebvIL-10 dimers “flipped” upon the reduction of IL-10R1 available for signaling by the addition of 3F9.

**DISCUSSION**

Studies from numerous laboratories support the idea that EBV uses normal B-cell biology to establish infection, persist, and replicate in a human host (37). More specifically, EBV infects the epithelium of the tonsils and subsequently naive B-cells, which upon expression of viral proteins (including ebvIL-10, LMP-1, and LMP-2) causes the cells to proliferate and differentiate extensively, leading to the establishment of a latent infection in the memory B-cell compartment (37, 38). Maintenance of viral pools occurs by reactivation of the virus from memory B-cells into plasma cells.

The functional properties of ebvIL-10 are remarkably suited to the life cycle of EBV. In particular, ebvIL-10 is more potent than hIL-10 in proliferating CD40-activated primary human B-cells, the mechanism of which is the focus of this study (8, 39). ebvIL-10 also selectively induces, to greater levels than hIL-10, immunoglobulin production and class switching in CD40-activated B-cells (8). The requirement for CD40-mediated B-cell activation to induce ebvIL-10 responsiveness is noteworthy because the EBV protein LMP-1 is a functional mimic of CD40 (40). Thus, it is hypothesized that LMP-1 and ebvIL-10 work together to drive EBV-infected B-cells toward the memory B-cell pool and also facilitate subsequent viral reactivation. In addition, unlike hIL-10, ebvIL-10 does not up-regulate MHC class II on B-cells, providing yet another reason why EBV encodes its own engineered IL-10 protein (10). Finally, ebvIL-10 still retains the ability to suppress proinflammatory cytokines and MHC class II/B7 on antigen-presenting cells that are recruited upon EBV infection (2).

It has been known for some time that ebvIL-10 exhibits ~1000-fold lower affinity for IL-10R1 than hIL-10 (21). This affinity difference has been shown to prevent ebvIL-10 from activating IL-10 responses on cells with low levels of IL-10R1 (22). Here, we were interested in understanding how receptor binding influences the activity of ebvIL-10 on cells with high levels of IL-10R1, where ebvIL-10 is more active than the tighter IL-10R1-binding hIL-10 (8, 21). To address this question, IL-10 chimeras were studied as monomers (M1s) that allowed a comparison of IL-10R1 and IL-10R1-IL-10R2 binding kinetics. SPR analysis of the resulting ebvIL-10M1 chimeras...
revealed that inserting single hIL-10 residues or entire loop sequences had a relatively small impact on IL-10R1 or IL-10R1/IL-10R2 binding affinity (0.7–11.5-fold). Thus, no amino acid change in ebvIL-10, relative to hIL-10, is classified as a hot spot residue that would disrupt or enhance binding by at least 2 kcal/mol (41). However, the combination of residues altered in the ebvIL-10 sequence leads to a substantial 3.5–4 kcal/mol change in IL-10R1 binding energy and significant differences in binding kinetics that are responsible for its unique biological profile.

Despite the relatively small impact of individual residue changes, the two chimeras that exhibited the greatest increase in IL-10R1 binding affinity are ebvIL-10^{V43L} and ebvIL-10^{A87I}, located in the AB and CD loops of IL-10, respectively. The SPR binding analysis is consistent with structural comparisons of the ebvIL-10/IL-10R1 and hIL-10/IL-10R1 complexes, which found that the AB loop of ebvIL-10, but not hIL-10, was partially disordered (36). The contribution of hIL-10^{Ile-87} in stabilizing the AB loop is clear because it packs in the hydrophobic core of the molecule against helix A residues Phe-36 and Phe-37, which precede the AB loop. Thus, in the ebvIL-10^{Ala-87} structure, packing in the molecular core is disrupted, which distorts the AB loop and presumably disrupts optimal ebvIL-10/IL-10R1 interface contacts. Similar to hIL-10^{Ile-87}, the side chain of hIL-10^{Ile-43} packs against residues in the EF loop. This packing arrangement is completely missing in ebvIL-10^{Val-43}. This structural difference is hypothesized to contribute to suboptimal contacts in the ebvIL10/IL-10R1 interface, resulting in lower IL-10R1 affinity.

As found in prior studies, the IL-10R2 chain contributes very little (2.5–11.6-fold) to the overall stability of the IL-10/IL-10R1 ternary complex (12, 22, 42). As might be expected, the contribution of the IL-10R2 chain to IL-10R1/IL-10R2 complex stability was greatest for chimeras with lower IL-10R1 affinity. Thus, hIL-10M1, with the greatest IL-10R1 affinity, exhibited the smallest increase in IL-10R1/IL-10R2 complex affinity (2.5-fold versus IL-10R1 alone). In contrast, ebvIL-10M1, which displays weak IL-10R1 binding, exhibited a ~7-fold increase in IL-10R1/IL-10R2 binding affinity. Interestingly, the chimera that exhibited the greatest increase in IL-10R1/IL-10R2 versus IL-10R1 binding affinity (11.6-fold) is ebvIL-10M1^{Val-43L}. Val-43 is located in the center of the IL-10 AB loop and within the IL-10R1 binding site, rather than in the CD-loop region, which forms part of the putative IL-10R2 binding site (26, 43). These results suggest that the IL-10 AB loop can alter the IL-10R2 binding site either by direct communication through the core of the molecule or by reorienting the cytokine on the IL-10R1 chain. Consistent with the later hypothesis, structural comparisons of ebvIL-10/IL-10R1 and hIL-10/IL-10R1 complexes revealed subtle differences in the orientation of ebvIL-10 and hIL-10 on IL-10R1 (36). Although these studies provided information on the mechanism of receptor engagement, no correlation between IL-10R2 binding and biological potency was observed.

