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Editor's Summary

Defender of Fertility

Defensins are a type of proteins that are produced by the human (and other animal) body to help fight off bacterial infections. Previous studies have demonstrated an association between male infertility and the presence of infection-fighting white blood cells in the semen, suggesting that infections may be somehow associated with infertility. Now, a study by Diao *et al*. explains this connection by showing that a specific type of defensin found in sperm plays a dual role, helping to fight off bacterial infection and promoting sperm motility. The authors also demonstrate a possible way to exploit this finding by treating sperm with recombinant defensin to increase the probability of successful in vitro fertilization.

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INFERTILITY

Deficient human β-defensin 1 underlies male infertility associated with poor sperm motility and genital tract infection

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Genital tract infection and reduced sperm motility are considered two pivotal etiological factors for male infertility associated with leukocytospermia and asthenozoospermia, respectively. We demonstrate that the amount of human β-defensin 1 (DEFB1) in sperm from infertile men exhibiting either leukocytospermia or asthenozoospermia, both of which are associated with reduced motility and reduced bactericidal activity in sperm, is much lower compared to that in normal fertile sperm. Interference with DEFB1 function also decreases both motility and bactericidal activity in normal sperm, whereas treatment with recombinant DEFB1 markedly restores DEFB1 expression, bactericidal activity, sperm quality, and egg-penetrating ability in sperm from both asthenozoospermia and leukocytospermia patients. DEFB1 interacts with chemokine receptor type 6 (CCR6) in sperm and triggers Ca^{2+} mobilization, which is important for sperm motility. Interference with CCR6 function also reduces motility and bactericidal activity of normal sperm. The present finding explains a common defect in male infertility associated with both asthenozoospermia and leukocytospermia, indicating a dual role of DEFB1 in defending male fertility. These results also suggest that the expression of DEFB1 and CCR6 may have diagnostic potential and that treatment of defective sperm with recombinant DEFB1 protein may be a feasible therapeutic approach for male infertility associated with poor sperm motility and genital tract infection.

INTRODUCTION

Infertility affects ~10 to 15% of couples worldwide, and male infertility contributes about 50% of these infertile cases (*1*). Male infertility is attributed to multiple factors, and defective sperm function is the most common cause of infertility (*2*). Sperm motility is considered one of the most important sperm functions that affect natural conception (*3*), and reduced sperm motility, also known as asthenozoospermia, is a common cause of infertility and accounts for about 18% of the male subfertility and infertility cases (*4*). Seminal tract infection is another common cause of infertility, which is observed in about 11% of infertile male patients (*5*). It has been postulated that the presence of leukocytes in semen, also known as leukocytospermia, which affects anywhere between 5 and 10% of the male population (*6*, *7*), is an indicator of seminal tract infection, but the correlation remains controversial (*8*). Asthenozoospermia is often associated with the presence of infection or leukocytes in semen (*8*, *9*); however, the pathogenesis and any association between asthenozoospermia and leukocytospermia remain largely unexplored.

After being produced from the testes, sperm acquire their motility and fertilizing capacity during their transit through the epididymis, in a process known as sperm maturation (*10*). Recently, members of the β-defensin family, a group of small secretory peptides with antimicrobial activities (*11*, *12*), have been implicated in the process of sperm maturation in the epididymis (*13*). Our previous studies have demonstrated that Bin-1b, a rat epididymis-specific β-defensin (*14*), is involved in the initiation of sperm motility (*13*), and that interfering with its function in an autoimmune rat model results in reduced sperm motility with compromised fertility (*15*). Multiple β-defensins with region-specific expression patterns in the epididymis have been found in humans and mice, suggesting that these secretory defensin peptides may contribute to the distinct microenvironments for sperm maturation in different segments of the epididymis (*16*). This notion is supported by the recent reports showing that epididymis-specific β-defensin 22 determines the fertilization capability of sperm through its heparin-binding activity (*17*), whereas β-defensin 15 is required for sperm motility and fertility (*18*). A recent report has also demonstrated that mutation in *DEFB126*, another epididymis-specific β-defensin (*19*), is correlated with decreased leptin-binding and egg-penetrating ability (*20*). Together, these findings make it clear that β-defensin family members may be involved not only in host defense but also in regulating sperm functions that are important to fertility. Although most of the studies have focused on the epididymis-specific β-defensins, the possible involvement of other β-defensin members, which are widely expressed in different epithelial tissues including the epididymis, in regulating sperm function and fertility has not been reported.

Human β-defensin 1 (DEFB1, also termed HBD-1) is the first identified member of the β-defensin family, with wide distribution in various epithelia throughout the body (*21*) and antimicrobial activities against viruses, bacteria, and fungi (*22*). Recent studies have shown that DEFB1 is not only expressed in the epithelia of the male genital tract but also present in seminal plasma and ejaculated spermatozoa (*23*), indicating

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that DEFB1 secreted by the male genital tract epithelia can bind to sperm. However, its role in regulating sperm function and defending male fertility has not been explored. The present study of sperm from infertile men diagnosed with asthenozoospermia or leukocytospermia has revealed a common cause for male infertility, associating the two abnormalities with reduced amount of DEFB1.

RESULTS

Deficiency of DEFB1 in sperm is associated with poor sperm motility and reduced bactericidal activity

We compared the expression of DEFB1 in sperm obtained from normal (n = 32) and infertile patients with either asthenozoospermia (n = 33; progressive sperm motility <32%) or leukocytospermia (n = 20; with leukocyte count >3 × 10^6 cells/ml and confirmed bacterial infection in the semen) (tables S1 and S2). Immunostaining showed that whereas DEFB1 was highly expressed in the lower head and midpiece of sperm from normal individuals, as previously described (*23*), DEFB1 immunostaining intensity was reduced in both types of abnormal sperm (Fig. 1A). Western blot analysis confirmed significantly lower level of DEFB1 in both types of abnormal sperm compared to normal sperm (Fig. 1B and fig. S1, $P < 0.01$). The specificity of the antibody was validated by preabsorption of the antibody with a recombinant DEFB1 (rDEFB1) peptide, which abolished the band detected in the Western blot (fig. S2). This effect was not observed with another defensin peptide, DEFB126 (fig. S2C), confirming the specificity of the antibody. The observed abnormal expression of DEFB1 in both asthenozoospermia and leukocytospermia suggests possible involvement of DEFB1 in the pathogenesis of the two abnormalities.

Because β-defensin family members have antimicrobial activities and some have been reported to promote sperm motility (*12*, *13*), we further investigated whether the low level of DEFB1 in abnormal sperm is associated with poor sperm motility and reduced bactericidal activity. As expected, the expression of DEFB1, as determined by Western blot (Fig. 1B), in sperm from both normal and asthenozoospermia patients was positively correlated with sperm motility ($r = 0.68$, $P = 3.71 \times 10^{-6}$) (fig. S3). On the contrary, the number of semen leukocytes, which is often proportional to the severity of bacterial infection, was found to be inversely correlated with the expression of DEFB1 in sperm from both normal and leukocytospermia patients ($r = -0.92$, $P = 8.27 \times 10^{-9}$) (fig. S4). The number of leukocytes was also inversely associated with sperm motility in normal and leukocytospermia patients ($r = -0.69$, $P = 1.56 \times 10^{-8}$) (fig. S5). The average sperm motility in leukocytospermia was significantly lower than that in the normal controls (34.0 ± 18.6% in leukocytospermia versus 72.4 ± 6.2% in normal, $P = 2.77 \times 10^{-9}$) (Fig. 1C), which was similar to the reduced sperm motility observed in asthenozoospermia (26.3 ± 9.7%).

