Published in final edited form as:

Science. 2014 December 12; 346(6215): 1362–1366. doi:10.1126/science.1259329.

Escape from bacterial iron piracy through rapid evolution of transferrin

Matthew F. Barber and Nels C. Elde*

Department of Human Genetics, University of Utah School of Medicine, Salt Lake City, UT, 84112, USA.

Abstract

Iron sequestration provides an innate defense termed nutritional immunity, leading pathogens to scavenge iron from hosts. Although the molecular basis of this battle for iron is established, its potential as a force for evolution at host-pathogen interfaces is unknown. We show that the iron transport protein transferrin is engaged in ancient and ongoing evolutionary conflicts with TbpA, a transferrin surface receptor from bacteria. Single substitutions in transferrin at rapidly evolving sites reverse TbpA binding, providing a mechanism to counteract bacterial iron piracy among great apes. Furthermore, the C2 transferrin polymorphism in humans evades TbpA variants from *Haemophilus influenzae*, revealing a functional basis for standing genetic variation. These findings identify a central role for nutritional immunity in the persistent evolutionary conflicts between primates and bacterial pathogens.

Iron is a precious cellular metal, sequestered by hosts and scavenged by pathogens (1-3). Vertebrate iron transport is mediated by serum transferrin, a protein that binds circulating iron and delivers it to cells via receptor-mediated endocytosis. Modern transferrin arose through a tandem duplication event in ancestral metazoans that produced two homologous domains, the N- and C-lobes, each of which binds a single iron ion with high affinity (4). Transferrin also contributes to host nutritional immunity by sequestering essential iron away from microbial pathogens. One hallmark of host immunity protein evolution is recurrent positive selection driven by diverse and rapidly evolving viruses (5). However, the essential nature of transferrin's role in iron transport necessitates functional conservation, which may impede its ability to adapt against iron piracy. Indeed, the impact of nutritional immunity on evolution at host-pathogen interfaces is unclear.

To determine if transferrin might be subject to pathogen-driven evolution in the primate lineage, we cloned and sequenced transferrin orthologs from 21 hominoid, Old World, and New World monkey species for phylogenetic analysis (Fig. 1A, Fig. S1). A combination of maximum likelihood-based algorithms to assess ratios of non-synonymous to synonymous substitution rates (dN/dS) revealed strong signatures of episodic positive selection in transferrin ($P < 0.0001$, Table S1) across several branches of the primate lineage (Fig. 1A, S2, Tables S2-S8). To date, such signatures of molecular "arms races" in mammals are primarily documented among cell surface receptors and innate pattern recognition proteins

*Correspondence to: nelde@genetics.utah.edu.

antagonized by viruses (6-8), reflecting the primacy of such host proteins as “front line” immune defenses or points of entry for viruses. These results indicate that primate transferrin has undergone bouts of rapid evolution reminiscent of canonical innate immunity factors engaged in host-pathogen arms races.

Our analysis of positive selection in transferrin revealed that 16 of 18 rapidly evolving sites map to the C-lobe (Fig. 1B), despite the fact that the N- and C-lobes are functionally homologous for iron binding and transport. This contrast was particularly clear in the hominoid lineage, where the C-lobe alone shows strong evidence of positive selection ($P < 0.0001$), while the N-lobe does not ($P > 0.99$; Table S4) and instead has evolved under purifying selection. The transferrin N- and C-lobes have thus been subject to very different selective pressures during their respective evolutionary histories, despite performing identical essential physiologic functions. Previous reports indicate that the transferrin receptor (Tf-R) in rodents and carnivores has been subject to positive selection driven by viral entry proteins (6, 9). However, Tf-R is subject to purifying selection in primates (Fig. S3A) and only one of 18 rapidly evolving sites in transferrin makes contact with Tf-R (Fig. S3B), indicating that, as expected, rapid evolution of transferrin has not been driven by co-evolution with its cognate receptor.