SPR analysis confirms that the ebvIL-10M1^{A87I} chimera exhibits the greatest increase in IL-10R1 affinity, relative to ebvIL-10M1, as previously reported for ebvIL-10^{A87I} on cells by Ding et al. (20). However, we found by SPR analysis that ebvIL-10M1^{A87I} enhances IL-10R1 binding only ~6-fold, not ~100-fold as reported by Ding et al. (20). Furthermore, the monomeric and dimeric ebvIL-10^{A87I} chimeras maintained “ebvIL-10-like” biological activities on TF-1/hIL-10R1 cells, rather than switching to a hIL-10-like phenotype. We hypothesize that these differences may have resulted from Ding et al. (20) evaluating ebvIL-10 chimeras on murine cell lines expressing murine IL-10 receptors. Because EBV is a human pathogen, it is likely that ebvIL-10 has evolved to specifically target the human IL-10R1 chain. If true, this suggests that interpreting the biological functions of ebvIL-10 in mouse model systems may not translate well to humans.

Optimal cellular responsiveness of TF-1/hIL-10R1 cells is obtained using the ebvIL-10 dimer, which presumably assembles 1:2 ebvIL-10/IL-10R1 complexes on the cell surface. In vitro analysis of receptor complex formation reveals that the ebvIL-10 dimer is significantly impaired in its ability to form 1:2 ebvIL-10/IL-10R1 complexes. Cells expressing high levels of IL-10R1 would increase the probability of ebvIL-10 forming the 1:2 ebvIL-10/IL-10R1 complex. However, hIL-10 is very efficient in forming 1:2 hIL-10/IL-10R1 complexes yet exhibits lower potency on TF-1/IL-10R1 cells. Thus, assembly of the 1:2 complex appears to be only part of the story. Notably, the SPR studies show that the initial 1:1 ebvIL-10/IL-10R1 complex dissociates 165 times faster than hIL-10/IL-10R1, and, once a 1:2 ebvIL-10/IL-10R1 complex is formed, it dissociates ~4 times faster than the 1:2 hIL-10/IL-10R1 complex. This suggests that ebvIL-10 signaling may occur by a mixture of 1:1 and 1:2 ebvIL-10/IL-10R1 complexes. It is also possible that ebvIL-10 engages cell surface IL-10R1 chains like a pinball game, continuously shuttling between different cell surface receptor chains. The fast dissociation of the ebvIL-10/IL-10R1 complex may reduce receptor internalization, compared with slowly dissociating hIL-10/IL-10R1 complexes, which could contribute to the enhanced signaling of ebvIL-10 on TF-1/hIL-10R1 cells used in this study as well as in primary human B-cells (8, 21, 44).

Interestingly, the mechanistic strategies used by ebvIL-10 to alter its functional properties are also used by the cell to regulate other cytokine signaling systems. In particular, IFNα and IFNβ both signal through a common IFNAR1/IFNAR2 receptor complex, but IFNα2 exhibits substantially lower affinity for the complex than IFNβ (45). Despite the lower affinity of IFNα2, relative to IFNβ, IFNα2 is more active than IFNβ on cells that express high levels of the IFNAR receptors (46). Interestingly, cell surface IFNAR1 levels have been shown to be regulated by ubiquitination that is mediated by βTrcp E3 ubiquitin ligase (47). IL-10R1 levels have also been shown to be regulated by βTrcp, although this regulation does not seem to be influenced by IL-10 binding (48). Nonetheless, these studies suggest that EBV has taken advantage of host cell signaling strategies to establish an optimal cellular/immune environment that promotes virus survival.

The mechanistic studies presented here provide additional insight into the complex signaling properties of ebvIL-10. In particular, the two-step assembly of ebvIL-10/IL-10R1 1:2 complexes establishes a mechanism for how ebvIL-10 avoids signaling on cells with low IL-10R1 levels yet is able to robustly activate cells with high IL-10R1 levels. Clearly, enhanced signaling...
potency of ebvIL-10, relative to hIL-10, depends on the formation of a significantly weaker ebvIL-10/IL-10R1 complex. Thus, ebvIL-10 uses an IL-10R1 affinity tuning strategy to alter its cellular targets and signaling potential. Although this is conceptually simple, the diverse levels of IL-10R1 on various cell types that are altered during an immune response (1, 30) suggest that ebvIL-10 exhibits very complex and dynamic signaling properties in an infected host.

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**Two-step IL-10R1 Binding Mechanism by ebvIL-10**

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**JOURNAL OF BIOLOGICAL CHEMISTRY 26595**