The inverse association of DEFB1 expression with the number of semen leukocytes in patients with confirmed genital tract infection suggests reduced bactericidal activity due to the reduced amount of DEFB1. Because DEFB1 itself is known to have bactericidal activity (*12*) through disrupting the cytoplasmic membrane of microorganisms (*24*), we suspected that varying levels of DEFB1 in sperm may affect the bacteria-killing capacity of sperm, although bactericidal activity of sperm per se had not been reported. We tested this possibility by incubating sperm with *Escherichia coli* and *Staphylococcus aureus*, two bacterial strains commonly found in male genital tract infection (*25*, *26*), for 1 hour and by counting the bacterial colony-forming units (CFUs) after overnight bacterial culture. The results showed that normal sperm exhibited strong bactericidal activity against both strains of bacteria, as indicated by the significantly reduced CFUs compared to the control without sperm treatment (Fig. 1D, $P < 0.01$). Notably, DEFB1 was detected in originally DEFB1-free bacteria after incubation with sperm (fig. S6), indicating transfer of sperm-bound DEFB1 to bacteria, which explains its bacteria-killing effect. Because DEFB1 level was decreased in both asthenozoospermia and leukocytospermia (Fig. 1, A and B), we suspected that both types of abnormal sperm would have reduced bactericidal activity. Indeed, the results showed that the sperm obtained from both asthenozoospermia and leukocytospermia patients exhibited reduced bactericidal activity, as reflected by the increased *E. coli* CFUs in the bacterial cultures after incubation with the abnormal sperm compared to that with normal sperm (Fig. 1E, $P < 0.01$). Together, these results indicate that reduced sperm motility and bactericidal activity in both types of abnormal sperm may stem from deficiency in DEFB1.

Immunodepletion of DEFB1 impairs sperm motility and bactericidal activity in normal human sperm

If abnormal expression of DEFB1 is associated with both asthenozoospermia and leukocytospermia, we should be able to mimic the two abnormalities by interfering with DEFB1 function in normal sperm. To test this, we treated normal human sperm with antibody against DEFB1 to neutralize its function, and then examined its effect on sperm motility and bactericidal activity, with immunoglobulin G (IgG) as the control antibody. In Fig. 2A, addition of DEFB1 antibody (20 μg/ml), but not control IgG, significantly reduced sperm motility in normal sperm (n = 5) ($P = 0.008$), but not in sperm obtained from asthenozoospermia and leukocytospermia patients (Fig. 2A). Notably, DEFB1 antibody did not cause agglutination of sperm (less than five sperm per agglutinate; fig. S7), suggesting that the effect of DEFB1 antibody on sperm motility was primarily due to the immunodepletion of DEFB1, but not agglutination of sperm. Because the members of the β-defensin family have similar protein conformation, it is important to demonstrate that the DEFB1 antibody does not cross-react with other β-defensins on sperm. Indeed, rDEFB1 abolished the effect of DEFB1 antibody on sperm motility, but recombinant DEFB126 had no effect (fig. S8), indicating that the effect of DEFB1 antibody on sperm motility is primarily attributed to its specific effect on DEFB1, but not other β-defensins. Similarly, the bactericidal activity of normal human sperm, but not the sperm from the two abnormal groups, was significantly reduced by the treatment with DEFB1 antibody, as indicated by the increased CFUs of *E. coli* after treatment (Fig. 2B, $P = 4.46 \times 10^{-4}$). DEFB1 antibody treatment in normal sperm also increased *S. aureus* CFUs, confirming a role of DEFB1 in mediating sperm bactericidal activity (Fig. 2C). No significant effect was observed with control IgG in all the experiments with DEFB1 antibody treatment (fig. S9). In addition, we used a separate source of monoclonal DEFB1 antibody and observed similar effects on sperm motility and bactericidal activity in sperm obtained from normal individuals (fig. S10), confirming that the effect of DEFB1 antibody was specific. Together, these results suggest that specifically interfering with functional DEFB1 by its antibody can mimic the two types of sperm abnormalities in infertile men, providing further support to the notion that deficiency in DEFB1 is associated with two sperm disorders that cause male infertility.