Remarkably, 14 of 16 rapidly evolving sites in the transferrin C-lobe form direct contacts with transferrin binding protein A (TbpA) from *Neisseria meningitidis* when mapped to a recently solved, high-resolution co-crystal structure of human transferrin bound to TbpA (Fig. 1B, C, Fig. S4; ref. 10). Several Gram-negative human pathogens including *Neisseria gonorrhoeae*, *Neisseria meningitidis*, and *Haemophilus influenzae* scavenge host iron via surface receptors that bind and extract iron exclusively from the C-lobe of transferrin (11-13). As the primary component of these bacterial receptors, TbpA is a transmembrane transporter that facilitates extraction and translocation of iron into the bacterial periplasm. Notably, the specificity of TbpA proteins for their respective host transferrin is hypothesized to restrict the host range of these bacteria (14, 15). The rapidly evolving sites we identified cluster within six of the seven sub-regions of the TbpA interface, proximal to the TbpA L3 helix finger and plug loop which have been defined as critical points of contact between these two proteins (10). In contrast, no rapidly evolving sites in transferrin form contacts with TbpB, a bacterial accessory co-receptor that coordinately binds the C-lobe of transferrin for iron acquisition (10, 16) (Fig. S5). Thus, positive selection of the transferrin C-lobe may have been solely driven by interactions with TbpA and ancestral TbpA-like proteins.

To define the functional consequences of rapid evolution in primate transferrin, we used competitive binding assays (see methods) to directly assess interactions between transferrin and TbpA (17, 18). For these experiments we purified recombinant transferrin proteins from a panel of primate species (Fig. S6) and expressed TbpA from the human-specific pathogens *N. gonorrhoeae* and *H. influenzae* (strains MS11, and Eagan, respectively) in non-pathogenic BL21 *E. coli*, which do not possess transferrin receptors (Fig. S7). Consistent with previous studies, TbpA from both pathogens bound strongly to recombinant human transferrin (17, 19) (Fig. 1D). TbpA variants also recognized transferrin from gorillas, but not chimpanzees, orangutans, gibbons, or baboons (Fig. 1D). The lack of binding between

TbpA and chimpanzee transferrin was particularly striking given that chimpanzees represent the closest primate relative to gorillas and humans. These observations were independently corroborated by competitive binding enzyme-linked immunosorbent assays (ELISA) recapitulating transferrin-TbpA interactions (Fig. 1D, bar graphs). Thus, transferrin divergence among great apes is sufficient to dictate distinct outcomes in bacterial TbpA binding interactions.

Among the four amino acid differences between human and chimpanzee transferrin, position 591 (glutamic acid in humans, lysine in chimpanzees) shows strong signals of recurrent positive selection (Fig. 2A, Table S6) and, intriguingly, also lies proximal to the interface with the L3 helix finger of TbpA, which plays a pivotal role in iron acquisition (10) (Fig. 2B, Fig. S4). Competitive binding assays revealed that a glutamic acid to lysine substitution at this position in human transferrin (E591K) is sufficient to impair binding to TbpA from both *N. gonorrhoeae* and *H. influenzae*, rendering its binding affinity similar to chimpanzee transferrin (Fig. 2c). In addition, introducing a glutamic acid to lysine substitution at position 591 (K591E) in chimpanzee transferrin is sufficient to restore TbpA binding (Fig. 2C). Variation at position 591 thus directly links positive selection of transferrin to recognition by bacterial TbpA. Furthermore, closely related bonobos share glutamic acid 591 with humans and gorillas (Fig. 2A), providing clear evidence of recent transferrin adaptation in chimpanzees. These findings are surprising given that previous studies concluded the transferrin-TbpA interaction is largely impervious to single point mutations in TbpA, likely owing to a substantial 2,500 Å² binding interface (10). This result highlights the predictive power of positive selection analyses to pinpoint residues that most drastically alter binding affinity at host-pathogen interfaces. Our findings also bolster the hypothesis that transferrin is one of a handful of factors limiting the host range of human-specific bacterial pathogens (15, 20), even among closely related primate species.

Given the ability of a single substitution to dictate TbpA recognition between primate transferrin orthologs, we were curious whether standing genetic variation of transferrin in human populations might provide similar protection. After the major C1 allele, C2 is the most abundant transferrin variant, found at roughly 6-26% allele frequency across human populations (21) (Fig. 2D, Tables S9, S10). C2 differs from C1 by a single C/T substitution that changes proline 589 to serine (Fig. 2D, Table S9). While this variation in transferrin has long been recognized, no appreciable differences in iron binding or other activities have been discerned between C1 and C2 (22). However, this polymorphism occurs only two amino acids away from position 591, which is sufficient to control TbpA binding (Fig. 2B, E). In competitive binding assays, the C2 variant was markedly resistant to recognition by TbpA from *H. influenzae* (Fig. 2F), providing a striking example of adaptive functional consequences for genetic variation in humans. This result highlights the evolutionary impact of nutritional immunity on primate evolution, from 40 million years of species divergence to a single polymorphism circulating in human populations.

To quantify differences in TbpA binding with transferrin human variants, we generated dissociation curves and calculated half maximal inhibitory concentrations (IC₅₀). IC₅₀ calculations revealed severely reduced binding by *H. influenzae* TbpA to C2 transferrin relative to C1 (Fig. S8, Table S11). Unlike TbpA from *H. influenzae*, the *N. gonorrhoeae*

variant bound C2 with nearly equal affinity to C1 transferrin as determined by competitive binding assays and IC₅₀ calculations (Fig 2F, S8, Table S11), indicating functional variability among pathogen TbpA orthologs.

To delineate functional outcomes among TbpA proteins, we sampled variants from additional pathogenic strains isolated in clinics. We found that TbpA from *H. influenzae* isolates Eagan and strain 11 are specific for recognition of the C1 variant, potentially at the expense of C2 recognition (Fig. 2f, 3A, B). In contrast, TbpA from *N. meningitidis* and *N. gonorrhoeae* displayed similar binding to both C1 and C2 transferrin, while *H. influenzae* strain 15 TbpA exhibited intermediate C2 binding affinity (Fig. 3A, B). Based on these findings we speculate that there is an evolutionary tradeoff between increased affinity to transferrin C2 versus increased breadth of transferrin recognition. Regardless of whether such a tradeoff constrains bacterial evolution, these findings reveal functionally distinct outcomes for transferrin recognition by TbpA among bacteria. Together our observations strongly suggest that both transferrin and TbpA have undergone repeated counter adaptations during the battle for iron over the course of primate evolution.

Observing functional consequences of both transferrin and TbpA evolution is consistent with the predictions of the *Red Queen* hypothesis, which posits that evolutionary arms races arise by recurrent episodes of positive selection between hosts and pathogens (5, 23). To directly investigate whether TbpA has been subject to positive selection, we compared gene sequences from a large group of human-derived *Neisseria* and *H. influenzae* isolates (Fig. 3B, S9). Horizontal gene transfer and recombination among bacterial strains notoriously compromise phylogenetic analyses of bacterial genes (24). Therefore, we relied on algorithms accounting for potential recombination break points and used combinations of structural insights and binding assays to substantiate predictions of positive selection (see Methods). We identified 10 sites among *Neisseria* and nine among *Haemophilus* displaying strong signatures of positive selection, with one site shared between the two groups (Fig. 3C, D, Tables S12-S17). Nearly every site, though non-overlapping between *Neisseria* and *Haemophilus*, lies in predicted extracellular loops of TbpA, which comprise the transferrin-binding interface (Fig. 3C, D). These domains display marked variation even among closely related pathogen isolates, in contrast to a high degree of conservation in transmembrane domains (Fig. S10, S11).

To assess the functional implications of TbpA substitutions at rapidly evolving sites, we mutagenized *N. gonorrhoeae* TbpA at three positions under selection to corresponding amino acids present in *H. influenzae* (strain Eagan) TbpA (Fig. S12). Of these three mutations, R365Q exhibited reduced binding to human transferrin (Fig. 3E). Thus, substitutions at rapidly evolving sites in transferrin as well as TbpA modulate interactions at this protein interface. By integrating phylogenetic analyses with high-quality structural data and experimental approaches, our results provide a high-resolution view of molecular genetic dynamics on both sides of this novel interface of host-pathogen evolutionary arms races.

In addition to the C1/C2 polymorphism in human transferrin, position 589 toggles exclusively between proline and serine across the primate lineage (Fig. 2E, Fig. S13), a

potential signature of antagonistic pleiotropy at a largely constrained position, as observed for other host-pathogen interfaces (7). Previous work has also implicated the C2 transferrin variant as a risk factor for disorders involving iron metabolism, including Alzheimer's disease, however these associations remain controversial and appear dependent on the populations tested and interactions with other susceptibility loci (25, 26). Our findings provide a functional basis for human transferrin variation and establish an important role for nutritional immunity in recent human evolution.