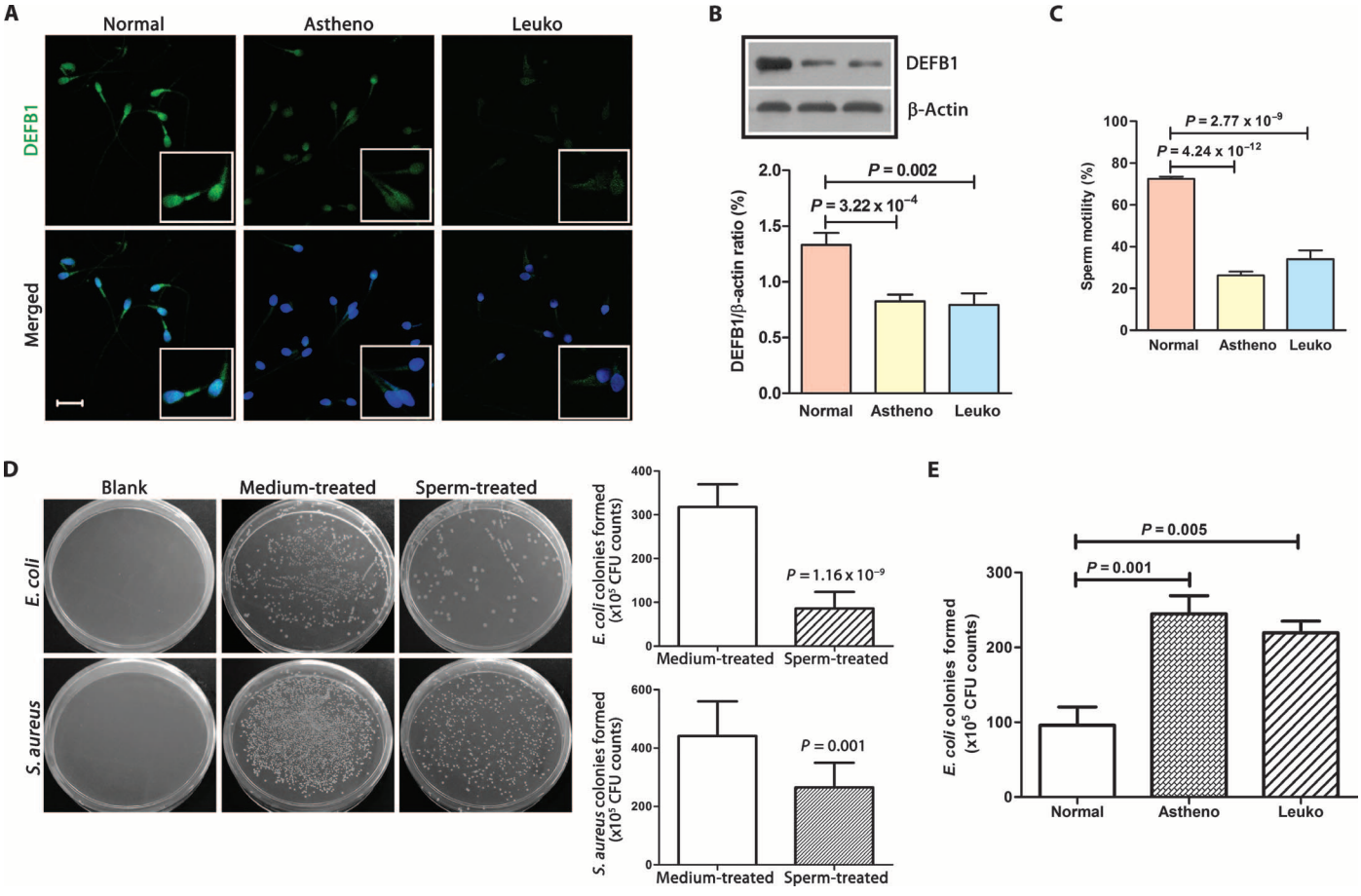

Fig. 1. Expression of DEFB1 in sperm is associated with poor sperm motility and antibacterial activity. (**A**) Representative confocal images showing the expression and localization of DEFB1 (green) in the head and midpiece of normal sperm and its comparatively lowered level in sperm obtained from infertile patients with asthenozoospermia and leukocytospermia. Scale bar, 5 μm. Nuclei were counterstained with DAPI (4′,6-diamidino-2-phenylindole) (blue). Enlarged images are shown in the inset. (**B**) Representative Western blot from pooled normal and patient samples (normal and leukocytospermia, n = 10; asthenozoospermia, n = 27), showing decreased DEFB1 in asthenozoospermia (Astheno) and leukocytospermia (Leuko) patients compared to normal individuals. The summary of individual Western blot results is shown below. β-Actin was used as loading control. (**C**) Summary of sperm motility assessed by CASA (computer-aided sperm analysis) in asthenozoospermia (n = 33), leukocytospermia (n = 20), and normal (n = 32) sperm. (**D**) Representative plates from overnight bacterial cultures of *E. coli* and *S. aureus* after incubation with human tubal fluid (HTF) medium or normal sperm for 1 hour. HTF medium without bacteria was used as blank control. Summaries of colonies formed per plate (CFU) are shown in the right panel. (**E**) Comparison of *E. coli* CFUs after incubation with sperm obtained from normal individuals, and asthenozoospermia and leukocytospermia patients. Data are presented as means ± SEM, compared by Student's t test (D), one-way analysis of variance (ANOVA) with Tukey post hoc test (B and E), or Kruskal-Wallis test (C).

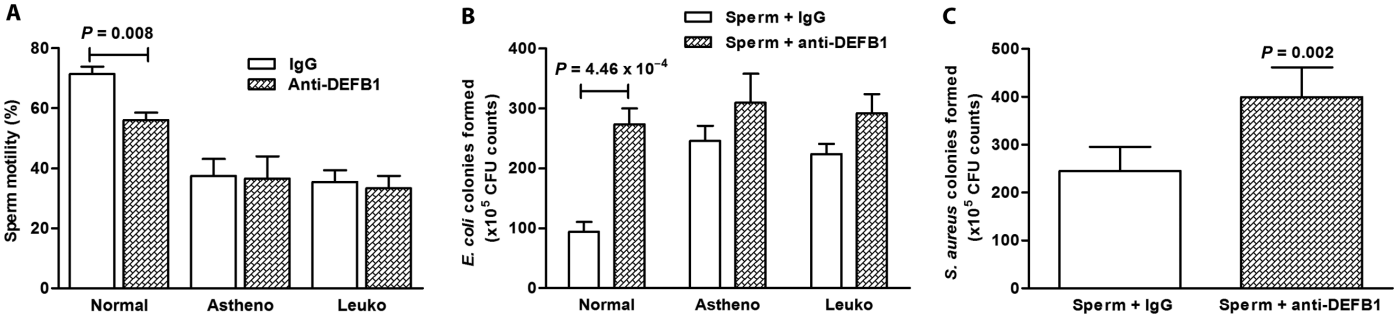

Fig. 2. Immunodepletion of DEFB1 impairs bactericidal activity and motility of normal sperm. (**A** and **B**) Motility of sperm (A) and summary of *E. coli* CFU (B) from normal, asthenozoospermia (Astheno), and leukocytospermia (Leuko) patients (n = 5 for each group), with anti-DEFB1 antibody (Santa Cruz, FL-68) or IgG (20 μg/ml) as control. (**C**) Summary of *S. aureus* CFU formed after incubation, with normal sperm and anti-DEFB1 antibody or IgG as control. Data are presented as means ± SEM, compared by Mann-Whitney test (A) or Student's t test (B and C).

rDEFB1 restores sperm motility and bactericidal activity in infertile sperm

If the impaired sperm motility and bactericidal activity observed in infertile patients with asthenozoospermia and leukocytospermia were indeed caused by an abnormally low level of DEFB1, restoring functional DEFB1 in sperm from the infertile patients should rescue the sperm functions. To test this, we first examined whether we could restore DEFB1 expression in abnormal sperm by incubating them with an rDEFB1, which contains 37 amino acid residues of the active region (32 to 68 amino acids) of DEFB1. We used free rDEFB1 at levels similar to the DEFB1 levels present in an ejaculate of sperm as determined by Western blot (fig. S11). In Western blot analysis, rDEFB1 treatment for 1 hour resulted in an increase in the levels of rDEFB1 protein (3.9 kD) in both normal and abnormal sperm, whereas the original sperm DEFB1 content did not change significantly (Fig. 3A). Quantification of the DEFB1 immunoreactivity in sperm after rDEFB1 treatment indicated a significant uptake of rDEFB1 (3.9 kD) in both types of abnormal sperm, as well as normal sperm (Fig. 3B, $P < 0.05$), although the total restored DEFB1 content in infertile sperm was still considerably lower than that of normal sperm.

We then examined whether rDEFB1 treatment could restore sperm functions in the abnormal sperm. In Fig. 3C, treatment with rDEFB1 (800 ng/ml) significantly improved sperm motility in sperm from asthenozoospermia and leukocytospermia patients compared with their counterparts before treatment ($P < 0.01$). Although the DEFB1-restored sperm motility in infertile sperm was still lower than that of normal sperm, it reached a level of >50% (52.4 ± 9.2% and 50 ± 10.4% in asthenozoospermia and leukocytospermia, respectively), with enhanced forward motility in both types of abnormal sperm (Fig. 3D).

We also conducted a hyaluronan binding assay (HBA), a method for sperm selection in in vitro fertilization clinics, where bound sperm was demonstrated to be correlated with high sperm motility (*27*) and improvement in clinical outcomes (*28*). The HBA results showed that treatment with rDEFB1 significantly increased the percentage of bound sperm in both groups of infertile sperm (Fig. 3E, $P < 0.01$). We also examined the egg-penetrating ability of rDEFB1-treated sperm in a human sperm–hamster oocyte penetration assay (SPA), which has long been shown to have prognostic value on fecundity rate (*29*). Indeed, rDEFB1 significantly increased the number of oocyte-penetrating sperm in both abnormal groups (Fig. 3F, $P < 0.01$), suggesting that rDEFB1 treatment may increase the fertilization rate of sperm obtained from asthenozoospermia and leukocytospermia patients in vitro. To elucidate how rDEFB1 increases the oocyte-penetrating capacity of sperm from two abnormal groups, we examined both sperm capacitation, a process that sperm must undergo to fertilize the oocyte, and hyperactivation, a specific beating pattern in the flagellum of sperm required for fertilization (*30*), after rDEFB1 treatment. A23187, a calcium ionophore, and IBMX, a phosphodiesterase inhibitor that elevates cAMP (adenosine 3′,5′-monophosphate) concentration, were used to induce capacitation and hyperactivation, respectively. The results showed that rDEFB1 treatment of sperm obtained from normal individuals did not induce acrosome reaction (fig. S12). Immunodepleting DEFB1 by neutralizing antibody significantly inhibited the hyperactivated sperm motility induced by A23187 or IBMX, but not the acrosome reaction induced by A23187 (fig. S13, $P < 0.05$). These results suggest that rDEFB1 may restore hyperactivated motility in abnormal sperm groups, leading to enhanced egg-penetrating ability.

We also examined whether rDEFB1 treatment could restore bactericidal activity in the two groups of infertile sperm. Treatment with rDEFB1 (500 ng/ml) significantly increased bactericidal activity (and reduced *E. coli* CFU counts) in sperm obtained from both asthenozoospermia and leukocytospermia patients, but not those from normal fertile individuals (Fig. 3G, $P < 0.01$). The effect of rDEFB1 on sperm motility and bactericidal activity in both types of abnormal sperm could be significantly reversed by antibody against DEFB1 (Fig. 3, H to K, $P < 0.01$). Furthermore, the reversal effect of DEFB1 antibody on sperm motility could also be counteracted by additional rDEFB1 in a concentration-dependent manner, as exemplified in the leukocytospermia patients (fig. S14), confirming the specificity of the effects of rDEFB1 and DEFB1 antibody. Together, our results demonstrated that restoring DEFB1 in sperm by rDEFB1 treatment could rescue sperm qualities, including motility, egg-penetrating ability, and bactericidal activity, in sperm obtained from infertile patients with asthenozoospermia and leukocytospermia.

DEFB1 interacts with chemokine receptor type 6 and induces Ca^{2+} mobilization in sperm

Like other defensins, DEFB1 is known to have the capacity to directly kill bacteria by disrupting the cytoplasmic membrane of microorganisms (*24*); however, its effect on sperm motility has not been reported. Since Ca^{2+} mobilization is known to play a central role in sperm motility (*31*), we suspected that the effect of DEFB1 on sperm motility might be mediated by an interacting protein in sperm that is linked to Ca^{2+} mobilization. Previous studies have demonstrated that chemokine receptor type 6 (CCR6) binds to DEFB1 and HBD-2 in immune cells (*32*) and links to Ca^{2+} mobilization in colonic epithelial cells upon its ligand stimulation (*33*). Moreover, CCR6 expression was recently found in sperm (*34*), suggesting that CCR6 may be a potential interacting protein of DEFB1 in sperm. We confirmed the expression of CCR6 in sperm by Western blot and immunofluorescence staining. The results showed that CCR6 protein was expressed in both human and mouse sperm (fig. S15). To confirm functional expression of CCR6 in sperm, we used its specific ligand CCL20 (*33*) to induce Ca^{2+} mobilization in sperm. In Fig. 4A, CCL20 (50 ng/ml) triggered a rise in intracellular Ca^{2+}, as indicated by the increase in fluorescence intensity ratio, in the lower sperm head and the midpiece ($P = 7.53 \times 10^{-13}$), which are major sites for Ca^{2+} mobilization during sperm activation (*31*). The CCL20-induced Ca^{2+} mobilization could be blocked by CCR6 antibody (20 μg/ml). Similarly, rDEFB1 (800 ng/ml) mimicked the effect of CCL20 in inducing Ca^{2+} mobilization in sperm (Fig. 4B), which could be blocked by CCR6 antibody (20 μg/ml), indicating that DEFB1 may bind to CCR6, just like its ligand CCL20, and trigger Ca^{2+} mobilization necessary for sperm motility. Furthermore, the rDEFB1- and CCL20-induced Ca^{2+} mobilization in sperm appeared to involve both extracellular Ca^{2+} influx and release of intracellular stored Ca^{2+}, because recombinant protein treatment in Ca^{2+}-free condition or in the presence of thapsigargin, which depletes the intracellular calcium store, could partially prevent the Ca^{2+} mobilization (Fig. 4C and fig. S16). The ability of rDEFB1 to mimic the effect of the CCR6 ligand CCL20 in inducing Ca^{2+} mobilization in sperm suggests its possible interaction with CCR6 in promoting sperm motility. In addition to Ca^{2+} mobilization, bicarbonate-mediated cAMP production is a common pathway known to regulate sperm motility (*35*). We found that DEFB1 also increases cAMP production in both normal and abnormal groups in the presence of bicarbonate (fig. S17), consistent with the role of DEFB1 in promoting sperm motility.