While canonical innate immunity factors have been appreciated as nodes of host-virus evolution, our work demonstrates that nutritional immunity has played a fundamental role in the survival of primate populations challenged by bacterial pathogens. *H. influenzae* and *N. meningitidis* remain a major source of morbidity and mortality in regions where vaccine coverage is poor (27, 28) and drug-resistant *N. gonorrhoeae* is developing into an urgent public health threat (29). By illuminating the battle for iron as a major driving force of host-pathogen evolution, from 40 million years of primate divergence to emerging human epidemics today, our studies reveal new reservoirs of genetic resistance to infectious diseases.

Materials and Methods

Primate genome sources

Total RNA was obtained (RNeasy kit; Qiagen) from the following cell lines from the Coriell Cell Repositories except where otherwise noted: *Homo sapiens* (human; primary human foreskin fibroblasts; gift from A. Geballe), *Gorilla gorilla* (western lowland gorilla; AG05251), *Pongo pygmaeus pygmaeus* (Bornean orangutan; AG05252), *Hylobates lar* (white-handed gibbon; PR01131), *Hylobates leucogenys* (white-cheeked gibbon; PR00712), *Hylobates syndactylus* (island siamang; PR00722), *Macaca mulatta* (rhesus monkey liver tissue; gift from Scott Wong), *Papio anubis* (olive baboon; PR00036), *Lophocebus albigena* (black crested mangabey; PR01215), *Allenopithecus nigroviridis* (Allen's swamp monkey; PR01231), *Cercopithecus ascanius* (red-tailed guenon; PR00566), *Colobus guereza* (colobus monkey; PR00240), *Trachypithecus francoisi* (Francois' leaf monkey; PR01099), *Saguinus fuscicollis* (Spix's saddle-back tamarin; AG05313), *Callithrix geoffroyi* (white-fronted marmoset; PR00789), *Lagothrix lagotricha* (common woolly monkey; AG05356), *Saimiri sciureus* (common squirrel monkey; AG05311), *Aotus nancyma* (night monkey; PR00627), *Callicebus moloch* (dusky titi; PR00742), *Alouatta sara* (Bolivian red howler monkey; PR00708).

cDNA cloning and sequencing

RNA (50 ng) from each primate was used for RT-PCR (SuperScript III; Invitrogen) with primers listed in Table S17. PCR products were TA-cloned into pCR2.1 (Invitrogen) and sequenced from three different clones. Gene sequences are deposited in Genbank (accession numbers: KM972645-KM972665; also see Supplemental Data). The human transferrin variant cloned was identical in sequence to the GenBank entry for the C1 variant (NM001063). *Pan troglodytes* (chimpanzee; NM001144835) transferrin was synthesized for

subsequent cloning (Genewiz). Primers and strain generated as part of this study are provided (Tables S18, S19).

Evolutionary analyses and structural observations

DNA sequences were aligned using MUSCLE with indels trimmed based on amino-acid comparisons (Supplemental Data). The generally accepted primate phylogeny (Fig. 1A) was used for evolutionary analysis and compared to a PhyML tree (atgc.lirmm.fr/phyml/) generated from the alignment of transferrin, which placed several species at different nodes (Fig. S1). Parallel analysis with the transferrin gene tree did not alter any results significantly (Table S2). Maximum-likelihood analysis of the transferrin data set was performed with codeml of the PAML software package(30). A free-ratio model allowing dN/dS variation along different branches of the phylogeny was employed to calculate dN/dS values between lineages. Two-ratio tests were performed with likelihood models comparing all branches fixed at dN/dS=1 or an average dN/dS value from the whole tree applied to each branch to varying dN/dS values according to branch. Complementary analysis grouping lineages according to dN/dS values with multi-model inference (GA-Branch; HyPhy software) was also applied to the data set.

To test for selection in transferrin, the multiple alignment was fitted to either F3x4 or F61 codon frequency models. Likelihood ratio tests (LRTs) were performed by comparing the following site-specific models (NS sites): M1 (neutral) with M2 (selection), M7 (neutral, beta distribution of dN/dS<1) with M8 (selection, beta distribution, dN/dS>1 allowed). Similar LRTs that also account for synonymous rate variation and recombination (FUBAR, SLAC, FEL, MEME, PARRIS; HyPhy software package) were performed.

Bacterial TbpA sequences were obtained from GenBank. Sequences were aligned using MUSCLE and manually trimmed based on amino acid comparisons among *Neisseria* or *H. influenzae* variants. Maximum-likelihood phylogenetic trees for TbpA were generated with PhyML and used for subsequent analysis to screen for positive selection. Maximum-likelihood analysis of *Neisseria* and *Haemophilus* TbpA variants was performed with codeml of the PAML software package, with a gene tree generated using PhyML. Additionally, positive selection in TbpA was assessed using four methods (MEME, FUBAR, SLAC, and FEL) from the HyPhy software package with phylogenies generated using the GARD algorithm, to search and account for recombination break points. Codons in TbpA with posterior probabilities of >0.95 in PAML that also passed at least two of four HyPhy-based tests for selection were reported to be under positive selection (Fig. 3D).

Human transferrin C2 allele frequencies were obtained from the 1000 Genomes Phase 3 dataset (www.1000genomes.org) Crystal structures were visualized using Chimera (www.cgl.ucsf.edu/chimera).

Protein purification

Primate transferrin orthologs were cloned into the pFASTBac vector (Invitrogen) containing an N-terminal Strep-Flag-Precision protease tag. Generation of baculoviral DNA from DH10Bac cells was performed using the Bac to Bac system (Invitrogen) per the

manufacturer's instructions. Sf9 insect cells (Expression Systems) were transfected with baculoviral DNA using Cellfectin II reagent (Invitrogen). After three passages to amplify viral titers, a one litre culture of Sf9 cells was infected, and cell pellets were collected 48 hours post-infection. Cell pellets were lysed in tris-buffered saline (TBS; 50 mM Tris pH 8.0, 150 mM NaCl) containing 1% Triton X100, protease inhibitors, iron-nitrilotriacetic acid (Fe-NTA), and 15 U avidin, then sonicated for three 30 second cycles with a needle sonicator (Branson). Lysates were centrifuged at >30,000xG for 90 minutes and passed through a 0.4 micron low protein binding filter. Clarified lysates were loaded onto a 5 mL StrepTrap HP column (GE Healthcare), washed with up to 1 M NaCl, and eluted in TBS containing 2.5 mM D-desthiobiotin (Sigma). Precision protease (Invitrogen) was added to samples and dialyzed against 1 L of TBS overnight.

TbpA binding assays

Competitive binding dot blot assays with transferrin and TbpA were performed similarly to previous studies (17). Briefly, TbpA variants from indicated strains were cloned into the pGEN vector containing a LAC promoter and ampicillin resistance marker (Table S18). Plasmids were transformed into BL21 *E. coli*, which were grown overnight in LB broth contain ampicillin. Bacteria were spotted on nitrocellulose using a BioDot apparatus (Bio-Rad), and membranes were blocked in 5% milk in phosphate buffered saline (PBS) for 30 minutes. After washing in PBS, membranes were incubated with 0.5 ug/mL of HRP-conjugated human transferrin (HRP-transferrin; Fitzgerald) in the presence of two-fold serial dilutions of recombinant primate transferrin (5, 10, and 20 ug/mL as determined by Bradford assay). Relative recombinant transferrin levels were also confirmed by coomassie stained SDS-PAGE (Fig. S5). Blots were washed three times in PBS, and HRP-transferrin bound to TbpA-expressing *E. coli* was detected by chemiluminescence. Testing transferrin binding to TbpA mutants was performed as described above, except that bacterial cultures were diluted two-fold before spotting to nitrocellulose and incubation with 0.5 ug/mL HRP-transferrin. Western blot samples were prepared by boiling *E. coli* in loading buffer and probing with anti-TbpA antibody (gift of Cynthia Cornelissen) or anti-*E. coli* (Biodesign).