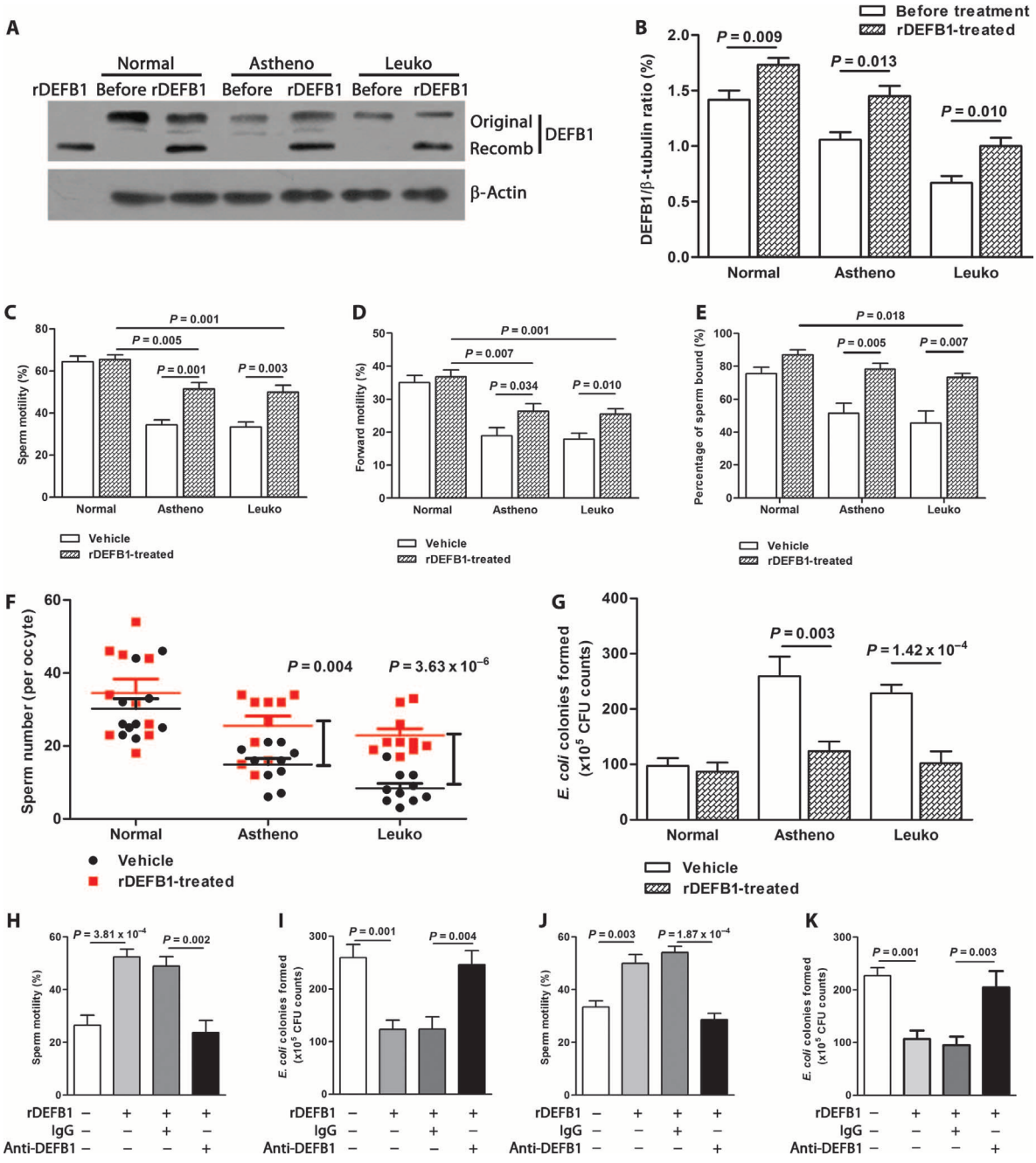

Fig. 3. rDEFB1 treatment restores DEFB1 expression and function in infertile sperm. (**A**) Representative Western blot showing the expression pattern of DEFB1 in sperm before and after rDEFB1 treatment (500 ng/ml) in normal, asthenozoospermia, and leukocytospermia samples (n = 10 in each group). Note the uptake of rDEFB1 (Recomb; 3.9 kD) after treatment, whereas the original DEFB1 expression remains unchanged. β-Actin was used as loading control. (**B**) Summary of Western blot results showing total DEFB1 immunoreactivity (both original and rDEFB1) before and after rDEFB1 treatment. (**C** to **G**) rDEFB1 treatment–induced restoration of sperm motility (C), forward motility (D), bound sperm in HBA (E), penetrated sperm number in human sperm–hamster oocyte penetration assay (SPA) (F), and bacteria-killing activity (G) (C and D: 800 ng/ml; E and F: 1 μg/ml; G: 500 ng/ml) in sperm obtained from asthenozoospermia and leukocytospermia patients compared to normal individuals (n = 10 in each group). (**H** to **K**) Effect of anti-DEFB1 antibody on rDEFB1-rescued sperm motility (H and J) and bactericidal activity (I and K) in samples from asthenozoospermia (H and I) and leukocytospermia patients (J and K) (n = 10 in each group). Anti-DEFB1 antibody (20 μg/ml), added after 1 hour of treatment with rDEFB1, abolishes the rescuing effect of rDEFB1 on sperm motility (800 ng/ml) and bacteria-killing activity (500 ng/ml). IgG was used as a control for antibody treatment. Data are presented as means ± SEM, compared by Student's t test (B and E to G), Mann-Whitney test (C and D), Kruskal-Wallis test (C, D, H and J), or one-way ANOVA with Tukey post hoc test (E, I, and K).

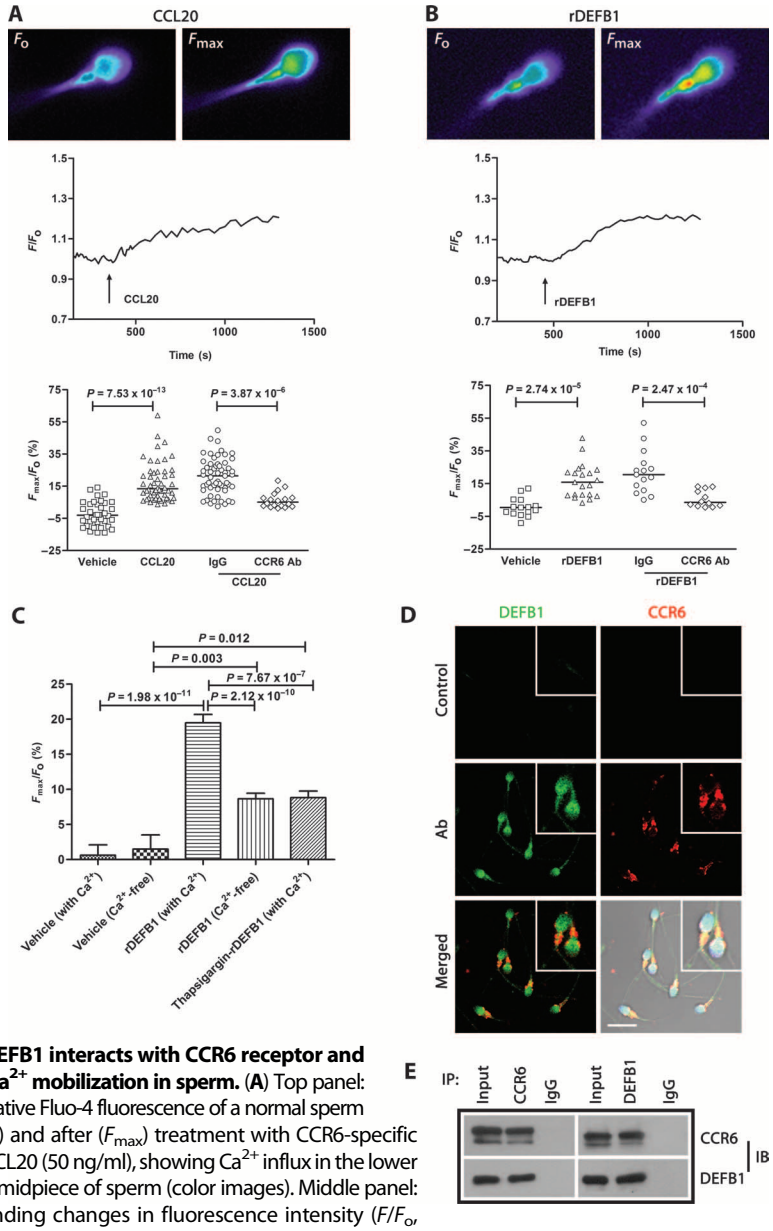

Fig. 4. DEFB1 interacts with CCR6 receptor and triggers Ca^{2+} mobilization in sperm. (**A**) Top panel: Representative Fluo-4 fluorescence of a normal sperm before (F_o) and after (F_{max}) treatment with CCR6-specific agonist, CCL20 (50 ng/ml), showing Ca^{2+} influx in the lower head and midpiece of sperm (color images). Middle panel: Corresponding changes in fluorescence intensity (F/F_o, where F_o is the baseline fluorescence intensity before CCL20 stimulation) over time, showing a gradually rising response about 100 s after CCL20 challenge (indicated by arrow). Lower panel: Scatter plot summarizing the percentage increase of $[Ca^{2+}]_i$, which was determined from the maximum fluorescence intensity after (F_{max}) and before (F_o) addition of CCL20 (50 ng/ml) alone or with CCR6 antibody (Ab) or IgG (20 μg/ml). (**B**) Top panel: Representative Fluo-4 fluorescence of a normal sperm before (F_o) and after (F_{max}) treatment of the normal sperm with rDEFB1 (800 ng/ml). Middle panel: A time course recording of the rDEFB1-induced response, showing a gradually rising response about 100 s after DEFB1 challenge (indicated by arrow). Lower panel: Scatter plot is the summary of responses (percentage increase of $[Ca^{2+}]_i$) in the absence or presence of rDEFB1 alone or with CCR6 antibody or IgG (20 μg/ml). (**C**) Quantification of intracellular Ca^{2+} after rDEFB1 treatment in Ca^{2+}-containing or Ca^{2+}-free condition, and in the presence of thapsigargin (2 μM). (**D**) Representative confocal images showing colocalization of DEFB1 (green) and CCR6 (red) in the midpiece of normal sperm. Single-channel images are shown in the middle panel. Merged fluorescent image is shown in bottom left. Merged phase-contrast and fluorescent image is shown in bottom right. Primary antibodies were omitted in the control (top panel). Nuclei were counterstained with DAPI (blue). Enlarged images are shown in inset. Scale bar, 5 μm. (**E**) Coimmunoprecipitation of CCR6 and DEFB1 in normal sperm. Sperm lysates were pulled down with either anti-CCR6 (left panel) or DEFB1 (right panel) antibody and immunoblotted for CCR6 or DEFB1 as indicated. IgG was used as control. Data are presented as means ± SEM, compared by one-way ANOVA with Tukey post hoc test (A to C).

To demonstrate possible interaction between DEFB1 and CCR6 in human sperm, we first performed immunolocalization of CCR6 in sperm and found that it was colocalized with DEFB1 primarily to the midpiece of sperm (Fig. 4D), suggesting that DEFB1 and CCR6 may physically interact in sperm. We then confirmed their interaction by coimmunoprecipitation. In this experiment, human sperm were lysed and endogenous CCR6 was pulled down by its antibody. As expected, DEFB1 was detected in the protein complex pulled down by CCR6 antibodies, but not the IgG control (Fig. 4E). Similarly, CCR6 was detected in DEFB1 pull-down complex (Fig. 4E), indicating protein-protein interaction between DEFB1 and CCR6 in sperm. Together, these results suggest that DEFB1 may induce Ca^{2+} mobilization important for sperm motility through its interaction with CCR6 in sperm.

Effects of DEFB1 on sperm motility and bactericidal activity depend on CCR6

DEFB1 has been shown to be expressed in the epithelia of the male genital tract and on ejaculated spermatozoa (*23*). Consistent with this previous finding, we have observed increasing expression of DEFB1 in sperm and epithelial cells along the length of the human epididymis, with a maximum level found in the cauda (tail) of the epididymis (fig. S18), suggesting that the epididymis-secreted DEFB1 is captured by the sperm during their transit through the epididymis. The demonstrated protein-protein interaction between DEFB1 and CCR6 raises the possibility that the epididymis-secreted DEFB1 may bind to sperm by first interacting with CCR6 in sperm. If this is the case, the amount of DEFB1 bound to sperm may be determined by the expression of CCR6 in sperm. To test this, we compared the expression of DEFB1 and CCR6 in normal and in the two types of abnormal sperm. In Fig. 5A, similar to the DEFB1 in different groups of sperm (Fig. 1), CCR6 was present in high amounts in normal sperm, but was reduced in asthenozoospermia and leukocytospermia (Fig. 5A). We conducted a correlation analysis and found a positive correlation between DEFB1 and CCR6 ($r = 0.69$, $P < 0.001$) (fig. S19), suggesting that the reduced amounts of DEFB1 in abnormal sperm may be due to the reduced amounts of its carrier/receptor CCR6.

We further examined whether the interaction with CCR6 is required for the function of DEFB1 in sperm. In Fig. 5B, immunodepletion of CCR6 decreased sperm motility in both normal and asthenozoospermia but not leukocytospermia sperm samples ($P < 0.01$). We excluded the possibility that the effect of CCR6 antibody on sperm motility was due to the agglutination of sperm (fig. S20). To further examine the specific involvement of CCR6 on DEFB1-mediated sperm motility, we treated sperm from all groups with rDEFB1 and rescued sperm motility in the two abnormal groups but not in the normal (Fig. 5, C to E), as previously observed (Fig. 3C). The effect of rDEFB1 could be reversed by CCR6 antibody but not IgG (Fig. 5, C to E, $P < 0.05$), indicating that CCR6 is required for mediating the effect of rDEFB1 on sperm motility. Although defensins are known to have direct bacteria-killing capacity, treatment of sperm with CCR6 antibody mimicked the effect of DEFB1 antibody (Fig. 2B) in significantly reducing the bactericidal activity in normal and in the two types of abnormal sperm, causing significantly increased CFU counts (Fig. 5F, $P < 0.01$). However, the direct bactericidal activity of DEFB1 is not sensitive to CCR6 antibody treatment in the absence of sperm (fig. S21). These results suggest that the bactericidal effect of sperm depends on both DEFB1 and CCR6 on sperm. Indeed, although rDEFB1 did not affect the bactericidal activity of normal sperm (Fig. 5G), it rescued the antibacterial activity in sperm obtained from both asthenozoospermia (Fig. 5H) and leukocytospermia (Fig. 5I) patients, and the rescuing effects could be significantly blocked by CCR6

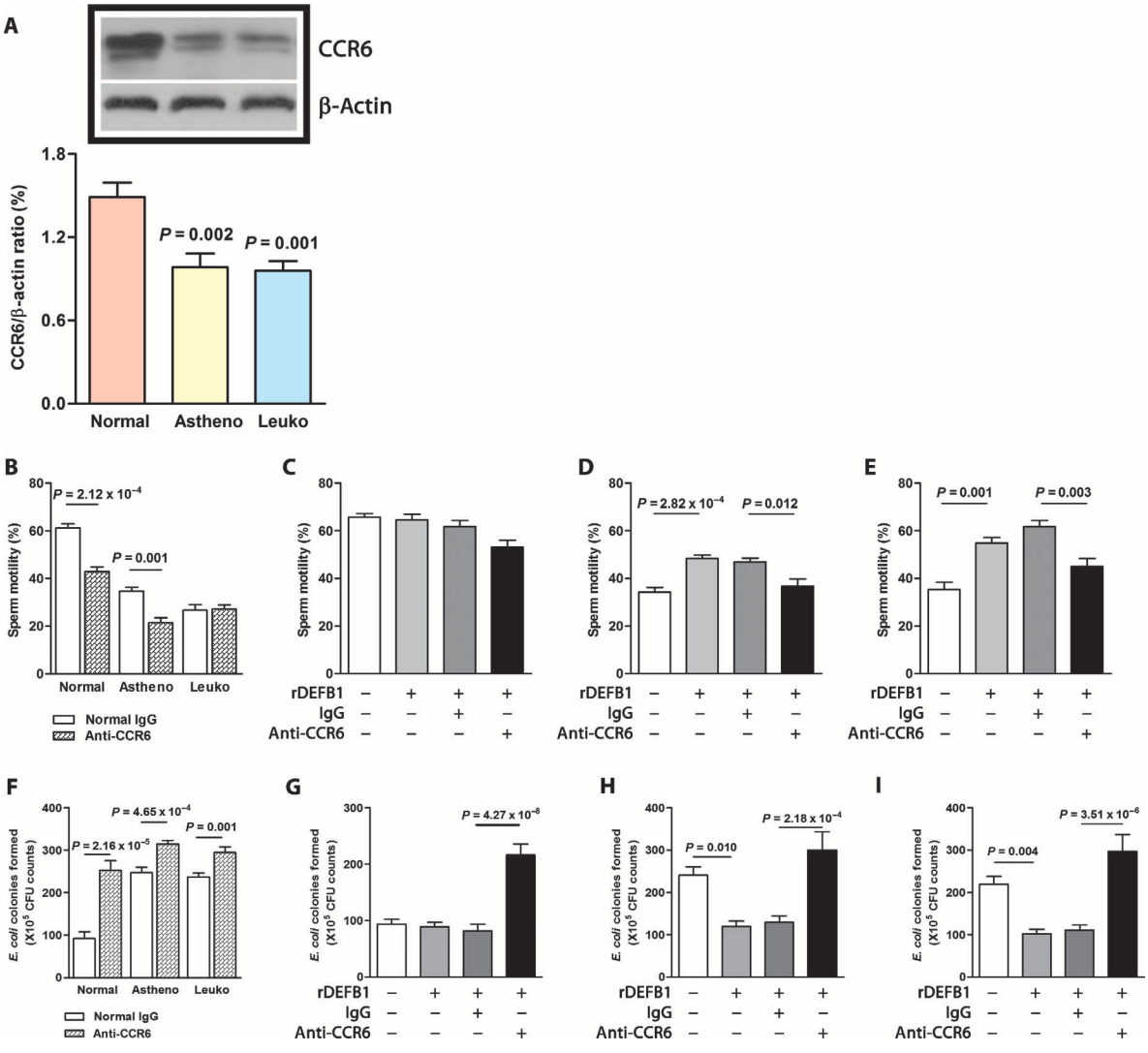

Fig. 5. CCR6 receptor mediates the effect of DEFB1 in sperm. (**A**) Representative Western blot from pooled normal and patient samples ($n = 10$ in each group) showing down-regulation of CCR6 in asthenozoospermia (Astheno) and leukocytospermia (Leuko) patients. The summary of individual Western blot results for each group is shown below. β-Actin was used as loading control. (**B**) Effect of anti-CCR6 antibody (20 μg/ml) on sperm motility in sperm from all three groups. (**C** to **E**) Effect of rDEFB1 alone (800 ng/ml) or rDEFB1 together with anti-CCR6 antibody on sperm motility in sperm from normal (C), asthenozoospermia (D), and leukocytospermia (E) patients ($n \geq 10$ in each group). (**F**) Effect of anti-CCR6 antibody (20 μg/ml) on bacteria-killing activity of sperm from all three groups. (**G** to **I**) Effect of rDEFB1 alone (500 ng/ml) or rDEFB1 together with anti-CCR6 antibody on bacteria-killing activity of sperm from normal (G), asthenozoospermia (H), and leukocytospermia (I) patients ($n \geq 10$ in each group). IgG was used as control for antibody treatment. Data are presented as means ± SEM, compared by Mann-Whitney test (B), Student's *t* test (F), Kruskal-Wallis test (C to E), or one-way ANOVA with Tukey post hoc test (A, G to I).

antibody (Fig. 5, G to I, $P < 0.001$), indicating that the rDEFB1-rescued bactericidal effect also requires CCR6. Together with the data demonstrating colocalization and protein-protein interaction between DEFB1 and CCR6 in sperm, these results indicate the importance of a DEFB1/CCR6 protein complex in mediating the dual action of DEFB1 in sperm host defense and motility.