Transferrin-TbpA binding ELISAs were performed by first adding 100 uL of log-phase cultures of TbpA-expressing *E. coli* to tissue culture treated 96-well plates at 37 degrees for three hours. Plates were subsequently washed with PBS and wells were incubated with PBS alone (blank), 0.5 ug/mL HRP-conjugated human transferrin, or HRP-conjugated human transferrin with recombinant primate transferrin for 30 minutes at room temperature. Plate wells were washed three times with PBS and ELISA was performed using 3,3', 5,5'-Tetramethylbenzidine (TMB) and stop solutions (Sigma). Absorbance at 450nm was measured on a BioTek Synergy HT plate reader. Data were normalized to signal from HRP-transferrin alone. Experiments were performed in triplicate, and bar graphs represent mean and standard deviation from four independent experiments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Matthew Mulvey and members of the Mulvey lab for assistance with bacterial genetics and helpful discussions; Viswanathan Chandrasekaran and Wesley Sundquist for assistance with baculoviral protein purification; Jerry Kaplan and Diane Ward for helpful advice and discussions; Joseph St. Geme III and Eric Porsch for *Haemophilus* strains and helpful discussions; Magdalene So for providing *Neisseria* DNA and strains; Scott Wong for providing rhesus macaque liver samples; Zev Kronenberg for assistance with human population genetics; and Cynthia Cornelissen for the gift of the TbpA antibody as well as helpful discussions. Janet Iwasa created the molecular animation of transferrin and TbpA evolution. We are grateful to Nicola Barber, Harmit Malik, Sara Sawyer, Nitin Phadnis, and members of the Elde lab for comments on the manuscript. Genbank accession numbers of transferrin and TbpA variants: KM972645 - KM972665; also see Supplemental Materials. This work is supported by awards from the Pew Charitable Trusts and the NIH to N.C.E. (GM090042) and M.F.B. (1F32GM108288). N.C.E. is a Pew Scholar in the Biomedical Sciences and Mario R. Capecchi Endowed Chair in Genetics.

References

- Weinberg ED. Nutritional immunity. Host's attempt to withhold iron from microbial invaders. *JAMA*. 1975; 231:39–41.
- Cassat JE, Skaar EP. Iron in Infection and Immunity. *Cell Host and Microbe*. 2013; 13:509–519. [PubMed: 23684303]
- Hood MI, Skaar EP. Nutritional immunity: transition metals at the pathogen–host interface. *Nature Reviews Microbiology*. 2012; 10:525–537.
- Park I, et al. Organization of the human transferrin gene: direct evidence that it originated by gene duplication. *Proc. Natl. Acad. Sci. U.S.A.* 1985; 82:3149–3153. [PubMed: 3858812]
- Daugherty MD, Malik HS. Rules of Engagement: Molecular Insights from Host-Virus Arms Races. *Annu. Rev. Genet.* 2012; 46:677–700. [PubMed: 23145935]
- Demogines A, Abraham J, Choe H, Farzan M, Sawyer SL, Sugden B. Dual Host-Virus Arms Races Shape an Essential Housekeeping Protein. *PLoS Biol.* 2013; 11:e1001571. [PubMed: 23723737]
- Elde NC, Child SJ, Geballe AP, Malik HS. Protein kinase R reveals an evolutionary model for defeating viral mimicry. *Nature*. 2009; 457:485–490. [PubMed: 19043403]
- Patel MR, Loo Y-M, Horner SM, Gale M, Malik HS, Hurst LD. Convergent Evolution of Escape from Hepaciviral Antagonism in Primates. *PLoS Biol.* 2012; 10:e1001282. [PubMed: 22427742]
- Kaelber JT, et al. Villarreal L. Evolutionary Reconstructions of the Transferrin Receptor of Caniforms Supports Canine Parvovirus Being a Re-emerged and Not a Novel Pathogen in Dogs. *PLoS Pathog.* 2012; 8:e1002666. [PubMed: 22570610]
- Noinaj N, et al. Structural basis for iron piracy by pathogenic *Neisseria*. *Nature*. 2013; 482:53–58.
- Schryvers AB, Morris LJ. Identification and characterization of the transferrin receptor from *Neisseria meningitidis*. *Mol. Microbiol.* 1988; 2:281–288. [PubMed: 3132585]
- Schryvers AB, Gray-Owen S. Iron acquisition in *Haemophilus influenzae*: receptors for human transferrin. *J. Infect. Dis.* 1992; 165(Suppl 1):S103–4. [PubMed: 1588135]
- Alcantara J, Yu RH, Schryvers AB. The region of human transferrin involved in binding to bacterial transferrin receptors is localized in the C-lobe. *Mol. Microbiol.* 1993; 8:1135–1143. [PubMed: 8361358]
- Schryvers AB, Gonzalez GC. Comparison of the abilities of different protein sources of iron to enhance *Neisseria meningitidis* infection in mice. *Infect. Immun.* 1989; 57:2425–2429. [PubMed: 2545624]
- Zarantonelli ML, et al. Transgenic Mice Expressing Human Transferrin as a Model for Meningococcal Infection. *Infect. Immun.* 2007; 75:5609–5614. [PubMed: 17893132]
- Calmettes C, Alcantara J, Yu R-H, Schryvers AB, Moraes TF. The structural basis of transferrin sequestration by transferrin-binding protein B. *Nat. Struct. Mol. Biol.* 2012; 19:358–360. [PubMed: 22343719]
- Cornelissen CN, Biswas GD, Sparling PF. Expression of gonococcal transferrin-binding protein 1 causes *Escherichia coli* to bind human transferrin. *J. Bacteriol.* 1993; 175:2448–2450. [PubMed: 8468302]

18. Schryvers AB, Lee BC. Comparative analysis of the transferrin and lactoferrin binding proteins in the family Neisseriaceae. *Can. J. Microbiol.* 1989; 35:409–415. [PubMed: 2543489]
19. Gray-Owen SD, Schryvers AB. The interaction of primate transferrins with receptors on bacteria pathogenic to humans. *Microb. Pathog.* 1993; 14:389–398. [PubMed: 8366816]
20. Lee BC, Schryvers AB. Specificity of the lactoferrin and transferrin receptors in *Neisseria gonorrhoeae*. *Mol. Microbiol.* 1988; 2:827–829. [PubMed: 2850444]
21. Kühnl P, Spielmann W. Transferrin: evidence for two common subtypes of the TfC allele. *Hum. Genet.* 1978; 43:91–95. [PubMed: 669722]
22. Zatta P, et al. The C2 variant of human serum transferrin retains the iron binding properties of the native protein. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease.* 2005; 1741:264–270.
23. Emerman M, Malik HS. Paleovirology--modern consequences of ancient viruses. *PLoS Biol.* 2010; 8:e1000301. [PubMed: 20161719]
24. Shapiro BJ, David LA, Friedman J, Alm EJ. Looking for Darwin's footprints in the microbial world. *Trends in Microbiology.* 2009; 17:196–204. [PubMed: 19375326]
25. Namekata K, et al. Association of transferrin C2 allele with late-onset Alzheimer's disease. *Hum. Genet.* 1997; 101:126–129. [PubMed: 9402955]
26. Wang Y, et al. Meta-analysis on the association between the TF gene rs1049296 and AD. *Can J Neurol Sci.* 2013; 40:691–697. [PubMed: 23968943]
27. O'Brien KL, et al. Burden of disease caused by *Streptococcus pneumoniae* in children younger than 5 years: global estimates. *Lancet.* 2009; 374:893–902. [PubMed: 19748398]
28. Halperin SA, et al. The changing and dynamic epidemiology of meningococcal disease. *Vaccine.* 2012; 30(Suppl 2):B26–36. [PubMed: 22178525]
29. Frieden T. Antibiotic Resistance Threats in the United States 2013. 2013 Centers for Disease Control and Prevention, US Department of Health and Human Services.
30. Yang Z. PAML 4: phylogenetic analysis by maximum likelihood. *Molecular Biology and Evolution.* 2007; 24:1586–1591. [PubMed: 17483113]
31. Studier FW, Moffatt BA. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* 1986; 189:113–130. [PubMed: 3537305]
32. Meyer TF, Mlawer N, So M. Pilus expression in *Neisseria gonorrhoeae* involves chromosomal rearrangement. *Cell.* 1982; 30:45–52. [PubMed: 6127165]
33. Nassif X, et al. Antigenic variation of pilin regulates adhesion of *Neisseria meningitidis* to human epithelial cells. *Mol. Microbiol.* 1993; 8:719–725. [PubMed: 8332064]
34. Anderson P, Johnston RB, Smith DH. Human serum activities against *Haemophilus influenzae*, type b. *J. Clin. Invest.* 1972; 51:31–38. [PubMed: 4536614]
35. Barenkamp SJ, St Geme JW. Identification of a second family of high-molecular-weight adhesion proteins expressed by non-typable *Haemophilus influenzae*. *Mol. Microbiol.* 1996; 19:1215–1223. [PubMed: 8730864]
36. Buscher AZ, Burmeister K, Barenkamp SJ, St Geme JW. Evolutionary and functional relationships among the nontypeable *Haemophilus influenzae* HMW family of adhesins. *J. Bacteriol.* 2004; 186:4209–4217. [PubMed: 15205423]

One Sentence Summary

The battle for iron is a principal force in the evolution of hosts and bacterial pathogens over millions of years of primate descent to modern humans.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 1. Primate transferrin has undergone recurrent positive selection at the binding interface with bacterial TbpA