DISCUSSION

The present study has demonstrated a dual role of DEFB1 in defending male fertility. These results also suggest that a deficiency of DEFB1 may contribute to the pathogenesis of male infertility associated with asthenozoospermia and leukocytospermia. It appears that DEFB1 creates an antimicrobial shield around the human sperm and, in addition, promotes sperm motility. This finding provides an explanation for the clinical observation that asthenozoospermia is often associated with leukocytospermia (*8*, *9*), indicating a common cause for the components of both reduced sperm motility and bactericidal activity. Although an early study has suggested that leukocyte concentration in semen is not associated with reduced sperm quality and conception rate in infertile patients (*36*), findings from recent studies show that oxidation stress caused by infection or inflammation may decrease sperm quality and lead to infertility (*37*). In light of the present finding, the infertility in leukocytospermia patients could be attributed to decreased sperm motility and/or increased infection-induced oxidative stress, both of which could stem from a deficiency of DEFB1 in sperm. A recent study has demonstrated the ability of human β-defensin 114, another β-defensin expressed in the epididymis, in preventing lipopolysaccharide (LPS)–induced sperm motility loss through its LPS-neutralizing ability, suggesting that the rescue effect of human β-defensin 114 on sperm motility can be secondary to its primary bacteria-suppressing action (*38*). However, this should not be the case for DEFB1 because the contaminating bacteria and leukocytes have been removed before immunodepleting DEFB1, and rDEFB1 can directly trigger calcium mobilization in normal sperm, indicating a primary effect of DEFB1 in promoting sperm motility. It should also be noted that the ability to swim is not an intrinsic property of sperm, but acquired during their transit through the epididymis (*10*). Recent studies have demonstrated the involvement of two epididymis-specific β-defensins, rat Bin-1b and human/mouse β-defensin 15, in initiating and maintaining sperm motility (*13*, *18*). Our observation that anti-DEFB1 antibody could not entirely abolish sperm motility in normal sperm indicates that sperm motility is not entirely determined by DEFB1. However, the increase in sperm motility induced by treatment with rDEFB1 in asthenozoospermia and leukocytospermia suggests an important role of DEFB1 in sperm motility and indicates a potential use for rDEFB1 to improve sperm quality in the treatment of these abnormalities.

Although the present results suggest a dual role of DEFB1 in defending male fertility in humans, the role of DEFB1 in mouse fertility is controversial. DEFB1 knockout mice appear to be fertile and breed well (*39*), but male mice with deletion in the β-defensin gene cluster encoding DEFB1 and eight other β-defensins (*Defb*Δ*9*) are sterile (*40*). These observations suggest that either DEFB1 functionally overlaps with other β-defensins in the same gene cluster or DEFB1 is dispensable for male fertility in mice. The latter would not be surprising because a number of proteins that are required for fertilization in humans have also been shown to be dispensable in gene knockout mice (*41*). Moreover, whereas human rDEFB1 increases intracellular Ca^{2+}, the deletion of β-defensin gene cluster in *Defb*Δ*9* mice elevates intracellular Ca^{2+} content (*40*), suggesting that the role of DEFB1 in human sperm might be distinct from that in mouse sperm. Note that although the genomic organization of the β-defensin cluster is retained, the high degree of peptide sequence variation in different orthologs implies extensive specialization and species-specific adaption during evolution (*42*). DEFB1 has been reported to have evolved in primate lineages (*43*), where it is quite distinct from its orthologs in rodents [TreeFam (http://www.treefam.org/); accession: TF336381]. Therefore, it is plausible that rodents may use other β-defensins for sperm motility, as reported previously (*14*, *18*). On the other hand, the reported evolution of DEFB1 in primate lineages,

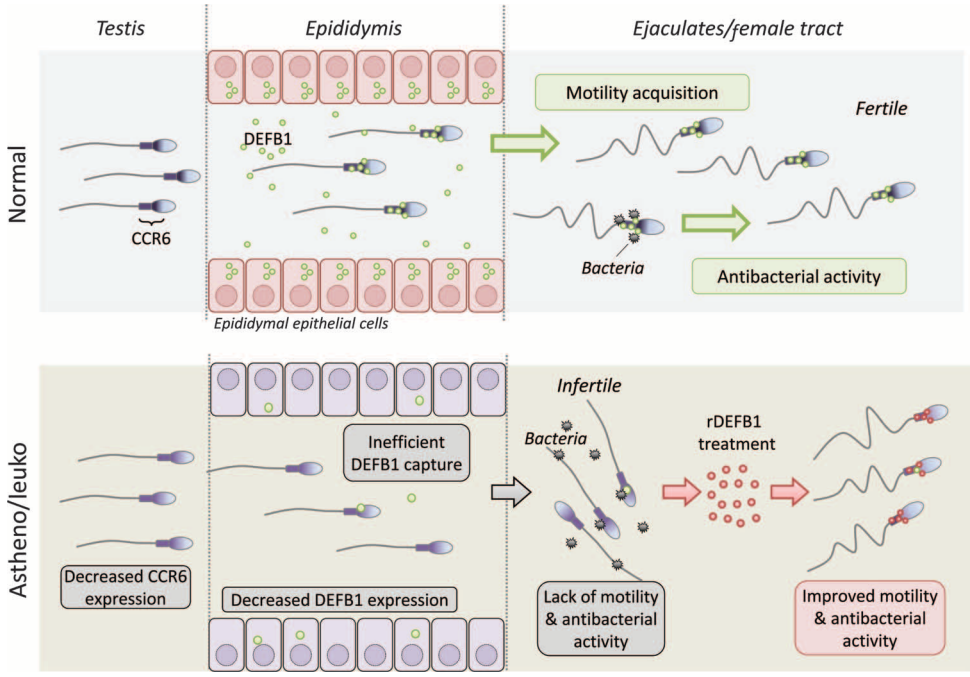

Fig. 6. DEFB1 plays a dual role in defending male fertility. This working model shows that in fertile individuals, testicular spermatozoa express CCR6 receptor. As the sperm passes through the epididymis, the epithelium releases DEFB1, which is captured by sperm through binding to the CCR6 receptor. Acquisition of DEFB1 confers motility and bacteria-killing activity of sperm in ejaculates or female reproductive tract (**upper panel**). In asthenozoospermia and leukocytospermia patients, sperm capture DEFB1 inefficiently because of reduced expression of CCR6 receptor in testicular spermatozoa and/or reduced expression of DEFB1 in epididymal epithelium. These sperm are infertile, with low motility and bacteria-killing activity (**lower panel**). rDEFB1 treatment of the infertile sperm restores sperm motility and bacteria-killing activity (lower panel).

together with the currently demonstrated role of DEFB1 in defending human fertility, as well as its well-known role in host defense in humans (*44*), suggests that DEFB1 may have acquired specialization and species-specific adaptation during evolution, which may be vital to the survival of human species. Further investigation along this line may provide insights into the molecular mechanism defending human fertility.

An unexpected finding from the present study is the involvement of CCR6 receptor in mediating the effect of DEFB1 on both sperm motility and bactericidal activity. CCR6 has recently been shown to be expressed in sperm and involved in sperm chemotaxis upon binding with its chemokine ligand CCL20; however, the underlying mechanism was not clear (*34*). The present finding that CCR6 is linked to Ca^{2+} mobilization in sperm and interacts with DEFB1, together with the observed inhibition of DEFB1-induced Ca^{2+} mobilization and sperm motility by CCR6 antibody, indicates a mechanism involving a DEFB1/CCR6 protein complex in regulating sperm motility. Together, these findings suggest that depending on the types of ligands, such as chemokines or β-defensins, CCR6 receptor may elicit different types of physiological responses required for sperm function and male reproduction.

Another surprise from the present study is that sperm have antimicrobial activity and that this activity requires both DEFB1 and its interacting partner CCR6. Although antimicrobial activity of human semen has been observed since 1949 (*45*), the contents of seminal fluid, including β-defensin members, but not sperm, were thought to be responsible (*46*). Here, we showed that the bactericidal activity of sperm requires CCR6, because treatment of sperm with CCR6 antibody greatly reduced their basal and rDEFB1-enhanced bactericidal capacity. Notably, whereas CCR6 is endogenously expressed in sperm, as indicated by its testicular expression in germ cells, DEFB1 is highly expressed in the epithelium of the epididymis, consistent with its well-known expression in various epithelial tissues (*21*). This, together with the demonstrated protein-protein interaction between CCR6 and DEFB1, prompts us to propose that CCR6 in sperm acts as a DEFB1 carrier/receptor that captures DEFB1 secreted during sperm transit through the epididymis (Fig. 6). That is, the binding of DEFB1 to sperm requires its interaction with CCR6. This notion is supported by the observed positive correlation between DEFB1 and CCR6 expression in sperm, both of which are reduced in asthenozoospermia and leukocytospermia compared to normal. The requirement of CCR6 for DEFB1 binding to sperm is further supported by the observation that the restored levels of DEFB1 in sperm from patients with asthenozoospermia and leukocytospermia are still lower than that of normal sperm after incubation with rDEFB1. This suggests that the binding of DEFB1 to sperm is CCR6-dpendent and that the reduced levels of DEFB1 in the two types of abnormal sperm may partly be due to reduced CCR6 expression in sperm. Therefore, the abnormalities in DEFB1-deficient sperm could result either from defective DEFB1 production/secretion from the male genital tract or from a possible defect in CCR6 expression in sperm (Fig. 6).

DEFB1 is expressed on almost every epithelial surface of the body at low concentration and has minor bacteria-killing activity compared to other defensins (*47*). It is debatable whether DEFB1 plays an important role in host defense. Here, we have shown that sperm in physiological HTF medium have bacteria-killing activity, which can be blocked by DEFB1-neutralizing antibody and rescued by rDEFB1, suggesting that DEFB1 may contribute, at least in part, to the intrinsic bacteria-killing activity of the sperm. That is, although DEFB1 may be present at a low amount in seminal plasma, it can be concentrated by binding to sperm to exert its bactericidal effect. However, this raises a question as to how sperm-bound DEFB1 can exert its bactericidal action because it is well established that the binding of defensins to the cell membrane of bacteria is a prerequisite for their bactericidal action, either by forming pores or by permeabilizing the cell through an electrostatic interaction without forming a pore (*44*). The likely scenario is that when bacteria come in contact with sperm, DEFB1, originally bound to sperm, can be transferred to the bacteria. In the present study, we detected the transfer of sperm-bound DEFB1 to bacteria after incubation with sperm, which explains its bacteria-killing effect.

The fact that both reduced sperm motility and bactericidal activity can be restored by treatment with rDEFB1 has considerable clinical implications (Fig. 6). Current treatment for male infertility includes empiric regimens with vitamins and minerals (*48*, *49*) or intracytoplasmic sperm injection, which are associated with increased risk of congenital abnormalities in the offspring (*50*). The present study has demonstrated a restoration of >50% sperm motility with enhanced forward motility, hyperactivated motility, and improved oocyte penetration ability in both types of abnormal sperm by rDEFB1 treatment. This may provide an effective treatment method to increase sperm motility and fertilization rate in assisted reproduction. In addition, rDEFB1 treatment also increases the number of sperm bound in the HBA assay, suggesting a potential role of DEFB1 in modulating sperm quality, maturity, and structural integrity apart from sperm motility, which may also contribute to improving clinical outcomes. The present finding warrants further investigation into the possible role of DEFB1 in modulating other sperm parameters.

Although the present study has provided promising results, which may form the basis for the potential development of rDEFB1 as a treatment method for infertile patients with asthenozoospermia and leukocytospermia, there are limitations in the present study that warrant further investigation. First, the observed rDEFB1-increased sperm-fertilizing capacity in the abnormal sperm was obtained from a "human sperm–hamster oocyte hybrid" system. Therefore, the fertilization-promoting effect of rDEFB1 on the two types of abnormal sperm for penetrating human oocytes remains to be tested. Second, the safety of using rDEFB1, particularly any potential adverse effects of rDEFB1 treatment on embryo development, has not been addressed in the present study. Further investigation is required to address these concerns.

In closing, the demonstrated dual role of DEFB1 in defending male fertility and the involvement of DEFB1 and its interacting protein CCR6 in the pathogenesis of asthenozoospermia and leukocytospermia have suggested potential diagnostic targets and a feasible treatment method for male infertility associated with poor sperm motility and genital tract infection.

SUPPLEMENTARY MATERIALS

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Materials and Methods

Fig. S1. Decreased expression of DEFB1 in sperm from asthenozoospermia and leukocytospermia patients.

Fig. S2. Specificity of anti-DEFB1 antibody.

Fig. S3. Correlation between DEFB1 expression and sperm motility in normal and asthenozoospermia sperm.

Fig. S4. Inverse correlation between DEFB1 expression and number of leukocytes in normal and leukocytospermia sperm.

Fig. S5. Inverse correlation between sperm motility and number of leukocytes in normal and leukocytospermia sperm.

Fig. S6. Transfer of sperm-bound DEFB1 to bacteria after incubation with sperm.

Fig. S7. Negative effect of DEFB-neutralizing antibodies on sperm agglutination.

Fig. S8. Reversal of anti-DEFB1 antibody effect by rDEFB1.
Fig. S9. Negative effect of normal IgG on bactericidal activity of sperm.
Fig. S10. Effect of immunodepletion of DEFB1 by a separate source of DEFB1 antibody on sperm motility and bactericidal activity.
Fig. S11. Comparison of expression of sperm-bound and recombinant DEFB1.
Fig. S12. Negative effect of rDEFB1 on acrosome reaction.
Fig. S13. Effect of immunodepletion of DEFB1 on hyperactivated motility.
Fig. S14. Counteracting the reversal effect of DEFB1 antibody on rDEFB1 treatment by addition of preabsorbed rDEFB1.
Fig. S15. Expression of CCR6 receptor in human and mouse sperm.
Fig. S16. Effect of CCR6 ligand CCL20 on intracellular Ca^{2+} concentrations in normal sperm.
Fig. S17. Effect of rDEFB1 on cAMP production in normal and abnormal sperm.
Fig. S18. Expression of DEFB1 in human epididymis.
Fig. S19. Correlation between expression of DEFB1 and CCR6 in normal and infertile sperm.
Fig. S20. Negative effect of neutralizing antibodies against CCR6 on sperm agglutination.
Fig. S21. Effect of rDEFB1 and anti-CCR6 antibody on bactericidal activity in the absence of sperm.
Table S1. Clinical parameters of normal, asthenozoospermia, and leukocytospermia patients' sperm used in DEFB1 expression profiling.
Table S2. Infection and fertility status of leukocytospermia patients used in DEFB1 expression profiling.
Table S3. Original data (provided as an Excel file).
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