(A) A primate phylogram highlighting rapid evolution of transferrin. dN/dS ratios along each branch of the primate lineage are listed, with dN/dS values >1 highlighted in blue. Branches with no synonymous changes display N:S substitution ratios in parentheses. (B) Schematic of the human transferrin protein (yellow). Amino acid positions showing evidence of positive selection (PAML, posterior probability > 0.95) are denoted by blue arrows. Regions of transferrin spanning the *N. meningitidis* TbpA binding interface (10) are highlighted in brown. (C) Ribbons representation of the structure (PDB: 3V8X) of human transferrin (yellow) in complex with *N. meningitidis* TbpA (gray), with the position of the bacterial outer membrane labeled. Amino acid side-chains in transferrin with strong evidence of positive selection are marked by blue spheres. The position of a ferric ion is modeled as a red dot. (D) Competitive TbpA binding assays performed as dot blot (left) or ELISA (right), using *E. coli* expressing indicated pathogen TbpA. Samples were incubated with horseradish peroxidase (HRP)-conjugated human transferrin alone (0.5 ug/mL), or HRP-transferrin in the presence of increasing concentrations of recombinant purified transferrin (5, 10, or 20 ug/mL) from indicated primates. Error bars represent standard deviation (SD) of four independent experiments. ** indicates $P < 0.01$ relative to human transferrin.

Fig. 2. Transferrin divergence in humans and chimpanzees impairs TbpA binding

(A) Schematic representation (top) showing divergent amino acid positions between human and chimpanzee transferrin. Blue arrows indicate amino acids that also display signatures of positive selection. Amino acid alignment (bottom) around position 591, highlighting the chimpanzee-specific E591 to K substitution. (B) Sites of positive selection in transferrin (blue) proximal to loop 3 of TbpA. Position 589, which is variable in human populations, is highlighted in green. (C) Competitive binding dot blots and ELISAs using recombinant human and chimpanzee transferrin, along with human E591K and chimpanzee K591E mutant proteins. Error bars represent SD of four independent experiments. ** denotes $P < 0.01$. (D) Distribution of the transferrin C2 polymorphism (green) across human populations. (E) Primate phylogeny and amino acid alignment displaying toggling of transferrin position 589 across primates. Colors denote variable amino acids at each position. Variability at position 591 is also highlighted. (F) Competitive binding dot blot and ELISAs using the major transferrin variant (C1), the transferrin P589S variant (C2), as well as chimpanzee and gorilla transferrin. Error bars represent SD of four independent experiments. ** indicates $P < 0.01$ relative to human (C1) transferrin.

Fig. 3. Rapid evolution and functional variation of TbpA among human pathogens

(A) Competitive binding dot blots and ELISAs assessing TbpA binding to the human transferrin C2 variant. Error bars represent SD of four independent experiments. ** indicates $P < 0.01$ relative to human (C1) transferrin, or C2 transferrin (vertical line). * indicates $P = 0.05$. (B) Gene tree of TbpA from *H. influenzae* isolates. TbpA from strain Eagan (magenta) as well as two divergent TbpA variants, strains 11 and 15 (green) are highlighted. (C) Structure of human transferrin (yellow) in complex with *N. meningitidis* TbpA (gray). Side-chains of amino acids under positive selection in transferrin (blue), *H. influenzae* TbpA (purple), and *Neisseria* TbpA (orange) are denoted by colored spheres. * indicates position under positive selection in both *Haemophilus* and *Neisseria* TbpA. A “top” view of TbpA (right) shows exposure of rapidly evolving sites at the transferrin binding interface. (D) Schematic representation of sites among either *H. influenzae* or *Neisseria* TbpA showing evidence of positive selection, overlaid on TbpA from *N. meningitidis*. Arrows denote sites that pass multiple tests of positive selection incorporating PhyML TbpA gene trees as well as phylogenies that account for recombination break points. Predicted extracellular loops are indicated in dark gray. * indicates a single amino acid position showing evidence of positive selection in both *Haemophilus* and *Neisseria*. (E) Indicated *E. coli* strains expressing mutations of TbpA were tested for interactions with human transferrin-HRP (left panel). A control blot was stained with ponceau (middle panel) as a loading control. Western blots using antibodies against *N. gonorrhoeae* TbpA or total *E. coli* were performed with cell lysates from indicated strains (right panels).